

Discovery and Characterization of Inhibitors of *Pseudomonas aeruginosa* Type III Secretion^{∇†}

Daniel Aiello,¹ John D. Williams,¹ Helena Majgier-Baranowska,¹ Ishan Patel,¹ Norton P. Peet,¹ Jin Huang,² Stephen Lory,² Terry L. Bowlin,¹ and Donald T. Moir^{1*}

Microbiotix, Inc., Worcester, Massachusetts 01605,¹ and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115²

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The type III secretion system (T3SS) is a clinically important virulence mechanism in *Pseudomonas aeruginosa* that secretes and translocates up to four protein toxin effectors into human cells, facilitating the establishment and dissemination of infections. To discover inhibitors of this important virulence mechanism, we developed two cellular reporter assays and applied them to a library of 80,000 compounds. The primary screen was based on the dependence of the transcription of T3SS operons on the T3SS-mediated secretion of a negative regulator and consisted of a transcriptional fusion of the *Photobacterium luminescens luxCDABE* operon to the *P. aeruginosa* *exoT* effector gene. Secondary assays included direct measurements of the T3SS-mediated secretion of a *P. aeruginosa* ExoS effector- β -lactamase fusion protein as well as the detection of the secretion of native ExoS by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of culture supernatants. Five inhibitors in three chemical classes were demonstrated to inhibit type III secretion selectively with minimal cytotoxicity and with no effects on bacterial growth or on the type II-mediated secretion of elastase. These inhibitors also block the T3SS-mediated secretion of a YopE effector- β -lactamase fusion protein from an attenuated *Yersinia pestis* strain. The most promising of the inhibitors is a phenoxyacetamide that also blocks the T3SS-mediated translocation of effectors into mammalian cells in culture. Preliminary studies of structure-activity relationships in this phenoxyacetamide series demonstrated a strict requirement for the *R*-enantiomer at its stereocenter and indicated tolerance for a variety of substituents on one of its two aromatic rings.

The type-three secretion system (T3SS) is a complex multi-protein apparatus that facilitates the secretion and translocation of effector proteins from the bacterial cytoplasm directly into the mammalian cytosol. This complex protein delivery device is shared by more than 15 species of gram-negative human pathogens, including *Salmonella* spp., *Shigella flexneri*, *Pseudomonas aeruginosa*, *Yersinia* spp., enteropathogenic and enteroinvasive *Escherichia coli*, and *Chlamydia* spp. (23, 25, 43). In the opportunistic pathogen *P. aeruginosa*, the T3SS is the major virulence factor contributing to the establishment and dissemination of acute infections (19). Four T3SS effectors have been identified in *P. aeruginosa* strains: ExoS, ExoT, ExoY, and ExoU. ExoS and ExoT are bifunctional proteins consisting of an N-terminal small G-protein-activating protein (GAP) domain and a C-terminal ADP ribosylation domain, ExoY is an adenylate cyclase, and ExoU is a phospholipase (reviewed in reference 11). In studies with strains producing each effector separately, ExoU and ExoS contributed significantly to persistence, dissemination, and mortality, while ExoT produced minor effects on virulence in a mouse lung infection model, and ExoY did not appear to play a major role in the pathogenesis of *P. aeruginosa* (51). While not a prototypical effector toxin, flagellin (FliC) also may be injected into the

cytoplasm of host cells from *P. aeruginosa* via the T3SS machinery, where it triggers the activation of the innate immune system through the nod-like receptor NLRC4 inflammasome (13, 33).

The presence of a functional T3SS is significantly associated with poor clinical outcomes and death in patients with lower-respiratory and systemic infections caused by *P. aeruginosa* (48). In addition, T3SS reduces survival in *P. aeruginosa* animal infection models (49) and is required for the systemic dissemination of *P. aeruginosa* in a murine acute pneumonia infection model (56). T3SS appears to contribute to the development of severe pneumonia by inhibiting the ability of the host to contain and clear the bacterial infection of the lung. The secretion of T3SS toxins, particularly ExoU, blocks phagocyte-mediated clearance at the site of infection and facilitates the establishment of an infection (9). The result is a local disruption of an essential component of the innate immune response, which creates an environment of immunosuppression in the lung. This not only allows *P. aeruginosa* to persist in the lung but also facilitates superinfection with other species of bacteria.

While several antibacterial agents are effective against *P. aeruginosa*, the high rates of mortality and relapse associated with serious *P. aeruginosa* infections even in patients with hospital-acquired pneumonia (HAP) receiving antibiotics active against the causative strain reflect the increasing incidence of drug-resistant strains and highlight the need for new therapeutic agents (10, 46, 52). Conventional bacteriostatic and bactericidal antibiotics appear insufficient to adequately combat these infections, and new treatment approaches such as inhib-

* Corresponding author. Mailing address: Microbiotix, Inc., 1 Innovation Dr., Worcester, MA 01605. Phone: (508) 757-2800, ext. 102. Fax: (508) 757-1999. E-mail: dmoir@microbiotix.com.

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TABLE 1. Strains and plasmids

Strain	Genotype or features	Reference or source
<i>P. aeruginosa</i>		
MDM852	PA01::pGSV3-' <i>exoT</i> '-luxCDABE	This study
MDM1355	PA01 Δ pscC::pGSV3-' <i>exoT</i> '-luxCDABE	This study
MDM973	PAK/pUCP24GW-lacI ^Q -lacPO- <i>exoS</i> ::blaM	This study
MDM974	PAK Δ pscC/pUCP24GW-lacI ^Q -lacPO- <i>exoS</i> ::blaM	This study
MDM1156	PAO-LAC/pUCP24GW-lacPO-luxCDABE	This study
PAK Δ C	PAK Δ pscC; T3SS defective	28
PAK Δ S	PAK Δ exoS; secretes ExoT as its only cytotoxic T3SS effector	28
PAK Δ ST _{exoU}	PAK Δ exoS::miniCTX- <i>exoU</i> - <i>spcU</i> ; secretes ExoU as its only cytotoxic T3SS effector	28
PAK Δ TY	PAK Δ exoT Δ exoY; secretes ExoS as its only T3SS effector	28
MDM1387	PA14 <i>xcpQ</i> ::MrT7; PAMr_nr_mas_02_2:H7; defective in type II secretion	29
<i>Y. pestis</i>		
JG153/pMM85	KIM Δ pgm pPCP1 ⁻ pCD1 ⁺ /pHSG576 <i>yopE</i> ::blaM	31, 44

itors of *P. aeruginosa* virulence determinants may prove useful as adjunctive therapies (58).

The potential for T3SS as a therapeutic target has prompted several groups to screen for inhibitors of T3SS in various bacterial species, including *Salmonella enterica* serovar Typhimurium, *Yersinia pestis*, *Y. pseudotuberculosis*, and *E. coli* (reviewed in references 5 and 25). However, only a single screen for inhibitors of *P. aeruginosa* T3SS inhibitors has been reported, and it yielded specific inhibitors of one of the T3SS effectors, ExoU (27), rather than inhibitors of the T3SS machinery. High levels of sequence conservation among various proteins comprising the T3SS apparatus suggest that inhibitors of T3SS in one species also are active in related species. The broad-spectrum activity of T3SS inhibitors identified in a screen against *Yersinia* has been demonstrated in *Salmonella*, *Shigella*, and *Chlamydia* (22, 57, 59). However, the need for new, potent anti-pseudomonal agents argues for additional direct screening for *P. aeruginosa* T3SS inhibitors. To address this unmet need, we developed and applied a cell-based bioluminescent reporter assay for the identification of inhibitors of the *P. aeruginosa* T3SS and qualified the hits through a series of secondary assays. In this report, we describe the features of the most potent and selective inhibitors from the screen, including a new phenoxyacetamide inhibitor that blocks T3SS-mediated secretion and the translocation of toxin effectors from *P. aeruginosa* and exhibits minimal cytotoxicity. This inhibitor also is active against *Yersinia* and *Chlamydia* T3SS.

Preliminary structure-activity relationships (SARs) indicate that the stereocenter is crucial for activity and suggest regions of the molecule that could be altered to optimize potency.

MATERIALS AND METHODS

Strains, plasmids, and growth media. Bacterial strains and plasmids used for assays are described in Table 1. All *P. aeruginosa* strains were derivatives of PAO1 (21), PAK (1), or PA14 (45). *E. coli* TOP10 (Invitrogen), *E. coli* DB3.1 (Gateway host, Invitrogen), *E. coli* SM10 (7), and *E. coli* S17-1 (ATCC 47055) were used as hosts for molecular cloning. Luria-Bertani (LB) medium (liquid and agar) was purchased from Difco. LB was supplemented with 30 μ g/ml gentamicin (LBG) with or without 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and 5 mM EGTA (LBGI and LBGIE, respectively).

