Pseudomonas aeruginosa is a leading cause of hospital-acquired infections by gram-negative bacteria and is responsible for chronic infections of individuals with cystic fibrosis (15). As P. aeruginosa and other bacterial pathogens become multidrug resistant (22), there is a continual need for the identification of compounds directed at novel targets that could be developed into therapeutic agents. The ability of P. aeruginosa to cause a number of distinct infections has been attributed to its large genome, which encodes a variety of virulence factors (35). Prominent among these is the type III secretion system (TTSS), which allows the bacteria to deliver proteins directly into the host cell cytoplasm (39). ExoU is one of the toxic effector proteins delivered by the type III secretion apparatus, and its expression is associated with cytotoxicity toward eukaryotic cells (33, 34). In contrast to eukaryotic phospholipases, which remodel cellular membranes and synthesize proinflammatory secondary messengers such as arachidonic acid and leukotrienes (2, 9), ExoU PL2 activity primarily causes disruption of the host cytoplasmic membrane, resulting in cell lysis (24, 32, 33).

We sought to identify compounds that inhibit type III secretion-mediated cytotoxicity by protecting tissue culture cells from infection by P. aeruginosa strains that elaborate ExoU as a sole cytotoxic effector. From a synthetic small-molecule library, we have identified compounds that protected Chinese hamster ovary (CHO) cells from the cytotoxic activity of P. aeruginosa expressing ExoU. One of the most potent compounds is pseudolipasin A (Pseudomonas phospholipase inhibitor A). Pseudolipasin A does not interfere with type III secretion in general, suggesting that the protection observed occurs downstream of the delivery of ExoU. Pseudolipasin A not only protected CHO cells from intoxication but also rescued the amoeba Dictostelium discoideum from ExoU-mediated killing by P. aeruginosa. Furthermore, the cytotoxicity of cytoplasmically expressed ExoU in Saccharomyces cerevisiae was inhibited by pseudolipasin A. Pseudolipasin A inhibited the PL2 activity of ExoU in vitro but did not affect any of the other eukaryotic PL2 enzymes tested, including the cytosolic phospholipase that promotes the generation of arachidonic acid and subsequent generation of a proinflammatory immune response. This screen of a small-molecule chemical library identified a specific inhibitor of the enzymatic activity of a toxin that could be a prototype of therapeutic agents targeting bacterial virulence factors.

**MATERIALS AND METHODS**

**Strains and media.** P. aeruginosa strain PAK-VL1 (PAK ΔexoU ΔmexAB att:exoU-spcU/pMMB-exoU) harbors a deletion of the exoU gene, a deletion in the mexAB genes encoding the major drug efflux pump, a chromosomal insertion of exoU-spcU from PA103 at the ctx site, and the type III transcriptional activator exsA under the control of isopropyl-β-D-thiogalactopyranoside (IPTG) on the pMMB67 plasmid (37). Strains PAK, PA103, and PA14 have been described elsewhere (4, 18, 27). P. aeruginosa strains were routinely grown in Luria-Bertani broth (LB) and induced for type III secretion by chelating Ca2+ with 5 mM EGTA. Plasmid pMMB-exoU was maintained with 50 μg/ml carbenicillin, and exoU expression was induced with 1 mM IPTG. Assays for secretion and injection utilized pVL710 and pVL712. pVL710 is a pMMB-Ap-based plasmid that contains an exoU promoter driving the expression of β-lactamase. pVL712 contains the promoter of exoU driving the expression of an ExoU(S142A)-β-lactamase fusion protein. S. cerevisiae BY4742 was grown on yeast-peptone-dextrose medium. Plasmid pDH105 was maintained in BY4742 by growth on minimal synthetic defined (SD) base medium supplemented with –Leu dropout supplement (SD - Leu; Clontech, Mountain View, CA). D. discoideum strain AX3 was propagated in HL5 medium (per liter, 10 g of dextrose, 5 g of yeast extract, 5 g of tryptone, and 0.5 g of sodium acetate) supplemented with 2 μM Ca2+ (17). B. malaya strain W64A was maintained on 0.2% yeast extract and 0.4% casamino acids.
of Thionite peptone, 0.67 g of Na₂HPO₄ - 7H₂O, 0.34 g of KH₂PO₄, and 0.05 g of streptomycin-sulfate.

