ABSTRACT

Title of Document:	IDENTIFICATION OF SMALL CHEMICAL INHIBITIORS OF BACTERIAL TYPE THREE SECRETION SYSTEM MEDIATED CELL DEATH
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Abstract

Current antibiotic therapies for bacterial pathogens target common components essential for viability of all bacteria. This strategy has been very effective in combating bacterial infections but fails to discriminate against non-pathogens and places tremendous pressure on organisms to evolve antibiotic resistance mechanisms. A promising alternative to targeting bacterial viability is to inhibit virulence mechanisms that are unique to pathogenic bacteria and modulate interaction with the host. Gram-negative bacteria utilize a type three secretion system (T3SS) to inject effector molecules from the bacterial cytoplasm directly into the host, where the effectors subvert and modify host cell function for colonization and immune evasion. Using a high-throughput cell based screen we identified three inhibitors of the Gram negative T3SS; quinolozone compound PA T3SS-9 blocks *P. aeruginosa* ExoU mediated cell death by targeting downstream of ExoU phospholipase A_2 activity, and phenylquinazolinamine compound PA T3SS-11 and 2-phenylbenzoxazole compound PA T3SS-19 are broad range inhibitors of T3SS mediated cell death.

INHIBITION OF BACTERIAL TYPE THREE SECRETION SYSTEM MEDIATED CELL DEATH BY SMALL CHEMICAL COMPOUNDS

By

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Chapter 1: Introduction

The development of antibiotics was arguably the most important event in the 20th century. Prior to the discovery of penicillin, bacterial infection claimed over 100,000 lives annually in the United States (CDC 1999). The discovery and clinical application of penicillin ushered in the "age of antibiotics" (Davies 2006), and by 1969 antimicrobial drugs were so successful in treating infection that then Surgeon General William Stuart famously declared "the book closed (sic) on infectious disease"*. Today, however, we face a different reality. Bacteria have evolved resistance mechanisms to all known antibiotics, and with pharmaceutical companies focusing drug development resources on so called "lifestyle drugs" (Charles 2004), there is an immediate need to find an alternative to traditional antibiotics. Historically, antibiotics were derived from chemicals secreted by fungi, like *Penicillum notatum* (penicillins) (Fleming 1929), and bacteria, like *Streptomyces griseus* (aminoglycosides) (Schatz 1944), for their own defense against and modulation of neighboring species (Foster 2010, Martinez 2008). Antibiotics work by targeting essential and conserved cellular processes like DNA replication, transcription, translation, and cell wall synthesis, or by disrupting a structure necessary for survival, like the cell membrane. Despite this successful strategy, there are drawbacks.

*According to a New York Times article by Douglas Martin, published April 29, 2008, this often cited quote lacks primary sources, and "the Surgeon General himself cannot remember saying it".

Because antibiotics target conserved cellular processes, there is no discrimination between pathogens and non-pathogens during a course of antibiotic treatment (Sullivan 2001). Non-pathogenic normal flora are a large part of host defense, and are pivotal in shaping and supporting our digestive and immune systems (reviewed in Hooper 2001). Upsetting the balance of species diversity can cause acute disease, like candidiasis and diarrhea, and chronic illness like inflammatory bowel disease and obesity (Sartor 2001, Wolowczuk 2008, Vijay-Kumar 2010). Additionally, the antibiotics themselves are sometimes toxic or immunogenic, with debilitating side effects (Kahlmeter 1984, Blanca 1995). Furthermore, by targeting essential cellular processes, antibiotics give a growth advantage to resistant species and select for resistant bacteria within susceptible species. Due to the rapid growth rate of most bacteria, species can compensate quickly to fix within the population the mutation or extra chromosomal genetic element that provided the selective advantage. In an environmental context, selective pressure is not new; any neighbor able to resist chemical modulation has a selective advantage (Martinez 2008). However, overuse in homes, hospitals, and commercial agriculture caused antibiotics to saturate the environment (Kumerer 2003, Kemper 2008), placing constant selective pressure on bacteria to evolve resistance.