PCR and primers. Synthetic oligonucleotide primers (from Operon, Inc.) were designed using the published genome sequence for *P. aeruginosa* (53) and web-based PRIMER3 (Whitehead Institute) (Table 2). Primers were used at 10 μ M in PCR amplifications with Failsafe polymerase (Epicentre), buffer G (Epicentre), and 4% dimethylsulfoxide (DMSO) for *P. aeruginosa* chromosomal DNA templates.

Screening compounds. Compounds screened in this study were purchased from Chembridge (San Diego, CA) and Timtec (Newark, DE), diluted in 96-well master plates at 2.5 mM in DMSO, and stored at -20°C.

Luciferase transcriptional reporter screen. A transcriptional fusion of the *Photobacterium luminescens* lux operon (*luxCDABE*) to effector gene *exoT* (PA0044) was constructed by inserting an internal fragment of the *exoT* gene (712 bp generated by PCR with primers *exoT*-F+EcoRI and *exoT*-R+EcoRI) (Table 2) into EcoRI-cut reporter plasmid pGSV3-lux-Gm (37) as described previously (35). The resulting plasmid was introduced into *E. coli* SM10 cells and transferred into *P. aeruginosa* PAO1 and PA01 Δ pscC cells by conjugation (35) to generate recombinant reporter strains MDM852 and MDM1355, respectively. Insertion at the *exoT* chromosomal locus was confirmed by PCR with a primer

TABLE 2. Primers used

No.	Name	Sequence
1	<i>exoT</i> -F+EcoRI	TACTACGAATTCCCAGGAAGCACCGAAGG
2	<i>exoT</i> -R+EcoRI	CATTACGAATTCCTGGTACTCGCCGTTGGTAT
3	<i>exoT</i> -out-F	TAGGGAAAAGTCCGCTGTTTT
4	luxC-R	CCTGAGGTAGCCATTCATCC
5	<i>exoS</i> -F+GWL	TACAAAAAAGCAGGCTAGGAAACAGACATGCATATTCAATCGCTTCAG
6	<i>exoS</i> (234)-R	ATCTTTTACTTTTACCAGCGTTTCTGGGTGACCGTCGGCCGATACTCTGCT
7	BLA-F	CACCCAGAAAACGCTGGTGAA
8	BLA-R+GWR	TACAAGAAAAGCTGGGTTTGGTCTGACAGTTACCAATGC
9	GW-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
10	GW-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT
11	lux-F+GWL	TACAAAAAAGCAGGCTAGGAAACAGCTATGACGAAGAAGATCAGTTTAA TAATTAACGGCCAGGTTGAAATC
12	lux-R+GWR	TACAAGAAAAGCTGGGTGTTTTCCAGTCACGACGTT

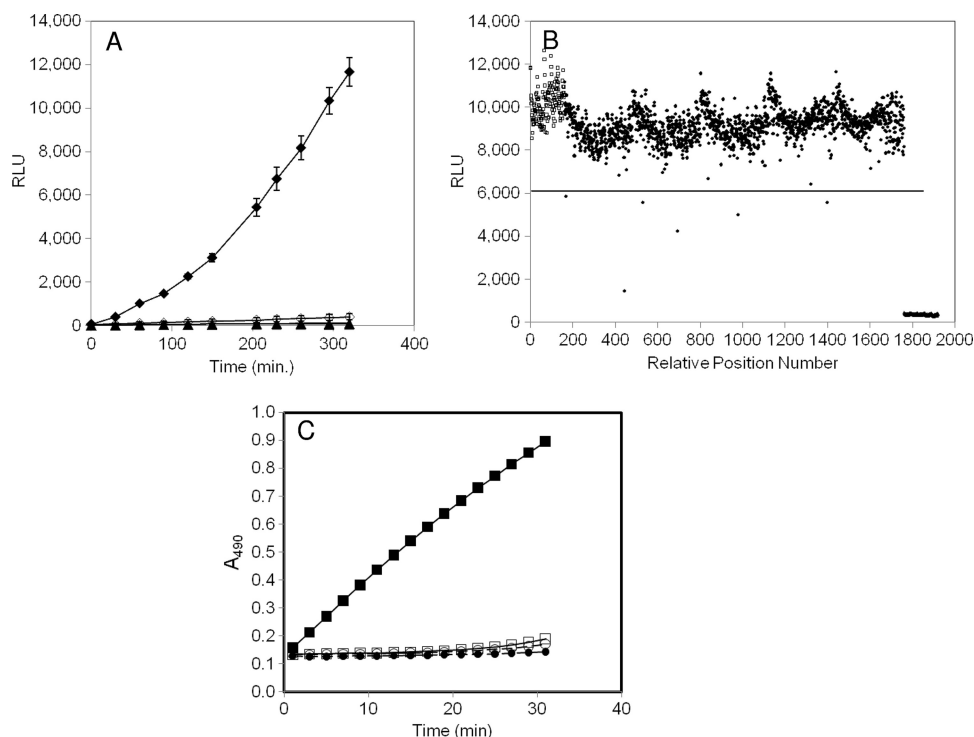


FIG. 1. Characterization of bioluminescent and chromogenic reporter strains for identification of T3SS inhibitors. (A) Luminescence (in relative light units, RLU) from a chromosomal transcriptional fusion of *exoT* to the *P. luminescens luxCDABE* operon in wild-type (strain MDM852) or $\Delta pscC$ (strain MDM1355) *P. aeruginosa* PAO1 cells. Overnight cultures were diluted at time zero to $A_{600} \sim 0.025$ and induced with 5 mM EGTA or left uninduced. RLU values were measured in 96-well opaque microplates throughout a 320-min time course. \blacklozenge , MDM852 with EGTA; \diamond , MDM852 without EGTA; \blacktriangle , MDM1355 with EGTA; \triangle , MDM1355 without EGTA. (B) Luminescence (RLU) from five 384-well microplates containing reporter strain MDM852 in a high-throughput screen for T3SS inhibitors. RLU values are shown at 300 min for 160 negative controls (\square ; fully induced by EGTA) in positions 1 to 160, for 160 positive controls (\blacktriangle ; no induction by EGTA) in positions 1,761 to 1,920, and for 1,600 samples (\bullet) in positions 161 to 1,760. Six samples were designated hits because their RLU values displayed Z scores of >4 (i.e., >4 standard deviations below the average sample value, denoted as a horizontal line at 6,084 RLU). Compound 1 at position 443 was the most potent hit (Z score = 10) (Table 3). (C) Detection of ExoS'- β LA secretion from *P. aeruginosa* strains MDM973 (PAK) and MDM974 (PAK $\Delta pscC$) carrying pUCP24GW-*lacI*^Q-*lacPO*-*exoS'*-*blaM*, as measured by the hydrolysis of nitrocefin. A_{490} values are plotted versus time for MDM973 in the presence (\blacksquare) and absence (\square) of 5 mM EGTA and for strain MDM974 in the presence (\bullet) and absence (\circ) of 5 mM EGTA.

outside the cloned locus (*exoT*-out-F) and a primer within the *luxC* gene (*luxC*-R) (Table 2).

For inhibitor screening, compound master plates were thawed at room temperature on the day of the screen, and 1 μ l of compound (final content, 45 μ M compound and 1.8% DMSO) was added to the 384-well opaque black screening plates using a Sciclone ALH 3000 liquid-handling robot (Caliper, Inc.) and a Twister II Microplate Handler (Caliper, Inc.). Reporter strain MDM852 was grown at 37°C in LBG1 to an optical density at 600 nm (OD_{600}) of ~ 0.025 to 0.05 and transferred into microplates (50 μ l/well) containing test compounds and EGTA (5 μ l of 0.1 M stock solution), which were covered with a translucent gas-permeable seal (catalog no. AB-0718; Abgene, Inc.). Control wells contained cells with fully induced T3SS (EGTA and DMSO, microplate columns 1 and 2) and uninduced T3SS (DMSO only, microplate columns 23 and 24). Plates were incubated at room temperature for 300 min. Luminescence was measured in an Envision Multilabel microplate reader (PerkinElmer) (Fig. 1A and B). The screening window coefficient, Z' factor (60), defined as the ratio of the positive- and negative-control separation band to the signal dynamic range of the assay, averaged 0.7 for the screen. All screening data, including the Z score, and confirmation and validation data were stored in one central database (CambridgeSoft's ChemOffice 11.0). Validated hits were reordered from the vendor and confirmed to be $>95\%$ pure and to be of the expected mass by liquid chromatography-mass spectrometry (LC-MS) analysis. Compounds for SAR analysis were ordered from Chembridge, Inc.