Chemical library and screening facility. The known PLₐₐₐ inhibitors methyl arachidonyl fluorophosphonate (MAFP) and bromoelcol lactone (BEL) were purchased from Cayman Chemical (Ann Arbor, MI). The Microenfram collection from ChemBridge (San Diego, CA) consists of 50,000 compounds and was screened in duplicate in a high-throughput screening assay. In this assay, the final concentration of compounds after pin transfer from a stock solution of 5 mg/ml is 12.5 µg/ml (approximately 25 to 50 µM depending on the molecular weight of each compound). The screening was performed at the Institute of Chemistry and Cell Biology at Harvard Medical School.

Inhibition of CHO cell killing by P. aeruginosa expressing ExoU. CHO cells were grown in F-12 medium supplemented with 10% fetal bovine serum and 2 mM glutamine. A total of 1 × 10⁶ CHO cells per well were seeded in 384-well plates. Compounds were pin transferred into 384-well plates, and the A₆₀₀ was read to exclude compounds with high absorbance at the output wavelength. CHO cells were infected at a multiplicity of infection (MOI) of 10 with PAK-VL1 grown to a mid-log-phase culture in 1 mM IPTG to induce expression of the type III secretion system. Antibiotics were added 2 h postinfection to restrict the cytotoxicity mediated by ExoU as well as to eliminate bacteria that would interfere with the readout. After an additional 5 h of incubation, a tetrazolium-based reagent, WST (Roche Applied Science, Indianapolis, IN), was added to the wells to detect the total reducing potential, which is an indicator of the number of live cells as measured by absorbance reading at 405 nm. Compounds that provided protection were retested in 96-well plates. In addition, compounds that protected cells from ExoU-mediated lysis were also tested by measuring the release of cellular lactate dehydrogenase (LDH; Roche Applied Science, Indianapolis, IN).

High-throughput secretion assay for the fusion of ExoU to the mature portion of β-lactamase lacking the signal peptide (BlabM). P. aeruginosa PAK or PAKαcmc containing either pVL710 and pVL712 was grown in LB containing 50 µg/ml carbenicillin. The bacteria were subcultured to an optical density at 600 nm (OD₆₀₀) of 0.005 in LB supplemented with 5 mM EGTA to induce the TTSS, and 25 µl was dispensed into 384-well plates and incubated for 3 h at 37°C. A 25-µl volume of CHO extract containing 50 µg/ml bovine serum albumin (EMD Biosciences, Madison, WI) was added to each well, and plates were read at 490 nm and 650 nm over a 60-min period.

High-throughput injection assay for ExoU-BlaM fusion. Confluent monolayers of CHO cells were grown in black, clear-bottom, 384-well plates. Cells were infected with 2 h at an MOI of 10 with P. aeruginosa PAK or PAKαcmc containing either pVL710 or pVL712. CCF4-AM (Invitrogen, San Diego, CA) was added according to the manufacturer's instructions for 30 min. The plates were read in a TECAN fluorescence plate reader using excitation at 360 nm and emission at 490 nm and 650 nm to detect the fusion of the lymphokines.

Protection of Dictyostelium discoideum from P. aeruginosa PA103-mediated killing. P. aeruginosa PA103-mediated killing of D. discoideum strain AX3 on SM/50 agar plates (per liter, 0.1 g of MgSO₄·1·9H₂O, 1·0 g of K₂HPO₄, 0.2 g of glucose, 0.2 g of Bacto peptone, 0.2 g of Bacto yeast extract, and 20 g of Bacto agar; Difco, Franklin Lakes, NJ) was carried out as described previously (17). PAK and PA103 were grown in LB containing 0.5 mM CuSO₄. Plates were incubated in a humidified chamber at 30°C for 24 h and resuspended by pipetting prior to spectrophotometer reading at 600 nm. Protection on SD – Leu agar was performed with 0.75 mM CuSO₄ for 10⁴ CHO cells per well.