Antibiotic resistance is now a major public health crisis (WHO 2001), and resistance mechanisms have been identified for all known antibiotics. Multi and extreme drug resistant strains of *Pseudomonas aeruginosa* (Levin 1999), *Staphylococcus aureus* (Barber 1961, Sharpe 2005), *Streptococcus pneumoniae* (Tomasz 1997), *Escherichia coli* (Edgar 1997), and *Mycobacteria tuberculosis* (Jassal 2009) have been identified,

and are treatable only with third or last line of defense drugs, often in supervised clinical settings (Brickner 1996, Sahm 2001, Shah 2007, Raviglione 2009). Slow development of novel antimicrobials underscores the threat to public health. In the last 40 years, only 3 new classes of antibiotics have been discovered: Cyclic lipopeptides (Debono 1988), Glycylcyclines (Testa 1993), and Oxazolidinones (Slee 1987). However, resistance to these drugs has already been reported (Dean 2003, Bersos 2004, Hayden 2005, Sabol 2005, Navon-Venezia 2007), exemplifying the need to develop novel treatments for bacterial borne illness.

With antibiotics quickly becoming obsolete, new strategies to combat bacterial infection are being explored and researchers are focusing on bacterial virulence factors as potential drug targets. Virulence factors, such as expression of virulence genes, structures that facilitate host contact, exotoxins and toxin delivery systems (reviewed in Clatworthy 2007), define pathogenic bacteria; they mediate interaction with the host by facilitating colonization, persistence, and immune evasion. Anti-virulence drugs in effect block the host-pathogen interaction and allow bacterial clearance by the host immune system. Anti-virulence drugs have a 2-fold benefit: targeting virulence mechanisms instead of essential factors reduces the selective pressure on bacteria, and since normal microflora lack virulence mechanisms they will remain unaffected by the treatment.

To date, chemical inhibitors have been identified for a variety of virulence mechanisms, such as transcription of virulence genes, enzymes that provide resistance to host defense, bacterial adhesion factors and secretion systems that alter host function. An example of a transcriptional inhibitor is virstatin, which inhibits *Vibrio*

cholerae transcriptional regulator ToxT from binding to the promoter of cholera toxin and the toxin co regulated pilus, rendering *V. cholerae* avirulent (Hung 2005). Examples of inhibitors of enzymes that aid in host defense evasion include chemicals from the bidentate benzofuran salicylic acid class of compounds, which have been shown to inhibit *M. tuberculosis* protein tyrosine phosphatase B (mPTB). Inhibition of mPTB recovers the hijacked host immune response, thus preventing *M. tuberculosis* survival within the macrophage (Chen 2010, Zhou 2010). Examples of adhesion factor inhibitors are alkyl mannosides, which competitively bind the mannose binding site of uropathogenic *E. coli* (UPEC) fimbriael adhesion protein FimH; this blocks ligation with host cell surface glycoproteins, effectively inhibiting the pathogen-host interaction (Bouckaert 2005). Similarly, a family of bicyclic 2-piradones was shown to bind to the type 1 pili chaperone PapD to block chaperone-usher interaction and therefore pili assembly, thus mitigating pili mediated virulence (Pinkner 2006). Likewise, many inhibitors of the Gram-negative type three secretion system (T3SS) have been identified (reviewed in Lee 2009). Chemicals in the salicylaldehyde family have been shown to abrogate T3SS mediated virulence by inhibiting transcription of *Yersinia pseudotuberculosis* positive regulator LcrF (Kaupi 2003), T3SS associated locus of enterocyte effacement (LEE) genes (Gauthier 2005), T3SS genes in *Chlamydia trachomatis* and *Chlamydia pneumoniae* (Bailey 2006, Muschiol 2006), as well as by inhibiting *Y. pseudotuberculosis* T3SS Yop effector protein secretion (Nordfelth 2005), *Salmonella typhimurium* T3SS effector protein secretion (Hudson 2007, Negrea 2007), and *Shigella flexneri* T3SS needle construction (Veenendall 2009). The compound 2-imino 5-arylidine thiazolidinone