Effector- β -lactamase secretion assays. (i) *P. aeruginosa*. A gene encoding an ExoS'- β -lactamase (β LA) fusion protein (comprised of 234 codons of *P. aeruginosa* effector ExoS fused to the TEM1 β -lactamase gene lacking secretion signal codons) was constructed by splicing by overlap extension PCR (SOE-PCR) (4)

using primers 5 to 10 (Table 2), sequence confirmed, cloned into *lacI*^Q-containing Gateway vector pUCP24GW (36) behind the *lac* promoter, and introduced into *P. aeruginosa* by electroporation (3). The secretion of fusion proteins was detected by measuring the hydrolysis of the chromogenic β -lactamase substrate nitrocefin in clear 96-well microplates in a modification of a previously described assay (27). Cells of strain MDM973 (PAK/pUCP24GW-*exoS':blaM*) were subcultured in the morning from overnight growths in LBG into 0.1 ml of LBGIE with or without test compounds and grown for 150 min. Nitrocefin (100 μ g/ml final) was added, and A_{490} measurements were taken every minute for 15 min in a Victor³V 1420 Multilabel HTS Counter (PerkinElmer). Slopes were calculated as a relative measure of the quantity of the effector- β LA fusion protein secreted and were absolutely dependent on induction with IPTG and EGTA and the presence of a functional *pscC* gene in the *P. aeruginosa* cells (Fig. 1C). Typical signal:background ratios were 6-10.

(ii) *Yersinia pestis*. Attenuated *Y. pestis* strain JG 153 (gift of Jon Goguen, University of Massachusetts Medical School, Worcester, MA) carrying plasmid pMM85 (*yopE::blaM*) was grown in LB plus 20 μ g/ml chloramphenicol at 30°C to prevent T3SS induction and the loss of the pCD1 plasmid encoding T3SS. To induce T3SS, cells were shifted from 30 to 37°C, and EGTA was added to a 1 mM final concentration. Cell culture (0.1 ml) was added to clear 96-well microplates containing test compound and incubated for 3 h at 37°C. Nitrocefin was added (100 μ g/ml final), and A_{490} measurements were taken every minute for 10 min in an Envision Multilabel microplate reader (PerkinElmer). Slopes were plotted versus the inhibitor concentration to determine the 50% inhibitory concentrations (IC_{50} s).

Counterscreen for inhibition of bioluminescence of *lac*-promoted *luxCDABE*. The complete *Photobacterium luminescens luxCDABE* locus was amplified from pGSV3-*lux* (37) by PCR with Phusion polymerase (NEB, Beverly, MA) and

primers lux-F+GWL and lux-R+GWR, followed by a second PCR with primers GW-attB1 and GW-attB2 to provide the full Gateway recognition sequence (Table 2). The ~5.8-kb product was gel purified and inserted into pDONR221 with BPClonase (Invitrogen) and then into pUCP24GW (36) with LRClonase (Invitrogen). The resulting pUCP24GW-*lacPO-luxCDABE* plasmid was introduced into the *P. aeruginosa* PAO-LAC strain carrying one chromosomal copy of the *lac* repressor, *lacI*^Q, at the *phiCTX* locus (20) by electroporation, selecting for gentamicin resistance (3). To measure the effects of T3SS inhibitors on *lac*-promoted luciferase production, the resulting strain MDM1156 was subcultured from overnight LBG growths into LBG1 at an A_{600} of ~0.05 and grown for 3 h in the presence or absence of inhibitors at 50 μ M. The percent inhibition by compounds of the luminescence (in relative light units [RLU]) produced by *lac*-promoted versus *exoT*-promoted luciferase was calculated and used as an indication of the T3SS selectivity of the screening hits.

Detection of inhibition of T3SS-mediated ExoS secretion into culture broths. *P. aeruginosa* strain PAK Δ TY, which produces the ExoS, but not the ExoT or ExoY, T3SS effectors, was grown overnight in LB and treated essentially as described previously (28). Bacteria were subcultured 1:1,000 in LB supplemented with 5 mM EGTA and grown for 3 h at 37°C with aeration in the presence or absence of inhibitors at the indicated concentrations. Bacteria were sedimented by centrifugation at $3,220 \times g$ for 15 min at 4°C. Culture supernatant was collected, and proteins were concentrated by precipitation with 12.5% trichloroacetic acid followed by a wash with acetone or by ultrafiltration. Proteins were resuspended according to original culture density (A_{600}), separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE), and stained with Coomassie blue. Stained gel image files were processed with ImageJ software (version 1.42q; NIH) by subtracting the background, inverting the image, and integrating the density of each band.

Inhibition of *P. aeruginosa* ExoU-dependent CHO cell killing. The rescue of CHO cells from the T3SS-mediated cytotoxicity of translocated effector protein ExoU was measured using a lactate dehydrogenase (LDH) release assay as previously reported (28), except that infection with *P. aeruginosa* was carried out for 2 h in the absence of gentamicin. Percent cytotoxicity (percent LDH release) was calculated relative to that of the uninfected control, which was set at 0% LDH release, and that of cells infected with *P. aeruginosa* unprotected by test compound (100% LDH release). LDH released from unprotected, infected cells reached at least 80% of the value obtained from complete lysis with 1% Triton X-100 in the 2-h timeframe of this experiment. Pseudolipasin, which acts by the direct inhibition of the ExoU phospholipase, was used as the control inhibitor (27).

Gentamicin protection assays of bacterial internalization. Experiments were carried out using a modification of a previously published method (18). A total of 2×10^5 HeLa cells were seeded into each well of a 12-well plate containing 2 ml per well of minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and incubated at 37°C in 5% CO₂ for 24 h. After two washes with PBS, 1 ml of MEM containing 1% FCS was added to the HeLa cells. MBX 1641 was added to half the wells at a 50 μ M final concentration (DMSO at 0.2% final concentration). *P. aeruginosa* strains PAK Δ C (negative control) and PAK Δ S (positive control) were grown overnight in LB medium at 37°C with shaking, diluted 1:1,000 in the morning, and grown to an OD₆₀₀ of 0.3 (~10⁸ cells/ml). Bacteria were washed in phosphate-buffered saline (PBS), resuspended in 1 ml of MEM, and added to the HeLa cells at a multiplicity of infection (MOI) of 10 in the presence or absence of MBX 1641. Infected HeLa cells were incubated at 37°C in 5% CO₂ for 2 h. After two washes with PBS, 1 ml of MEM containing 50 μ g/ml gentamicin was added, and cells were incubated for an additional 2 h. After three washes with PBS, the cells were lysed in PBS containing 0.25% Triton X-100, and dilutions were plated on LB agar plates to count the number of bacteria internalized within HeLa cells.

Elastase secretion assay. The effect of test compounds on the type II-mediated secretion of elastase from *P. aeruginosa* was determined by a modification of a previously described method (42). *P. aeruginosa* PA14 cells were cultured from a starting density of $A_{600} \sim 0.05$ for 16 h to saturation in LB in the presence or absence of test compound at 50 μ M. Cells were removed by centrifugation in a microcentrifuge, and 0.2 ml of cleared supernatant was added to 0.4 ml of a suspension of elastin-Congo Red (5 mg/ml; Sigma) in buffer consisting of 0.1 M Tris-HCl, pH 7.4, and 1 mM CaCl₂ in capped microcentrifuge tubes. Tubes were incubated at 37°C with shaking for 6 h. A volume of 0.4 ml of buffer consisting of 0.7 M sodium phosphate (pH 6.0) was added, tubes were centrifuged in a microcentrifuge to remove undigested elastin-Congo Red, and the A_{495} of the cleared supernatants was measured. Readings were normalized to the original cell density (OD₆₀₀), and the percent inhibition of elastase secretion was determined relative to that of untreated PA14 (no-inhibition control) and untreated

type II secretion-defective PA14 *xcpQ::MrT7* (29) (strain MDM1387) (Table 1) (complete inhibition control).

***Chlamydia trachomatis* growth inhibition assay.** The inhibition of the growth of *Chlamydia trachomatis* strain L2 by compounds was measured in 24-well plates essentially according to the method of Wolf et al. (59). Confluent monolayer Hep-2 cells were infected with L2 at an MOI of 0.5 and treated with compounds at the indicated concentrations for 48 h. The cultures then were collected and sonicated. Entire lysates were used for counting inclusion-forming units (IFUs) as a measurement of the production of chlamydia progeny elementary bodies (EBs) by replating onto fresh HeLa monolayers. An uninhibited control (DMSO only) and a complete inhibition control (200 μ g/ml chloramphenicol) were included. Experiments were done in triplicate.

MIC. MIC determination was done by the broth microdilution method described in the CLSI (formerly NCCLS) guidelines (39) and expressed in μ M to facilitate comparisons with IC₅₀ and cytotoxic concentration (CC₅₀) values.