RESULTS

Screening of a compound library for inhibitors of P. aeruginosa cytotoxic activity. To identify inhibitors that prevent intoxication of mammalian cells by P. aeruginosa expressing ExoU, we developed an assay that measures the total reducing potential of cells after P. aeruginosa infection as an indication of their viability. For this assay, we engineered strain PAK-VL1 (PAK ΔmexAB ΔexoS att:exoU scep/ pMMB-EXO4) which carries a deletion of the genes encoding the MexAB efflux pump such that the bacteria would be sensitized to small molecules acting on intracellular targets. exoS, the gene native to strain PAK that also encodes a type III secreted cytotoxin, is deleted, and the exoU operon from PA103 is placed in the ctx site on the chromosome (17). PAK-VL1 injection of ExoU via the TTSS resulted in a rapid cytotoxic effect on CHO cells (17, 36).

The high-throughput assay of ExoU-mediated cytotoxicity allowed rapid screening of 50,000 compounds in ChemBridge Microformat Library E. Cells infected with P. aeruginosa PAK-VL1 (Fig. 1A, 1st and 2nd columns) are intoxicated and unable to reduce WST, whereas cells protected by gentamicin (Fig. 1A, 23rd and 24th columns) are viable and reduce WST to the same extent as uninfected controls. Active compounds that can
Small-Molecule Inhibitors of P. aeruginosa ExoU

Pseudolipasin A does not inhibit type III secretion or type III injection into mammalian cells. We conducted several additional assays for type III-dependent secretion and injection by utilizing a β-lactamase reporter that fused BlaM to the C terminus of ExoU (Fig. 3A). As a result, type III secretion can be detected by β-lactamase cleavage of nitrocefin in the calcium-depleted TTSS-induced culture (23). Secretion of the ExoU-BlaM fusion (from plasmid pVL712) can be readily measured using the nitrocefin assay (Fig. 3B). Neither BlaM alone (expressed from pVL710), which lacks the type III secretion signal, nor a type III-defective strain (ΔpscC) can secrete BlaM via the TTSS. These data demonstrate that the purity of pseudolipasin A were confirmed by liquid chromatography-mass spectrometry and 1H nuclear magnetic resonance (see Fig. S1 in the supplemental material). To test whether pseudolipasin A is toxic to eukaryotic cells, CHO cells were propagated in 20 μg/ml of pseudolipasin A; they grew at the same rate as cells treated with the DMSO carrier over the course of 7 days. Additionally, no morphological changes were observed in the treated cells compared to cells grown in medium only (data not shown). These data suggest that pseudolipasin A is not toxic to mammalian cells.

Pseudolipasin A protects CHO cells from lysis mediated by ExoU delivered by various strains of P. aeruginosa. Protection of CHO cells is calculated as the inverse of the percentage of LDH release from infected cells. The following strains of P. aeruginosa were tested: PA14 (light blue), PAK ΔexoS att::exoU (red), PAK ΔmexAB ΔexoS att::exoU (yellow), PAK ΔexoS att::exoU pMMB67EH-exoA (green), PAK ΔmexAB ΔexoS att::exoU pMMB67EH-exoA (dark blue), and PA103 (orange). Strains containing pMMB67EH-exoA were induced with 1 mM IPTG.

FIG. 2. Pseudolipasin A protects CHO cells from lysis mediated by ExoU delivered by various strains of P. aeruginosa. Protection of CHO cells is calculated as the inverse of the percentage of LDH release from infected cells. The following strains of P. aeruginosa were tested: PA14 (light blue), PAK ΔexoS att::exoU (red), PAK ΔmexAB ΔexoS att::exoU (yellow), PAK ΔexoS att::exoU pMMB67EH-exoA (green), PAK ΔmexAB ΔexoS att::exoU pMMB67EH-exoA (dark blue), and PA103 (orange). Strains containing pMMB67EH-exoA were induced with 1 mM IPTG.

FIG. 1. Identification of pseudolipasin A. (A) A total of 50,000 compounds were screened in a 384-well plate format for compounds that protect CHO cells from cytotoxicity mediated by P. aeruginosa type III secretion pathway-delivered ExoU. Each screening plate included negative controls in which CHO cells were mock treated with DMSO only (1st and 2nd columns) and positive controls in which the antibiotic gentamicin was added to the wells prior to the addition of P. aeruginosa PAK-VL1 (23rd and 24th columns). Yellow wells indicate compounds that were able to protect CHO cells from P. aeruginosa infection, with a Z score for the plate of >3. (B) Compound structure of pseudolipasin A.
cells (Fig. 3C). These results suggest that pseudolipasin A acts at a step downstream from TTSS-mediated delivery of ExoU.