was shown to inhibit assembly of the T3SS machine in *P. aeruginosa*, *S. typhimurium*, and *Yersinia* species, likely by targeting the outer membrane "secretin" structural subunit (Felise 2009). Chemicals in the benzimidazole family have been shown to abolish DNA binding of *Y. pseudotuberculosis* positive regulator LcrF, (Oak 2009, Garrity-Ryan 2010) eliminating expression of T3SS genes. Finally, two inhibitors of *P. aeruginosa* exotoxins have been identified: pseudolipasin A, which specifically abolishes activity of phospholipase ExoU, blocking ExoU mediated cell death (Lee 2007), and exosin, a specific inhibitor of ExoS ADP-ribosyltransferase activity that prevents ExoS mediated apoptosis (Arnaldo 2008). This list, while not exhaustive, is representative of the work done so far to establish "proof of concept" for virulence factors as a novel class of drug targets. As such, the T3SS is of particular interest due to its prevalence among Gram-negative pathogens.

The T3SS is a virulence factor found in a variety of Gram-negative animal and plant pathogens including respiratory pathogens *P. aeruginosa* and *Bordetella bronchiseptica* (Skinner 2005), enteric pathogens *Salmonella*, *Vibrio*, *Escherichia* and *Shigella* spp and *Y. pseudotuberculosis*, obligate intracellular pathogens *C. trachomatis* and *C. pneumoniae*, biodefense pathogens *Yersinia pestis*, *Burkholderia mallei* and *pseudomallei,* and plant pathogens *Pseudomonas syringae, Xanthomonas campestris*, *Erwinia amylovora*, and *Ralstonia solanacearum* (reviewed in Cornelis 2006, Galan 2006, Lee 2009). Pathogens use the T3SS to inject effector proteins directly into the host, in an effort to subvert or modify host cellular functions. The T3SS is functionally conserved across genera; although each species uses unique effector proteins to promote infection, the proteins that make up the T3S apparatus

are largely structurally conserved (Yip 2006) (Protein nomenclature varies among our organisms of interest; please refer to Table 1). The T3S machine is composed of three main parts: a structure, known as the basal body, which spans the inner and outer membrane of the bacterial envelope, a needle-like hollow conduit through which effector proteins are transported, and a set of proteins termed the "translocon" that sit at the tip of the needle and are responsible for pore formation and effector translocation into the host (Galan 2006, Yip 2006). The basal body spans the bacterial envelope, peptidoglycan, periplasmic space, and outer membrane, and is homologous to the core flagellar structure (Blocker 2003). It is composed of a set of rings embedded in the inner membrane (MS and putative C rings), an outer membrane ring called the secretin, a periplasm spanning rod connecting the inner and outer membrane rings, as well as several accessory proteins implicated in secretion substrate specificity and machine engagement (reviewed in Cornelis 2006). Attached to the basal body is the needle complex, a ~60 nm long polymeric structure with an inner diameter of 2.5 nm (Hoyczyk 2001), through which effector proteins are thought to travel (Kubori 1998, Burghout 2004). The basal body and needle complex are sufficient for secretion of proteins into the extracellular milieu (Lee 1999), but additional translocator proteins (the "translocon") are necessary for translocation of effectors into the host (Håkansson 1996, Lee 1999, Goure 2004, Edqvist 2006). The translocon consists of a pentameric protein complex that forms the needle tip (Mueller 2005, Gebus 2008), and two putative pore forming proteins (Schoehn 2003, Gebus 2008). Together, these structures provide a one-way protein export channel

into the host cytosol, and offer many potential molecular targets for chemical inhibition of the T3SS.