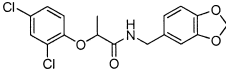
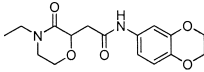
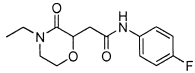
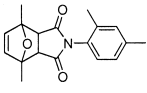
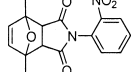
Determination of mammalian cytotoxicity. The CC₅₀ of a compound for cultured mammalian cells (HeLa; ATCC CCL-2; American Type Culture Collection, Manassas, VA) was determined as the concentration of compound that inhibits 50% of the conversion of MTS to formazan (32). Briefly, 96-well plates were seeded with HeLa cells at a density of 4×10^3 per well in VP-SFM medium without serum (14) in the presence or absence of serial dilutions of a compound dissolved in DMSO. Following incubation for 3 days at 37°C in VP-SFM, cell viability was measured with the vital tetrazolium salt stain 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide according to the manufacturer's instructions (Promega, Madison, WI). Values were determined in duplicate using dilutions of inhibitory compound from 100 to 0.2 μ M.

Chemistry. Phenoxyacetamides MBX 1685, MBX 1684, and MBX 1686, related to screening hit MBX 1641, all were prepared from 2,4-dichlorophenol. The alkylation of 2,4-dichlorophenol with ethyl 2-bromo-2-methylpropanoate (K₂CO₃, CH₃CN) provided ethyl 2-(2,4-dichlorophenoxy)-2-methylpropanoate, which was hydrolyzed (KOH, ethyl alcohol) and coupled (HOAT, EDCl, DMF, DIPEA) (2) with 3,4-methylenedioxybenzylamine to provide MBX 1685. The Mitsunobu coupling (34) of 2,4-dichlorophenol with (*S*)-ethyl 2-hydroxypropanoate (PPh₃, DIAD, THF) provided ethyl (*R*)-2-(2,4-dichlorophenoxy)propanoate, which was hydrolyzed (LiOH · H₂O, CH₃CN, H₂O) and then coupled as described above with 3,4-methylenedioxybenzylamine to give MBX 1684. The corresponding *S*-enantiomer (MBX 1686) was prepared in precisely the same fashion but using methyl (*R*)-2-hydroxypropanoate with 2,4-dichlorophenol in the Mitsunobu coupling protocol. Hit compound MBX 1641 and desmethyl compound MBX 1668 were prepared directly from commercially available 2-(2,4-dichlorophenoxy)propanoic acid and 2,4-dichlorophenoxyacetic acid, respectively, by being coupled with 3,4-methylenedioxybenzylamine as described above.

RESULTS

Identification of inhibitors of *P. aeruginosa* T3SS. A *P. aeruginosa* cell-based bioluminescent reporter screen for the identification of T3SS inhibitors was constructed in a fashion analogous to that described previously for *Yersinia* (24). Due to the tight coupling of T3SS gene regulation in *P. aeruginosa* with the type III secretion of the negative regulator ExsE, a reduced type III secretion capability results in the decreased expression of all T3SS operons (47, 54). We constructed *P. aeruginosa* strains carrying a transcriptional fusion of the T3SS effector gene *exoT* to the *luxCDABE* operon of *Photobacterium luminescens* and evaluated their luminescence production under T3SS-inducing and -repressing conditions. When Ca²⁺ levels remain high (no EGTA addition [55]) or a key component of the T3SS assembly is deleted (the *pscC* gene encoding the secretin component of T3SS [27]), T3SS is not functional and luminescence is significantly reduced compared to that of wild-type cells grown in low levels of free Ca²⁺ (addition of 5 mM EGTA) (Fig. 1A). The application of the wild-type transcriptional fusion strain was optimized for screening in 384-well microplates, and about 80,000 discrete chemical compounds were screened at 50 μ M to identify inhibitors of T3SS. Screening results are shown graphically for five representative 384-

TABLE 3. Summary of selective inhibitors of type III secretion

Compound no.	Series	Structure	RLU selectivity ^a	Serum effect ^b	ExoS-βLA IC ₅₀ ^c	HeLa CC ₅₀ ^d	CC ₅₀ /IC ₅₀ ^e	YopE-βLA IC ₅₀ ^f
1	A		7	3.7	12.5	102	8.1	22
2	B		4.1	4.8	20	>100	>5.0	16
3	B		3.9	4.2	13	100	6.2	6
4	C		9.2	3.1	15	>100	>6.7	103
5	C		2.4	4.7	21	>100	>4.8	51

^a Percent inhibition of *exoT-lux* RLU/percent inhibition of *lac-lux* RLU, both at 50 μM compound.

^b Percent inhibition of *exoT-lux* RLU in the absence of serum/percent inhibition of *exoT-lux* RLU in the presence of 10% fetal calf serum, both at 50 μM compound.

^c Compound concentration at which secretion of ExoS-βLA fusion protein from *P. aeruginosa* strain MDM973 is reduced by 50%. All IC₅₀ and CC₅₀ values are presented in μM.

^d Compound concentration at which the viability of HeLa cells cultured in serum-free medium is reduced by 50%.

^e Selectivity of T3SS inhibition as measured by the ratio of potency of the compound in the HeLa cell viability assay vs the T3SS inhibition assay.

^f Compound concentration at which secretion of YopE-βLA fusion protein from *Y. pestis* strain JG153/pMM85 is reduced by 50%.

well assay plates in Fig. 1B). The substantial signal-to-background ratio (>20) and the very modest coefficients of variation (standard deviation/average signal) for samples as well as positive and negative controls (all <10%) are representative of those observed in the entire screen. A total of 331 compounds (0.4% of the library) were detected as primary hits due to the inhibition of RLU values by at least 4 standard deviations below the sample average (Z score ≥ 4 ; solid line in Fig. 1B), and more than 60% of them (208 compounds) were confirmed as inhibitors when retested in the same assay in triplicate. However, more than 80% of these putative inhibitors were eliminated by requiring that they inhibit luminescence from the *exoT-lux* screening strain >2-fold more potently than that from a non-T3SS-regulated *lux* strain (*lac*-regulated *luxCDABE* in strain MDM1156). The absence of T3SS specificity observed for most screening hits likely is the result of the many non-T3SS-related mechanisms capable of reducing luminescence (e.g., inhibition of growth, energy metabolism, transcription, or translation).

Validation of inhibitors of *P. aeruginosa* T3SS-mediated secretion. The remaining T3SS-selective hits were evaluated directly for the inhibition of T3SS-mediated secretion. Measurements were carried out using a cellular assay consisting of an effector-reporter fusion protein. Codons for the type III secretion signals (8) and the GAP domain of *P. aeruginosa* ExoS (17) were fused to the TEM1 β-lactamase gene lacking its secretion signal. The construct was cloned into the exogenously replicating plasmid pUCP24GW, resulting in the production of ExoS'-βLA fusion protein under *lac* regulation in *P. aeruginosa* cells. In this assay, secreted β-lactamase activity is detected by the hydrolysis of the β-lactamase chromogenic substrate nitrocefin, resulting in an increased A_{490} . Signal generation is dependent on the presence of EGTA and IPTG and is eliminated

in T3SS-defective $\Delta pscC$ mutant cells (Fig. 1C). Almost all (41 of 43) of the T3SS-selective inhibitors identified in the transcriptional fusion reporter assays also inhibited the secretion of the effector-reporter fusion protein by at least 50% when added at a concentration of 50 μM during the induction of T3SS and the effector fusion. No inhibition was observed when compounds were added after induction at the time of chromogenic substrate addition, indicating that the compounds inhibit the appearance of extracellular β-lactamase rather than β-lactamase catalysis itself.

Finally, the inhibitors were evaluated for the potency of ExoS'-βLA fusion protein secretion inhibition (IC₅₀) and counterscreened for cytotoxicity (CC₅₀), yielding five validated T3SS inhibitors with IC₅₀s of ≤ 25 μM and CC₅₀s of ≥ 100 μM (Table 3). These five inhibitors exhibited no detectable MIC (MIC > 100 μM) against *P. aeruginosa* and did not inhibit the growth rate of *P. aeruginosa* cells (data not shown), confirming that they are not reducing luminescence or β-lactamase secretion by inhibiting bacterial cell growth or viability. These five validated T3SS inhibitors can be categorized into three structural classes, indicated in Table 3 as series A (phenoxyacetamides), B (malic diamides), and C (*N*-phenyl maleimide adducts).