**Protection of Dictyostelium discoideum from *P. aeruginosa* expressing ExoU.** In order to assess whether pseudolipasin A interference with *P. aeruginosa*-mediated killing is specific for CHO cells, we examined other eukaryotic models that are sensitive to type III-mediated killing. The amoeba *D. discoideum* has previously been shown to be susceptible to killing by *P. aeruginosa* PA103 in a type III secretion-dependent manner, and the PLA₂ activity of ExoU was shown to be responsible for this toxic activity (25). In this assay, amoebae fed on a lawn of bacteria will form plaques unless the *D. discoideum* is killed. The presence of pseudolipasin A in the agar protected *D. discoideum* from *P. aeruginosa* PA103, allowing the formation of visible plaques (Fig. 4A). Pseudolipasin A protected *D. discoideum* with an IC₅₀ of 0.2 μM. Addition of MAFP, a substrate analog inhibitor for cytosolic PLA₂ that also inhibits ExoU PLA₂ activity (24, 34), did not protect *D. discoideum* from PA103 (Fig. 4B). Although MAFP did not permit the growth of *D. discoideum* on *E. coli*, MAFP did not affect growth in liquid medium (data not shown). These data suggest that a PLA₂ is required for the amoeba to utilize bacteria as a food source and that MAFP, rather than inhibiting an unknown essential target, has an activity that is highly specific against *D. discoideum* PLA₂. Thus, pseudolipasin A acts to protect the eukaryotic cell from ExoU-dependent killing in a system in which the known PLA₂ inhibitor MAFP cannot, suggesting that pseudolipasin A has a greater specificity for ExoU. These results also demonstrate that protection by pseudolipasin A occurs in a number of eukaryotic cells.

**Protection of Saccharomyces cerevisiae from intracytoplasmically expressed ExoU.** Since pseudolipasin A appears to act downstream of type III injection of ExoU, we asked whether the activity of this compound requires a functional TTSS. We used an *S. cerevisiae* expression system in which exoU transcription was controlled by the copper-inducible promoter-1 (cup1). Addition of Cu²⁺ to the culture medium leads to expression of exoU and subsequent cell death, thus providing a method for detecting protection against ExoU activity in the absence of bacterial delivery. The PLA₂ inhibitor MAFP was able to protect yeast induced with copper from ExoU-mediated killing at an IC₅₀ of 7 μM for liquid medium and 1.5 μM for solid medium (Fig. 5A and B). Thus, pseudolipasin A acts directly on ExoU within the eukaryotic cell, independently of the bacterial type III secretion mechanism.

**Inhibition of the PLA₂ activity of ExoU in vitro.** Inhibition of ExoU PLA₂ activity by pseudolipasin A was examined using two different enzymatic assays. The first assay is based on the cleavage of arachidonyl-TEPC, resulting in the generation of a fluorescent compound that emits at 465 nm and 535 nm. Addition of Cu²⁺ to the culture medium leads to expression of exoU and subsequent cell death, thus providing a method for detecting protection against ExoU activity in the absence of bacterial delivery. The PLA₂ inhibitor MAFP was able to protect yeast induced with copper from ExoU-mediated killing at an IC₅₀ of 7 μM for liquid medium and 1.5 μM for solid medium (Fig. 5A and B). Thus, pseudolipasin A acts directly on ExoU within the eukaryotic cell, independently of the bacterial type III secretion mechanism.
used to test inhibition of the PLA$_2$ activity of ExoU by pseudolipasin A and MAFP, pseudolipasin A had an IC$_{50}$ of 7 μM (Fig. 6A), whereas the PLA$_2$ inhibitor MAFP irreversibly inhibited ExoU with an IC$_{50}$ of 20 nM (Fig. 6A).

The second assay is based on detection of cleavage products of 14C-labeled phosphatidylcholine by thin-layer chromatography (34). ExoU activity was also assessed by measuring the release of 14C-labeled fatty acid from palmitoyl oleoyl phosphatidylcholine as described previously (34). MAFP inhibited this reaction by 75%, while BEL inhibited this reaction by 60% (Fig. 6B). Pseudolipasin A inhibited 80% of the reaction at concentrations similar to those of MAFP and BEL (Fig. 6B). These results indicate that pseudolipasin A inhibits the PLA$_2$ activity of ExoU in vitro.