Structural Element	***Yersinia* spp**	***S. typhimurium***	***P. aeruginosa***
Inner ring (MS ring)	YscJ	PrgH*	PscJ
Inner ring (C ring)	YscQ	InvK	PscQ
Rod	YscI	PrgJ	PscI
Outer ring (secretin)	YscC	InvG	PscC
Needle	YscF	PrgI	PscF
Translocon (Tip)	LcrV	SipD	PcrV
Translocon (Pore)	YopB, YopD	SipB, SipC	PopB, PopD

*Not conserved (Cornelis 2006)

Table 1. Proteins of the T3SS

To identify compounds that inhibit T3SS mediated cell death, we adapted a method developed by Lee et al, (2007) through which inhibition of *P. aeruginosa* T3SS effector ExoU mediated cytotoxicity of epithelial cells is correlated to reducing potential of live epithelial cells in culture. In our screen, cell death was measured as activity of lactate dehydrogenase (LDH) released from lysed epithelial or murine macrophage-like cells into the extracellular media. Using this screen, we identified quinolozone, phenylquinazolinamine, and 2-phenylbenzoxazole compounds that were able to reduce ExoU mediated epithelial cell death by >90%. Additionally, the phenylquinazolinamine and 2-phenylbenzoxazole compounds were also able to inhibit *Y. pseudotuberculosis* and *S. typhimurium* T3SS mediated death of murine macrophage-like cells, suggesting these compounds have the potential for broad range application.

Chapter 2: Methodology

Bacteria culture

See Table 2 for species, strains, and plasmids. Unless otherwise noted, all bacteria were cultured in Luria-Burtani (LB) broth and aerated at 200 rpm. *Yersinia* spp were grown at 26°C. To induce T3S, *Y. enterocolitica* w22703 and isogenic T3SS mutant Δ*lcrD* (Kum1, Kumaran Ramamurthi) were subcultured and incubated for two hours at 37°C. *Y. pseudotuberculosis* YPIII and isogenic T3SS mutant Δ*yopHEJMOB* were subcultured with 5 mM $MgCl_2$ and 5 mM EGTA (ethylene glycol tetraacetic acid) and incubated for one hour at 26°C followed by two hours at 37°C to induce T3S.
S. typhimurium SL1344 and isogenic T3SS mutant Δ*prgH* (Cathy Lee) were grown at 37°C. To induce T3S, these bacteria were subcultured in LB broth supplemented 5 g NaCl/liter (10 g NaCl/liter final) and incubated for three hours at 37°C followed by a half-hour incubation at 37°C without aeration. *Pseudomonas* spp were grown at 37°C. To induce T3S, *P. aeruginosa* PAK Δ*mexAB att::pro-exoU-spcU* pMMB-*exsA* and isogenic T3SS mutant Δ*pscC* were subcultured with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and incubated for two hours at 37°C.
P. aeruginosa PAK, isogenic effector mutant Δ*exoS* and isogenic T3SS mutant Δ*pscC* were grown at 37°C. To induce T3S, bacteria were subcultured with 5 mM EGTA and incubated for two hours at 37°C to induce T3S.
For each assay, duration of infection and MOI are dependent on ability of bacteria to adhere to the host, efficiency of T3SS machine construction, secretion, and effector

activity. In light of this, duration of infection and MOI were empirically determined prior to testing with experimental compounds.

Species/Strain	Plasmid	Antibiotic Resistance[1]	Media[2]
P. aeruginosa PAK	-	-	LB
P. aeruginosa PAK Δ*pscC*	-	-	LB
P. aeruginosa PAK Δ*xcp*	-	-	LB
P. aeruginosa PAK Δ*fliC*	-	-	LB
P. aeruginosa PAK Δ*pilA*	-	-	LB
P. aeruginosa PAK Δ*exoS*	pVL710 *(exoU-spcU-β-lactamase)*	*amp*	
P. aeruginosa PAK Δ*exoS*	pVL712 *(exoU* S142A-*spcU-β-lactamase)*	*amp*	
P. aeruginosa PAK	pVL712	*amp*	LB
P. aeruginosa PAK Δ*pscC*	pVL712	*amp*	LB
P. aeruginosa PAK Δ*mexAB att::pro-exoU-spcU*	pMMB-*exsA* (transcriptional activator)	*amp*	LB
P. aeruginosa PAK Δ*mexAB* Δ*pscC att::pro-exoU-spcU*	pMMB-*exsA*	*amp*	LB
P. syringae DC3000	-	-	LB
P. syringae DC3000 Δ*hrc*	-	*tet*	LB
S. typhimurium SL1344	-	-	LB 10g NaCl/L
S. typhimurium SL1344 Δ*prgH*	-	-	LB 10g NaCl/L
S. typhimurium SL1344	pJK17 (*exoU-spcU-β-Lactamase)*	*cat*	LB 10g NaCl/L
S. typhimurium SL1344 Δ*prgH*	pJK17	*cat*	LB 10g NaCl/L
Y. enterocolitica w22703	-	-	LB
Y. enterocolitica w22703 Δ*lcrD* (Kum1)	-	-	LB
Y. enterocolitica w22703	pJK17	*cat*	LB
Y. enterocolitica w22703	pVL127 (*exoU-spcU*)		
Y. enterocolitica w22703 Δ*lcrD* (Kum1)	pVL127	*cat*	LB
Y. enterocolitica w22703	pMM83 (*yopM-β-lactamase*)	*cat*	LB
Y. enterocolitica w22703 Δ*lcrD* (Kum1)	pMM83	*cat*	LB
Y. pseudotuberculosis YPIII	-	-	LB
Y. pseudotuberculosis YPIII Δ*yopHEJMOB*	-	-	LB