Inhibition of T3SS-mediated secretion of native effectors. To confirm that inhibitors identified by the cell-based reporter assays inhibit the T3SS-mediated secretion of natural effectors, we concentrated conditioned culture medium from a *P. aeruginosa* ExoS-secreting strain exposed to each of the five T3SS inhibitors at 50 μM during growth for 3 h under T3SS-inducing conditions and visualized the secreted effectors on SDS-PAGE (Fig. 2A). All five compounds inhibited the secretion of ExoS from *P. aeruginosa* cells by at least 75%. The concentration

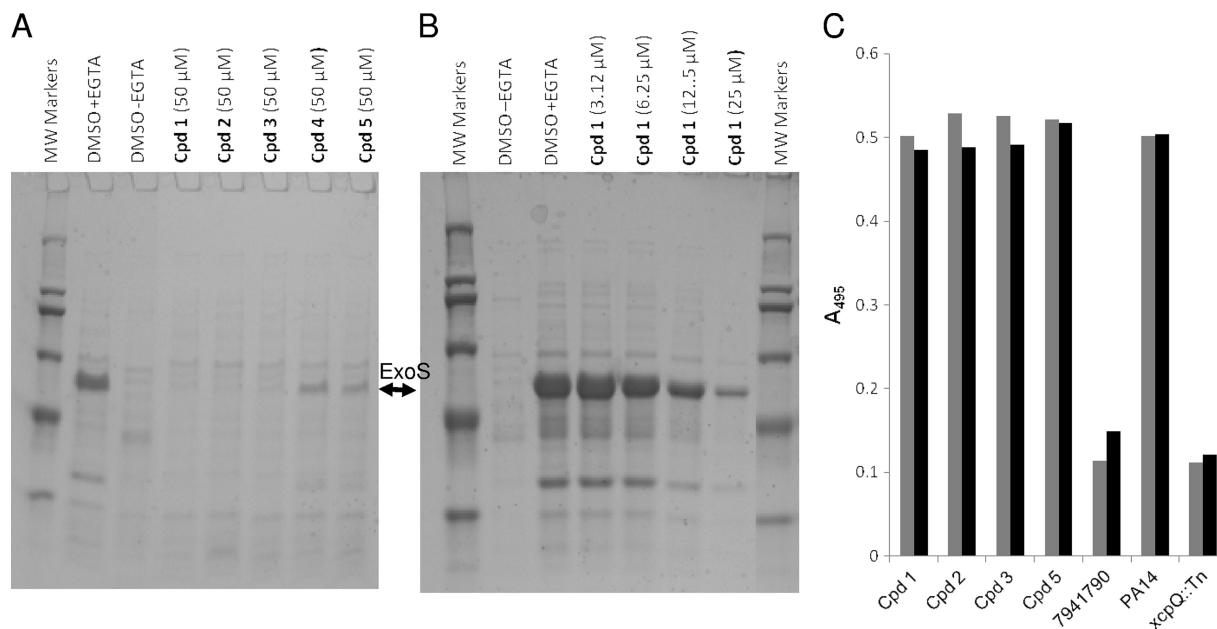


FIG. 2. Evaluation of inhibition of type III and type II secretion in *P. aeruginosa*. *P. aeruginosa* ExoS-secreting strain PAKΔTY was grown under T3SS-inducing conditions (LB plus 5 mM EGTA) for 3 h in the presence of the indicated concentrations of compounds, and culture medium (1 ml) was concentrated in SDS-PAGE sample buffer, separated by SDS–12.5% PAGE, and stained with Coomassie blue. The positive control, DMSO plus EGTA, was treated with 5 mM EGTA but not inhibitors, and the negative control, DMSO without EGTA, was treated with neither EGTA nor inhibitors. The identity and molecular weights of protein markers are as follows: porcine myosin (200K), *E. coli* β-galactosidase (116K), rabbit muscle phosphorylase B (97K), bovine albumin (66K), ovalbumin (45K), and bovine carbonic anhydrase (29K). (A) Secreted proteins from cells treated with EGTA and the five validated T3SS inhibitors (Table 3). The band corresponding to 49K ExoS is marked. (B) Secreted proteins from cells treated with EGTA and serial dilutions of T3SS inhibitor compound 1. (C) Effects of T3SS inhibitors on type II secretion of elastase. *P. aeruginosa* PA14 cells were grown in LB medium for 16 h in the presence of 50 μM of the indicated compounds. As controls, PA14 and PA14 *xcpQ::Tn* cells were grown in LB in the presence of the equivalent concentration of DMSO, and PA14 was grown in the presence of a type II secretion inhibitor (compound 7941790; Chembridge, Inc.). Culture medium corresponding to equivalent numbers of cells was harvested by centrifugation and incubated with shaking for 6 h with Congo Red-elastin. Digested soluble Congo Red was measured by the A_{495} in two independent assays and plotted (gray and black bars).

dependence of the inhibition of native ExoS secretion was examined in detail for compound 1 and was found to be very similar to that observed in the ExoS'-βLA inhibition assay (IC_{50} of ~12.5 μM) (Fig. 2B). The inhibitory effect appeared specific for type III secretion, since members of all three structural classes failed to inhibit type II-mediated elastase secretion when added to type II secretion-competent *P. aeruginosa* PA14 cells at 50 μM (Fig. 2C). Control inhibitor 7941790 (Chembridge, Inc.) reduced elastase secretion to the level observed in a type II-deficient PA14 strain carrying a transposon insertion in the secretin gene *xcpQ*, while the three series of T3SS inhibitors had no detectable effect.

Inhibition of T3SS-mediated effects on mammalian cells. To assess their effects on the T3SS-mediated translocation of effectors, the five specific inhibitors of type III secretion were tested in a cellular activity assay for T3SS effector translocation into mammalian cells (27). The compounds were added to CHO cells simultaneously with the addition of *P. aeruginosa* ExoU-producing cells to determine whether the inhibitors were capable of blocking CHO cell death due to the cytotoxic activity of translocated ExoU. Only compound 1 was capable of reproducibly rescuing CHO cells from the ExoU-secreting *P. aeruginosa* cells (Fig. 3A), and its potency in this assay (IC_{50} of ~15 μM) was similar to its potencies in the ExoS'-βLA assay (Table 3) and in the inhibition of the secretion of native

ExoS (Fig. 2B). These results demonstrate that the phenoxyacetamide compound 1 not only blocks the T3SS-mediated secretion of effectors from *P. aeruginosa* into culture medium but also blocks the translocation of effectors into mammalian cells. Rescue from ExoU cytotoxicity by compound 1 was limited somewhat due to the cytotoxicity of the compound itself in the absence of *P. aeruginosa* cells, which reaches about 30% at 25 μM (Fig. 3A, open circles) and 50% at 75 μM (not shown). This CC_{50} is somewhat lower than the values obtained with HeLa cells (102 μM) (Table 3; also see Fig. 5C) and 293T cells (110 μM) (data not shown) in the absence of serum. The difference probably reflects the facts that three different cell types were employed and that the CHO cells were under stress due to the sudden reduction in serum levels from 10 to 1% just prior to infection with *P. aeruginosa* cells. In any case, there is a clear margin of efficacy for compound 1 in this CHO rescue experiment. A known ExoU inhibitor, pseudolipasin (27), also rescued CHO cells from ExoU toxicity with a similar potency. We resynthesized compound 1 and verified that the resulting compound, MBX 1641, exhibits the same T3SS inhibition potency and selectivity as the original compound 1.

ExoS and ExoT appear to block the uptake of *P. aeruginosa* cells by both epithelial and phagocytic cells in culture, suggesting that the T3SS functions as a virulence factor by preventing the phagocytic cell clearance of *P. aeruginosa* cells during in-