Chemically related pseudolipasin A analogs and inhibitory activity. Compounds with structures similar to that of pseudolipasin A were identified (Table 1) and tested for their abilities to inhibit ExoU PLA$_2$ activity in vitro. Modification of the primary amine in pseudolipasin A resulted in inactive compounds. Furthermore, hydrophilic modification or addition of a fluorene ring also produced inactive compounds. The inability of structural analogs to act on the same process supports the finding that pseudolipasin A, identified in this screen, has a defined chemical specificity toward ExoU-mediated cytotoxicity. Interestingly, the primary screen and analysis of structur-


**DISCUSSION**

In this study, we describe a cell-based assay for the detection of inhibitors of mammalian cell killing by the cytotoxins delivered by bacterial type III secretion systems. Since the assay is based on cell viability in the presence of cytotoxin-producing bacteria, cytotoxic compounds were excluded. An additional attractive feature of this screening format was the ability to identify compounds that target individual components of a complex pathway, beginning with the synthesis of the effectors by the bacteria, their delivery, and their intracellular activity.

The screen yielded several active compounds, some of which were structurally related. We then used additional assays to define the specificity of the target of the compounds. A subset of the compounds protected CHO cells against the lethal action of *P. aeruginosa* expressing ExoU, but not against an isogenic strain expressing ExoS, an unrelated cytotoxin also delivered by the type III secretion system (Fig. 2). We therefore concluded that this group of molecules targeted ExoU following its entry into CHO cells by interfering with its activation or PLA2 enzymatic activity. The ability of these compounds to protect *S. cerevisiae* from killing by plasmid-encoded pseudolipasin A was tested against a panel of sPLA2 to determine its range of biological inhibition. The activity of sPLA2 was measured by the substrate diheptanoyl-TEPC (28). The cleavage of diheptanoyl-TEPC results in generation of free thiol at the sn-2 position, which can be measured by the reduction of DTNB. Pseudolipasin A did not inhibit bee venom PLA2 (group IV) or human group IID, IIE, V, X, or XII PLA2 at 81 μM concentrations (see Table S3 in the supplemental material).

**FIG. 6.** In vitro inhibition of ExoU PLA2 activity. (A) Inhibition of ExoU PLA2 activity in vitro by pseudolipasin A. Serial dilutions of pseudolipasin A were added to a reaction mixture containing six-His-tagged ExoU (inset), CHO cellular extract, arachidonic TEPC substrate, and Ellman’s reagent (DTNB). ExoU cleavage of arachidonyl-labeled ExoU (inset), CHO cellular extract, arachidonic TEPC substrate, and Ellman’s reagent (DTNB). ExoU cleavage of arachidonyl-labeled POPC-POPS mixed micelles. ExoU activity was determined by increased mobility of cleaved 14C-labeled oleic acid by thin-layer chromatography and reported as a percentage of ExoU PLA2 activity.

**FIG. 8.** Inhibition of PLA2 activity by molecular hydrophilicity. Given the conservation of some of the structural features of these compounds, it is conceivable that they inhibit ExoU by similar mechanisms.

**Pseudolipasin A is an ExoU-specific inhibitor of PLA2.** A number of eukaryotic PLA2 have been implicated in immune function by elicitng a proinflammatory response via the generation of secondary molecules such as leukotrienes (LTC4) and arachidonic acid (2, 9). In addition, sPLA2 have demonstrated antibacterial activity (8, 13, 16, 30). Pseudolipasin A was tested against a panel of sPLA2 to determine its range of biological inhibition. The activity of sPLA2 was measured by use of the substrate diheptanoyl-TEPC (28). The cleavage of diheptanoyl-TEPC results in generation of free thiol at the sn-2 position, which can be measured by the reduction of DTNB. Pseudolipasin A did not inhibit bee venom PLA2 (group IV) or human group IID, IIE, V, X, or XII PLA2 at 81 μM concentrations (see Table S3 in the supplemental material).