Table 2. Bacteria species, strains, and plasmids

[1] *amp*, resistance to 30μg carbenicillin per ml. *tet*, resistance to 10μg tetracycline per ml. *cat*, resistance to 30μg chloramphenicol per ml.

[2]10g NaCl/L necessary for induction of *S. typhimurium* T3SS.

Tissue culture

Unless otherwise stated, CHO (*C*hinese *H*amster *O*vary) K1 cells were grown in Ham's F12 with L-glutamine and 10% FBS, and CHO pro-5 cells were grown MEMα with ribonucleosides and deoxyribonucleosides, supplemented with 1% glutamine and 10% FBS. CHO K1 and CHO pro-5 cells were used interchangeably in our experiments. J774a.1 murine macrophage-like cells were grown in 1:1 DMEM and RPMI with 10% heat inactivated FBS, and RAW murine macrophage-like cells were grown in DMEM supplemented with 10% heat inactivated FBS. J774a.1 and RAW cells were used interchangeably in our experiments. All lines were incubated at 37°C in 5% CO_2 and seeded for confluency 16 hours prior to infection, unless otherwise noted.

Compounds

Structures of chemical compounds used in this study are depicted in Tables 3-6. Quinolozone compounds PA T3SS-1, PA T3SS-2, PA T3SS-3, and PA T3SS-4 were made by Dr. Kyuangae Lee at Harvard medical school. Quinolozone, phenylquinazolinamine, and 2-phenylbenzoxazole compounds PA T3SS 5-20 were obtained from ChemBridge (http://www.hit2lead.com/). Compounds stocks were in dimethyl sulfoxide (DMSO), and unless otherwise stated were diluted in LB or cell culture media and added 30 minutes prior to addition of bacteria. Final compound concentration was 20 µg/ml with 2% DMSO, or as indicated.