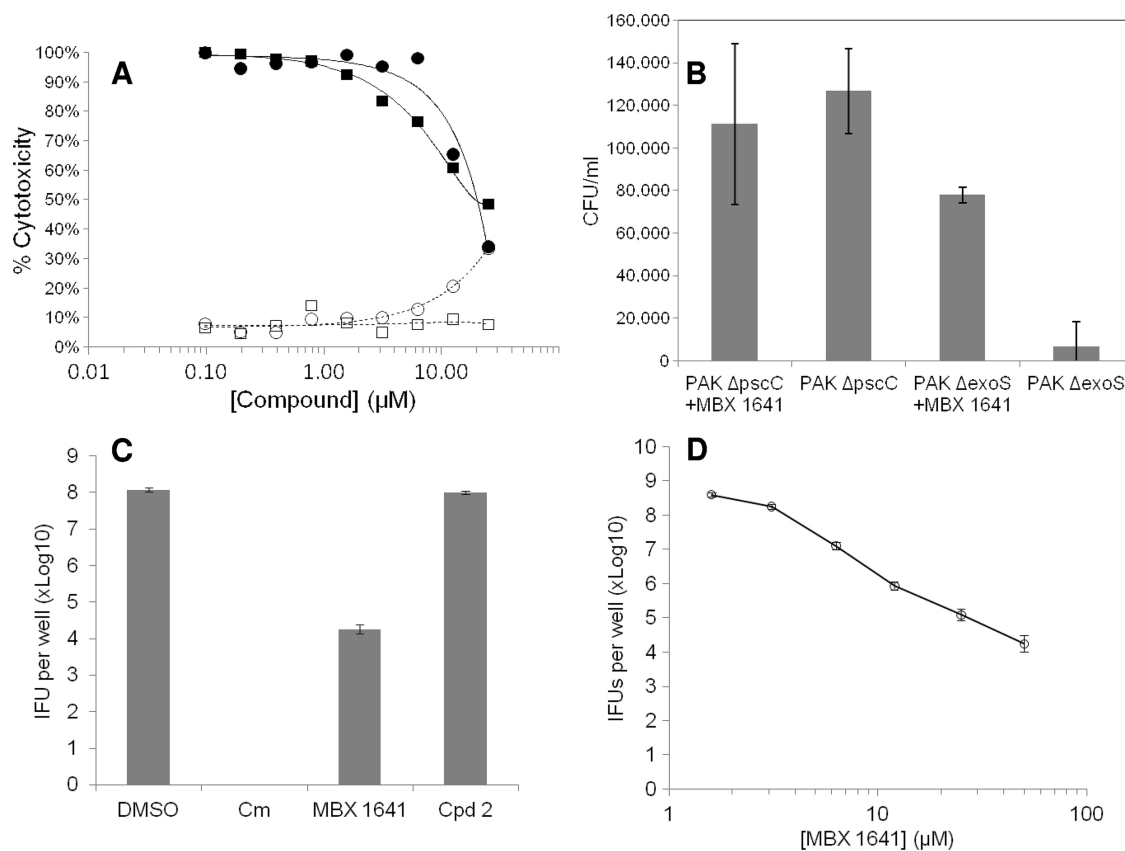


FIG. 3. Inhibition of T3SS-mediated effects on mammalian cells in culture. (A) Concentration-dependent rescue of CHO cells from ExoU cytotoxicity by T3SS inhibitor MBX 1641. ExoU-secreting *P. aeruginosa* strain PAKΔSTYexoU was mixed with CHO cells at an MOI of 5 in the presence of MBX 1641 (●) or the known ExoU inhibitor pseudolipasin (■) (27) at various concentrations as indicated. The percent cytotoxicity is calculated as the percentage of LDH released from cells intoxicated with *P. aeruginosa* with or without inhibitor compared to LDH released from intoxicated cells that were not treated with inhibitor. The effects of pseudolipasin (□) and MBX1641 (○) also are shown in the absence of *P. aeruginosa* cells to evaluate the inherent cytotoxicity of the compounds themselves. (B) T3SS inhibitor MBX 1641 relieves the ExoT block of the HeLa cell internalization of *P. aeruginosa*. HeLa cells were infected with *P. aeruginosa* PAK strains secreting ExoT (PAKΔS) or deficient in T3SS (PAKΔC) at an MOI of 10. MBX 1641 was added at 50 µM to half the wells containing each strain. After 2 h, cultures were treated with gentamicin (50 µg/ml) for an additional 2 h. HeLa cells were lysed with Triton, and serial dilutions were plated to determine the number of *P. aeruginosa* cells that had been protected from gentamicin by internalization. The CFU/ml of *P. aeruginosa* cells from lysed HeLa cells were determined in triplicate and plotted as the averages \pm standard deviations. (C) MBX 1641 but not compound 2 inhibits the growth of *C. trachomatis* L2 cells in Hep-2 cells in culture. Confluent monolayer Hep-2 cells were infected with L2 at an MOI of 0.5 and treated with compounds at the indicated concentrations, followed by sonication and the measurement of IFUs on HeLa monolayers. Experiments were done in triplicate, and averages \pm standard deviations are shown. Chloramphenicol (Cm) was used at 200 µg/ml as a positive control, and compound diluent (DMSO) was used as a negative control. (D) Concentration dependence of the inhibition of *C. trachomatis* L2 growth in Hep-2 cells by MBX 1641.

fection (6, 15). The inhibition of the T3SS-mediated secretion and translocation of ExoS or ExoT by mutation results in the increased internalization of bacteria (6, 15, 18, 50). MBX 1641 was tested to determine if its T3SS inhibition facilitated the internalization of *P. aeruginosa* cells by HeLa cells in culture. The addition of the compound at 50 µM to HeLa cells simultaneously with the addition of ExoT-producing *P. aeruginosa* cells at a multiplicity of infection of 10 resulted in a stimulation of the internalization of bacterial cells by more than 11-fold, as measured by the protection of bacteria from gentamicin (Fig. 3B, columns 3 and 4). In the presence of MBX 1641, the number of internalized *P. aeruginosa* ExoT-secreting cells increased to nearly the number of T3SS-deficient ΔpscC cells taken up by HeLa (Fig. 3B, columns 2 and 3). As expected, MBX 1641 had no significant effect on the already-high levels

of the uptake of a T3SS-deficient ΔpscC mutant strain (Fig. 3B, columns 1 and 2).

Bacterial spectrum of activity. The intracellular pathogen *Chlamydia trachomatis* expresses a T3SS thought to be responsible for injecting effectors into the host cytosol (23). Recently, *Yersinia* T3SS inhibitors INP0007 and INP0400, both members of an acylated hydrazone series (40), were demonstrated to arrest the growth of *C. trachomatis* in mammalian cell hosts (38, 59), suggesting that the T3SS plays an essential role in the *Chlamydia* development cycle. MBX 1641 and compound 2 (Table 3) were tested for the ability to block the growth of *C. trachomatis* L2 in Hep-2 cells. The results reveal that MBX 1641, but not compound 2, significantly reduced the growth of *C. trachomatis* when added at 50 µM (Fig. 3C). In addition, MBX 1641 exhibited a concentration-dependent effect on *C.*

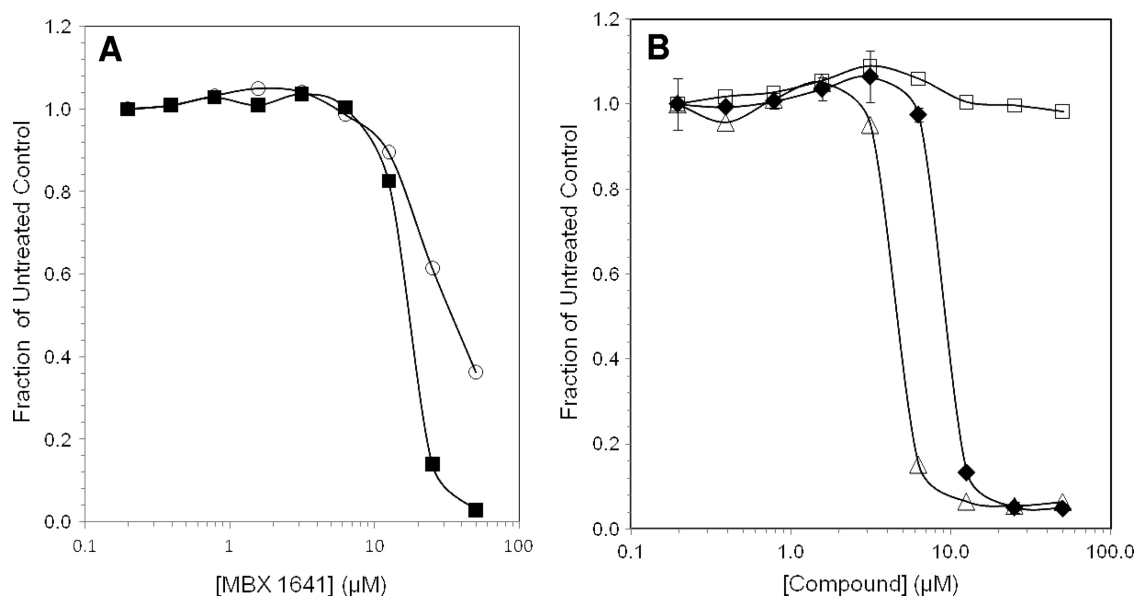


FIG. 4. Inhibition of T3SS-mediated secretion of effector-β-lactamase fusion proteins. (A) Cells growing under T3SS-inducing conditions were treated for 3 h with MBX 1641, and secreted β-lactamase activity was measured by the cleavage of nitrocefin as the $\Delta A_{490}/\text{min}$. The rate of nitrocefin cleavage as a fraction of that of the untreated control is plotted versus the compound concentration. Bacterial species and effector βLA fusions were *P. aeruginosa* ExoS'-βLA (■) and *Y. pestis* YopE-βLA (○). (B) Effects of MBX 1641 and its *R*- and *S*-enantiomers on ExoS'-βLA secretion from *P. aeruginosa*. Concentration dependence for MBX 1641 and its two stereo isomers, MBX 1684 and MBX 1686, were determined by the rate of nitrocefin cleavage by secreted ExoS'-βLA and calculated as the fraction of cleavage in the absence of inhibitor. Racemic mixture MBX 1641 (◆), *R*-enantiomer MBX 1684 (△), and *S*-enantiomer MBX 1686 (□) are indicated.

trachomatis growth in Hep-2 cells (Fig. 3D). These results suggest that MBX 1641 is capable of inhibiting T3SS in *Chlamydia*.

We also examined the ability of MBX 1641 to inhibit the T3SS of *Yersinia pestis*. As shown in Fig. 4A, MBX 1641 inhibits the T3SS-dependent secretion of a YopE-βLA effector fusion protein from attenuated *Y. pestis* strain JG153 with a potency about 3-fold poorer (IC₅₀ of ~38 μM) than that observed for its inhibition of ExoS'-βLA secretion from *P. aeruginosa*. It is interesting that the other four validated T3SS inhibitors of *P. aeruginosa* type III secretion also inhibit *Y. pestis* T3SS-mediated secretion (Table 3), which is consistent with the fact that the structural components of these two T3S systems share considerable sequence homology (23).