**cPLA2 share several conserved sequence features with ExoU, including the same residues in the catalytic dyad (34).** The activity of cPLA2 was measured indirectly in murine bone marrow-derived mast cells as the generation of the secondary molecule LTC4 from arachidonic acid released by cPLA2. The addition of pseudolipasin A up to 50 μM had no effect on LTC4 production, whereas the addition of 1 μM MAFP resulted in a decrease in LTC4 production (Fig. 7A). These results suggest that pseudolipasin A is a specific inhibitor for ExoU PLA2 activity.

A consequence of the proinflammatory response is the recruitment of neutrophils to the site of PLA2 activation, and this recruitment is an immediate host response to *P. aeruginosa* infections (20, 31). The compounds identified from our screen were tested for their abilities to provide protection of primary peripheral blood neutrophils from lysis mediated by ExoU-expressing *P. aeruginosa*. Peripheral blood neutrophils were infected with strain PAK-VL1 opsonized with serum at an MOI of 10. In the absence of compounds, the neutrophils were lysed rapidly (Fig. 7B). In the presence of the known inhibitor MAFP, 60% of the neutrophils were protected from lysis (Fig. 7B). Pseudolipasin A provided 40% protection at approximately twice the concentration of MAFP (Fig. 7B). These results indicate the possibility of these compounds and their derivatives may act as therapeutic agents for in vivo infection models.
<table>
<thead>
<tr>
<th>Source</th>
<th>Compound</th>
<th>Structure</th>
<th>% PLA2 activity&lt;sup&gt;a&lt;/sup&gt; at 200 μM</th>
<th>% Yeast growth&lt;sup&gt;b&lt;/sup&gt; at 20 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChemBridge</td>
<td>5633885</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>1.8</td>
<td>90.7</td>
</tr>
<tr>
<td>Maybridge</td>
<td>BTB10979SC</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>81.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Maybridge</td>
<td>BTB13111SC</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>61.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Maybridge</td>
<td>BTB14448SC</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>64.4</td>
<td>8.4</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>5635520</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>56.5</td>
<td>1.1</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>5305847</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>57.0</td>
<td>8.9</td>
</tr>
<tr>
<td>Maybridge</td>
<td>BTB13068SC</td>
<td><img src="image7.png" alt="Structure" /></td>
<td>50.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Sigma</td>
<td>S349925</td>
<td><img src="image8.png" alt="Structure" /></td>
<td>62.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Maybridge</td>
<td>CC28302CB</td>
<td><img src="image9.png" alt="Structure" /></td>
<td>103.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> PLA2 activity

<sup>b</sup> Yeast growth
ExoU confirmed this hypothesis (Fig. 5). Pseudolipasin A acts by inhibiting ExoU-catalyzed PLA₂ activity in vitro.

Depending on the assay system used, the potency of pseudolipasin A compared to that of known inhibitors of PLA₂ varied, and their activities in in vitro and in vivo assays did not always correlate well. A number of factors may contribute to these observed differences in pseudolipasin A activities. One of the rate-limiting steps in the activity of an inhibitor active in the cytoplasm is efficient uptake and accumulation of sufficient inhibitory levels. Therefore, any differences in the permeability of PLA₂ inhibitors in various cell types would be reflected in their relative potencies, which would be significantly different from those observed in in vitro enzymatic assays. Alternatively, different compounds could have different fates, depending on their interactions with various cellular constituents, leading to interference with their inhibitory activities. Finally, we cannot exclude the possibility that the target of pseudolipasin A is the unknown cellular activator of ExoU, while the known PLA₂ inhibitors very likely interfere directly with the enzymatic activity. Therefore, target specificity would also account for different efficiencies of PLA₂ inhibitors in various assays.