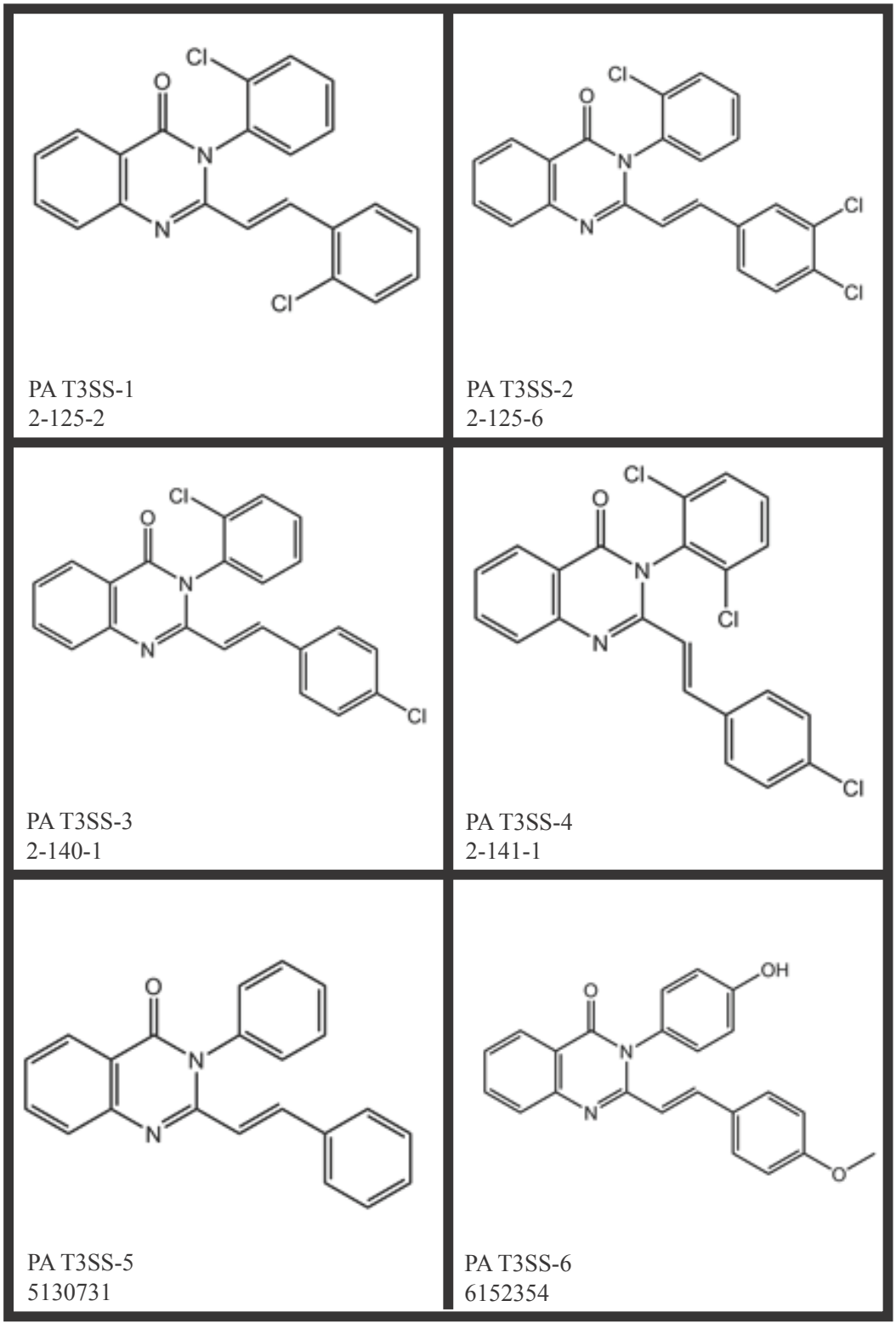


Table 3. Quinolozone compounds PA T3SS 1-6

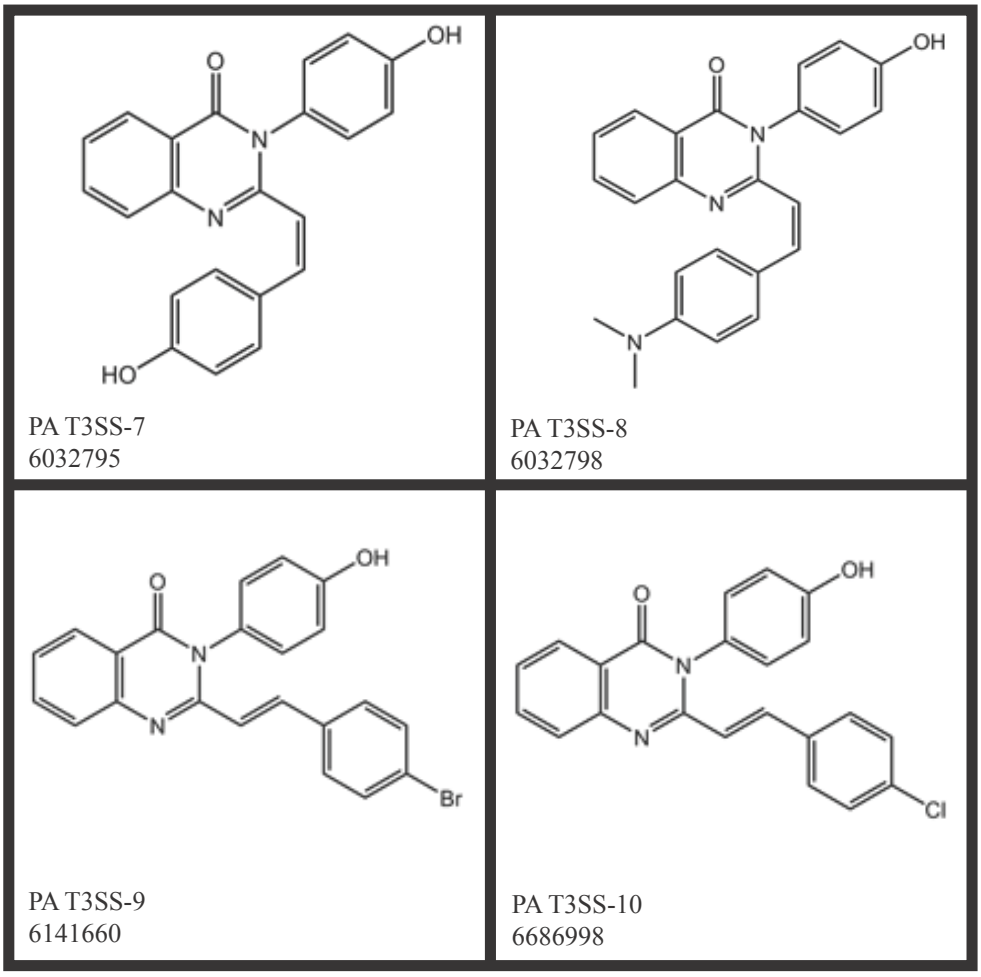


Table 4. Quinolozone compounds PA T3SS 7-10

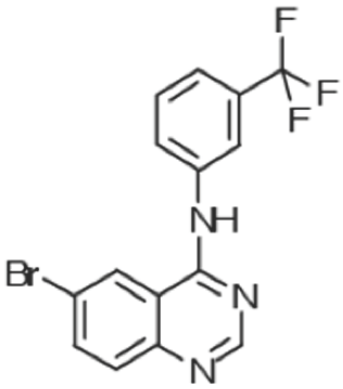