Preliminary SAR for phenoxyacetamide T3SS inhibitors. Results described above demonstrate that MBX 1641 inhibits both T3SS-mediated secretion and translocation. In addition, it does so with minimal effects on the extent (Fig. 5A) and rate (Fig. 5B) of the growth of *P. aeruginosa* cells and on the viability of HeLa cells (Fig. 5C), yielding a favorable selectivity index (CC₅₀/IC₅₀) of approximately 8. To explore the structure-activity relationships (SAR) of the phenoxyacetamide series represented by MBX 1641, a total of 114 analogs were purchased (Chembridge, Inc.) and assayed for T3SS inhibition at a single concentration (50 μM) (see Table S1 in the supplemental material). IC₅₀s were determined for several key analogs by using the ExoS'-βLA assay (Table 4). The results indicate that very few alterations are acceptable on ring A, but there is considerable flexibility in the substituents tolerated on ring B. Results also suggest that the linker region cannot be lengthened by one methylene unit, but a tertiary amine is

tolerated with some loss of activity. The discovery of inhibitory analogs in series A supports the validity of this chemotype as a T3SS inhibitor and provides a basis for the further optimization of the potency of this class of inhibitors.

Further SAR studies focused on the single stereocenter of MBX 1641, which is a racemic mixture. Since pure enantiomers were not available for purchase, we synthesized the two stereoisomers, MBX 1684 (*R*-isomer) and MBX 1686 (*S*-isomer). Also, to evaluate the effect of eliminating the stereocenter, we synthesized analogs of MBX 1641 lacking the methyl group at the stereocenter in the linker region (MBX 1668) and containing two methyl groups at the stereocenter (MBX 1685). The concentration-dependent inhibition of T3SS by these compounds was measured in the ExoS'-βLA reporter assay, and the results unambiguously establish the importance of the stereocenter for T3SS inhibitory activity. Only the *R*-isomer was active, and it was almost twice as potent as the racemic mixture (IC₅₀ of ~6 μM for MBX 1684 and ~10 μM for MBX 1641) (Fig. 4B, Table 4). Both analogs lacking the stereocenter, the desmethyl and dimethyl compounds, were inactive (IC₅₀s of >100 μM) (Table 4), as was the *S*-isomer (Fig. 4B).

DISCUSSION

In this study, a bioluminescent cellular reporter screen and multiple secondary assays were employed to identify and validate five new selective inhibitors of *P. aeruginosa* T3SS-mediated secretion and one inhibitor of T3SS-mediated translocation. These inhibitors display minimal cytotoxicity (CC₅₀ ≥ 100 μM) and moderate potency (IC₅₀s ≤ 15 μM) and exhibit no

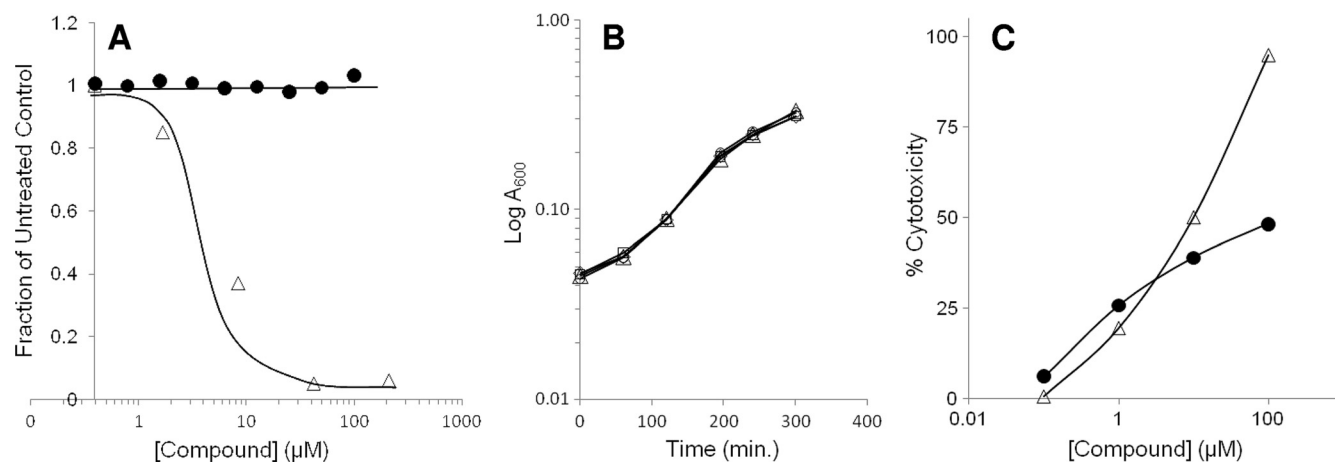


FIG. 5. Evaluation of the effects of MBX 1641 on bacterial and mammalian cell growth. (A) Determination of the MIC of MBX 1641 for *P. aeruginosa*. *P. aeruginosa* PAO1 cells were grown in the presence of the indicated concentrations of MBX 1641 (●) or tetracycline (△) for 16 h in clear 96-well microplates, and the OD₆₀₀ was determined. The OD₆₀₀ as a fraction of that of DMSO-treated control cells is plotted. (B) Growth rate of *P. aeruginosa* cells treated with MBX 1641. *P. aeruginosa* PAO1 cells were grown in the presence of three different concentrations of MBX 1641 for 5 h in clear 96-well microplates, and the OD₆₀₀ was measured periodically as indicated as a measure of cell density. MBX 1641 was present at 100 (□), 50 (●), or 25 μM (○), or cells were treated with an equivalent concentration (2%) of DMSO only (△). (C) HeLa cell cytotoxicity of MBX 1641. HeLa cells were cultured in VP-SFM medium without serum in the presence of the indicated concentrations of MBX 1641 (●) or novobiocin (△) for 3 days, and cytotoxicity was determined by the ability of remaining live cells to reduce a vital tetrazolium salt stain. Results are plotted as the percentage of cytotoxicity relative to levels for DMSO-treated and Triton X-100-lysed control cells.

significant effects on the extent or rate of the growth of *P. aeruginosa* cells, nor do they inhibit the type II secretion system as determined by measurements of secreted elastase. The compounds represent three different chemotypes (series A, B, and C) (Table 3), but series A and B appear to be structurally

related and contain a stereocenter, which was demonstrated to be critical for activity for series A. Compound 1 (MBX 1641) in series A reproducibly inhibits both T3SS-mediated secretion and translocation and was an effective antagonist in three mammalian cell assays that depend on T3SS intoxication of

TABLE 4. Preliminary structure-activity relationships

Vendor no. ^a	IC ₅₀ (μM) (ExoS ^T -βLA assay)	Stereocenter	Ring A	Linker modification	Ring B
MBX 1641	10	Racemic	2,4-Dichlorophenyl	None	3,4-Methylenedioxyphenyl
MBX 1684	6	<i>R</i> -isomer	2,4-Dichlorophenyl	None	3,4-Methylenedioxyphenyl
MBX 1686	>100	<i>S</i> -isomer	2,4-Dichlorophenyl	None	3,4-Methylenedioxyphenyl
MBX 1668	>100	None	2,4-Dichlorophenyl	Desmethyl	3,4-Methylenedioxyphenyl
MBX 1685	>100	None	2,4-Dichlorophenyl	Dimethyl	3,4-Methylenedioxyphenyl
6109233	5	Racemic	2,4-Dichlorophenyl	None	4-Methylphenyl
6380194	9	Racemic	2,4-Dichlorophenyl	None	4-Fluorophenyl
6375680	10	Racemic	2,4-Dichlorophenyl	None	4-Methoxyphenyl
6374948	12	Racemic	2,4-Dichlorophenyl	None	2-Methoxyphenyl
6468028	21	Racemic	2,4-Dichlorophenyl	<i>N</i> -methyl	Phenyl
5685325	25	Racemic	2,4-Dichlorophenyl	None	Furan-2-yl
6374984	45	Racemic	2,4-Dichlorophenyl	None	Pyridine-2-yl
6372013	59	Racemic	2,4-Dichlorophenyl	None	Pyridine-4-yl
8804126	61	Racemic	2,4-Dichlorophenyl	None	1,3-Dimethylpyrazol-4-yl
7229146	100	Racemic	2,4-Dichlorophenyl	Constrained <i>tert</i> -amine	1,2,3,4-Tetrahydroisoquinoline
6467504	>100	Racemic	2,4-Dichlorophenyl	+CH ₂ after NH	2-Cyclohexen-1-ylmethyl
7271715	>100	Racemic	2,4-Dichlorophenyl	None	3,4-Dichlorophenyl
7314595	>100	Racemic	2,4-Dichlorophenyl	+CH ₂ after NH	2-Chlorophenyl
9153915	23	Racemic	2-Chlorophenyl	None	3,4-Methylenedioxyphenyl
6116488	98	Racemic	2-Methyl-4-chlorophenyl	None	3,4-Methylenedioxyphenyl
7339628	>100	Racemic	2-Fluorophenyl	None	3,4-Methylenedioxyphenyl
7303859	>100	Racemic	3-Chlorophenyl	None	3,4-Methylenedioxyphenyl

^a See Table S1 in the supplemental material for structures.