Identification of ExoU PLA₂ inhibitors provides leads to three areas of research: identification of novel functions for PLA₂ enzymes, dissection of the enzymatic activity of ExoU, and development of novel therapeutics. An interesting observation is that the reaction intermediate PLA₂ inhibitor MAFP prevented *D. discoideum* from forming plaques on bacteria but does not affect the growth of the amoebae in liquid medium (V. T. Lee, unpublished data). Since MAFP is a potent inhibitor of ExoU-mediated cytotoxicity, the inability of *D. discoideum* to form plaques indicates that MAFP inhibits a PLA₂, an arachidonate binding protein, or another off-target function required for the phagocytosis of bacteria. A survey of phospholipase genes in the *Saccharomyces* genome database (6) and the *D. discoideum* database (dictyBase) (7) revealed only one PLA₂ in yeast, which has been shown to be involved in meiotic but not in haploid growth, whereas in *D. discoideum* no PLA₂ could be identified. Data from this study confirm that PLA₂ activity is dispensable for *S. cerevisiae* growth (19). Thus, the MAFP-mediated inhibition of *D. discoideum* growth on a bacterial lawn may be due to other arachidonate-utilizing proteins that could be inactivated by MAFP. A search of the dictyBase for arachidonate revealed a putative arachidonate 12-lipoxygenase (*lipA*) that is transcriptionally induced in the early response to *Legionella* infection (10). Further characterization of *lipA* will reveal whether it is essential for phagocytic utilization of bacteria as a food source. Future experiments determining the inhibitory target of MAFP that prevents the growth of *D. discoideum* on bacteria could reveal a novel function of PLA₂ in the phagocytic process.

Pseudolipasin A could be a useful tool for probing the mechanism of the intracellular PLA₂ activity of ExoU. Studies of other PLA₂ suggest that there are two sequential steps for activity, binding to the aqueous-lipid interface to gain access to the substrate followed by enzymatic cleavage of lipids along the interface, which could also apply to ExoU (3). In the scenario of type III secretion-delivered ExoU, the incoming toxin may act on the lipids located around the injection needle, thus

<table>
<thead>
<tr>
<th>Source</th>
<th>Compound</th>
<th>Structure</th>
<th>% PLA₂ activity at 200 μM</th>
<th>% Yeast growth at 20 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChemBridge</td>
<td>513508</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>41.0</td>
<td>-0.7</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>5162800</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>66.2</td>
<td>1.6</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>5768352</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>36.9</td>
<td>0.6</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>6687996</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>52.7</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Calculated as a percentage relative to ExoU activity in the absence of inhibitors.

* Calculated as a percentage relative to yeast growth in the absence of Cu²⁺ induction of *exoU* expression.
bypassing the requirement for interfacial binding. However, ExoU expressed directly with cells also results in cytotoxicity, suggesting that a cytosolic protein or a protein in the inner leaflet of the plasma membrane can recruit ExoU. Alternatively, the host activator could be required for activating the PLA2 activity of ExoU. One possible mechanism to account for the inhibition of the PLA2 activity of ExoU and the lack of any discernible effect on eukaryotic PLA2 is that pseudolipasin A, in preventing either the recruitment or the activation of PLA2, interferes with the interaction of ExoU with this unknown host factor. A prediction from this proposed mechanism is that pseudolipasin A is a noncompetitive allosteric inhibitor that does not occupy the active site. Testing of this hypothesis awaits the identification of the host activator. An in vitro assay with purified components will allow elucidation of the mechanism of PLA2 inhibition by pseudolipasin A and other ExoU inhibitors identified in this screen. In addition, the inhibitors should distinguish the function of the host factor in interfacial binding or activation of PLA2 activity. Thus, pseudolipasin A represents a novel class of inhibitor that is specific for the phospholipase A2 activity of a bacterial virulence factor.

In analogy with recent work targeting virulence mechanisms in pathogenic bacteria (14), pseudolipasin A represents another member of the emerging group of anti-infectives that do not kill or inhibit the growth of the microorganisms but instead attenuate their virulence. P. aeruginosa has been shown to require ExoU in a number of infection models, and expression of ExoU correlates with severe outcomes of certain human infections. Pseudolipasin A may be a lead compound for a therapeutic agent, due to its specificity for the PLA2 activity of the ExoU toxin and its lack of identified effects on a variety of eukaryotic cells. Increasing the potency of analogous compounds could provide suitable candidates for testing in animal models.

ACKNOWLEDGMENTS

V.T.L. was supported by a NIAID postdoctoral research fellowship. S.L., A.A.K., and J.H. were supported by NIH grant R21-HL079393. J.P.A. and E.K. were supported by NIH grant R01-HL070946. D.W.F. was supported by NIH grant R01-AI49577.

We thank Stephen Juris and Roger S. Smith for critical reading of the manuscript.

REFERENCES


Lee et al. INFECT. IMMUN.


Editor: V. J. DiRita