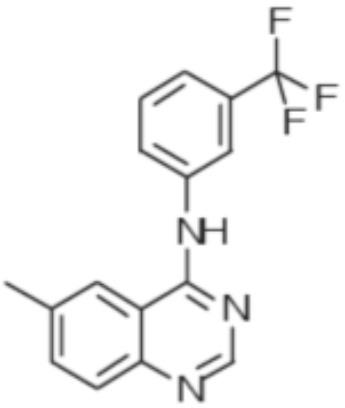
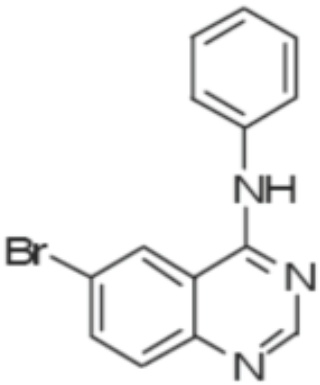
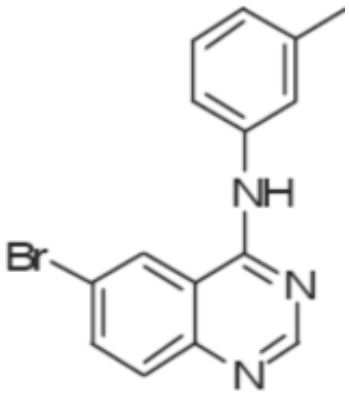
 PA T3SS-11 6145938	 PA T3SS-12 6133504
 PA T3SS-13 6142523	 PA T3SS-14 6158039