CHO cells by ExoU-producing *P. aeruginosa*, blockage by *P. aeruginosa* of HeLa cell internalization, and the growth of *C. trachomatis* in Hep-2 cells. The potency and selectivity of inhibitors in series A suggest that this class of T3SS inhibitors is suitable for further chemical optimization to produce a clinically useful inhibitor.

It is unclear why the other four validated inhibitors of T3SS-mediated secretion failed to inhibit T3SS-mediated translocation as measured by the rescue of CHO cells from ExoU intoxication. Most secretion inhibitors would be expected to inhibit translocation, since many aspects of T3SS-mediated secretion also are required for translocation. At least four possible explanations could account for this discrepancy. First, the inhibitors may interact with the T3SS apparatus at a site that is inaccessible when the *P. aeruginosa* needle is docked to the mammalian cell membrane. Second, the inherent cytotoxicity of the inhibitors may preclude our ability to detect the rescue of CHO cells from ExoU-mediated cytotoxicity. Some cytotoxicity was evident even in the successful inhibition by MBX 1641, and it limited our ability to achieve the complete rescue of CHO cells. While the four secretion inhibitors do not appear to be more cytotoxic than MBX 1641, even subtle increases in cytotoxicity may be sufficient to mask CHO cell rescue in this assay. Third, the secretion inhibitors may bind extensively to serum proteins and be unavailable for activity in the mammalian cell-based translocation assay. In fact, compounds 2, 3, and 5 do display greater loss of activity in the presence of serum than does compound 1 (MBX 1641) (Table 3, serum effect). A fourth formal possibility is that the inhibitors block T3SS induced by low levels of Ca^{2+} but not by mammalian cell contact. However, the speeds with which the inhibitors function seem to preclude action at the level of transcription regulation (see below).

The phenoxacetamide MBX 1641 does not appear to be related structurally to any of the T3SS inhibitors reported previously. Results have been described for T3SS inhibitor screens of *Yersinia pseudotuberculosis* (24, 41), *Y. pestis* (44), enteropathogenic *Escherichia coli* (EPEC) (16), *Salmonella enterica* serovar Typhimurium (12), and *P. aeruginosa* (27). All have utilized cell-based assays, both for the direct identification of compounds active against whole cells and because the complexity of the molecular machine renders biochemical screens of component parts of T3SS particularly challenging. The only previously described screen for *P. aeruginosa* T3SS inhibitors was based on the reducing potential of remaining live CHO cells and consequently could detect inhibitors of any step in the secretion, translocation, and toxin activity leading to mammalian cell death (27). The validated inhibitors identified in the screen were shown to inhibit the ExoU toxin directly rather than the T3SS process itself. However, one series of hits described in that study displays structural similarity to MBX 1641. Two compounds in that series, 5929052 and 5925831 (27 and Table S2 in the supplemental material), failed to exhibit detectable inhibition in the ExoS'- β LA assay described here (IC_{50} s > 100 μM ; unpublished results). The absence of detectable inhibition is not surprising, since those compounds were identified as ExoU inhibitors and since they lack the stereocenter demonstrated to be crucial for the T3SS-inhibitory activity of MBX 1641 (e.g., see the desmethyl analog in Table 4). It is particularly interesting to compare the previously reported

inhibitors of *Y. pseudotuberculosis* and *Y. pestis* T3SS to the inhibitors identified in this study, because the *Pseudomonas* T3SS proteins exhibit more sequence similarity to those of *Yersinia* than to those of any other genus (23). Two *Y. pseudotuberculosis* T3SS inhibitors, compounds 8 and 11 (41), were present in our screening collection. While they do inhibit *P. aeruginosa* T3SS moderately, they failed to inhibit the *exoT-lux* primary reporter screen with sufficient potency to be selected as primary hits (unpublished observations). One *Y. pestis* T3SS inhibitor, compound 2 (44), also was present in our screening collection, and it proved to be a potent inhibitor of *P. aeruginosa* T3SS in the primary and secondary screens applied here (IC_{50} of $\leq 10 \mu\text{M}$ in the ExoS'- β LA assay) but was not pursued due to high serum protein binding. The ability of three different *Yersinia* T3SS inhibitors to block *P. aeruginosa* T3SS is consistent with the high sequence homology observed for T3SS components in the two genera and with the ability of the five *P. aeruginosa* T3SS inhibitors described in this study to inhibit *Y. pestis* T3SS-mediated secretion.

The molecular target(s) of these *P. aeruginosa* T3SS inhibitors is not known; however, the results described here provide some evidence that these compounds specifically inhibit the activity of the T3SS apparatus. First, we have shown that the compounds are not simply inhibiting one of the effector toxins, because they specifically affected the secretion or the translocation of three different effectors: ExoS (SDS-PAGE), ExoT (HeLa cell internalization), and ExoU (rescue of CHO cells). Second, the inhibitors do not affect the extent (MIC) or rate of growth of *P. aeruginosa* cells. Third, the compounds do not appear to be general inhibitors of gene expression or virulence gene expression, because they demonstrate differential effects on the generation of luminescence by strains carrying *exoT-lux* and *lac-lux* transcriptional fusions, and they do not inhibit the production or secretion of another virulence factor, elastase, which utilizes the type II secretion mechanism. Fourth, the inhibition of ExoS'- β LA secretion by MBX 1641 is equally potent when measured in a multiple-efflux-pump knockout strain, *P. aeruginosa* strain PAO397 (26 and unpublished observations) (provided by Herbert Schweizer, Colorado State University). This suggests that T3SS inhibitors are not effluxed and/or do not need to enter *P. aeruginosa* cells to act, and the latter possibility is more likely, since few small molecules enter and are retained in *P. aeruginosa* cells (30). Fifth, MBX 1641 acts equally potently to block ExoS'- β LA secretion whether administered during or after the 2.5-h EGTA induction of T3SS, suggesting that the compound is not blocking T3SS gene expression or the assembly of the type III apparatus (unpublished observations). Finally, the strict requirement for the *R*-isomer configuration at the stereocenter of the phenoxacetamide series indicates that the inhibitor is interacting with a specific target or targets and is not acting by a promiscuous nonspecific mechanism. The observed spectrum of activity against T3SS in three bacterial species points to a conserved target, but the sequence conservation is high across species among many of the T3SS gene products.

In addition to establishing the importance of the stereocenter in the linker region of the phenoxacetamide series (series A), the initial SAR described here provides some clear directions for improving the potency of the inhibitor. The low tolerance for alterations to ring A (Table 4) suggests that this

region of the molecule, together with the stereocenter, is involved in important contacts with the target. The further chemical optimization of these regions may provide improved potency. In contrast, the considerable tolerance demonstrated for various substituents on ring B (Table 4) suggests that few target contacts are made on that side of the compounds, perhaps providing a location for a tethered photoreactive group for target identification or for other modifications to provide pharmacologic or toxicologic benefits.

The results of this study suggest that MBX 1641 is capable of inhibiting the T3SS of three different bacterial species, *P. aeruginosa*, *Y. pestis*, and *C. trachomatis*. Multiple different assays demonstrate the inhibition of *P. aeruginosa* T3SS, while the inhibition of T3SS in the other two species is based on a single assay in each case. Nevertheless, effector- β -lactamase fusion proteins appear to be reliable reporters of T3SS function. In the absence of a manipulable genetic system in *Chlamydia*, it has not been possible to firmly establish the essentiality of the T3SS for intracellular growth. We cannot rule out the possibility that MBX 1641 is arresting *C. trachomatis* growth by mechanisms other than T3SS inhibition, but the compound has not demonstrated promiscuous behavior in a variety of assays and does not appear to be overtly cytotoxic or to block gene expression.

In summary, the potency and selectivity of the phenoxyacetamide compound MBX 1641 to block both the T3SS-mediated secretion and translocation of *P. aeruginosa* effectors justifies further study of this class of T3SS inhibitors. The absolute requirement for the *R*-stereoisomer indicates that the phenoxyacetamides target a specific component required for type III secretion. The structure-activity relationships demonstrated here suggest approaches to optimize this compound series to achieve higher potency and reduced cytotoxicity. Such optimized compounds could be evaluated in animal models either alone or in combination with antibiotics to determine their benefit in potential therapeutic applications.

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