Table 5. Phenylquinazolinamine compounds PA T3SS 11-14

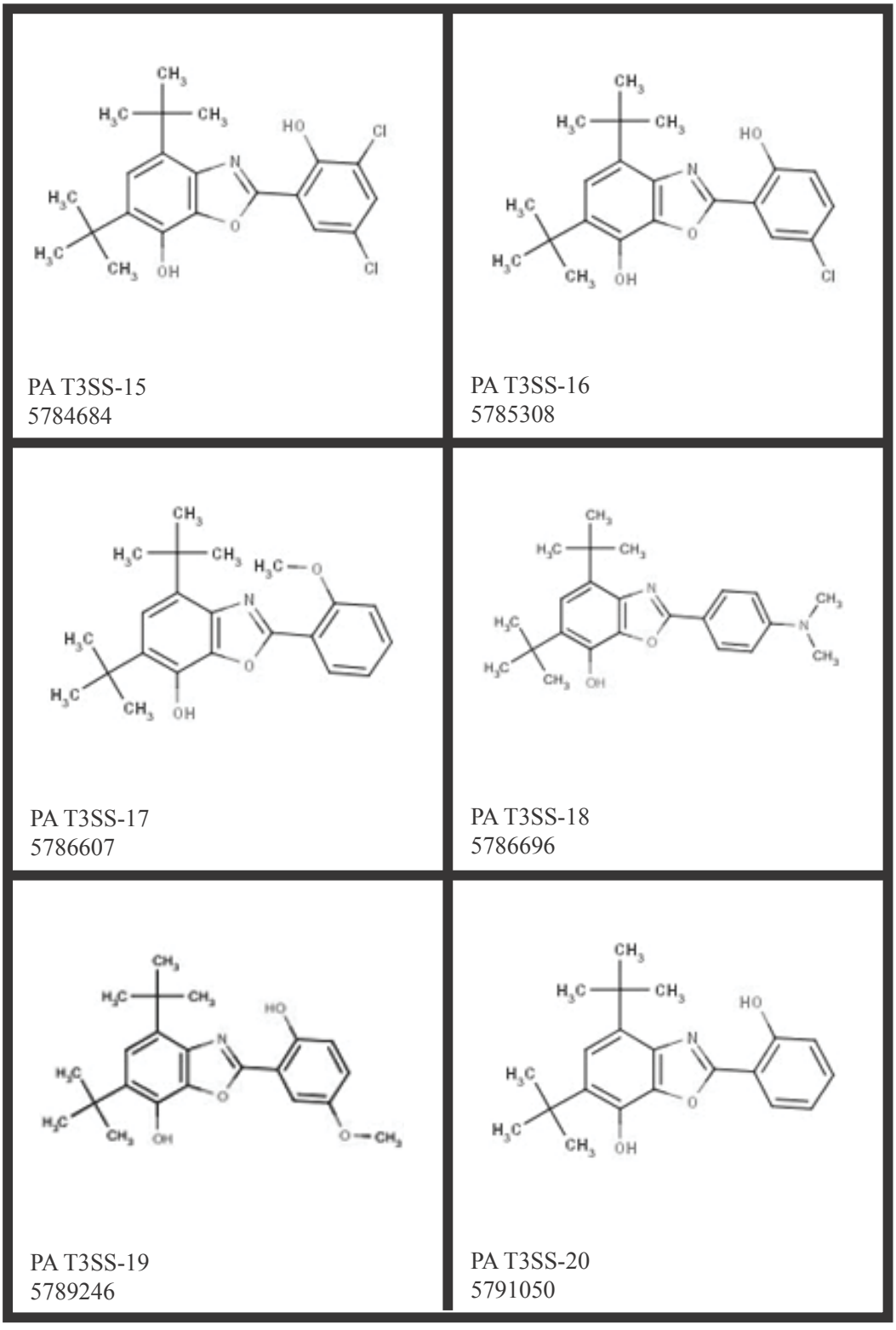


Table 6. 2-phenylbenzoxazole compounds PA T3SS 15-20

Compound screens

To identify compounds that inhibit T3SS mediated cell death, we adapted a method developed by Lee et al, 2007, through which inhibition of T3SS mediated cytotoxicity is correlated to reducing potential of compound-protected live cells in culture, relative to cells treated with DMSO carrier control, after infection with $T3SS^+$ or $T3SS^-$ *P. aeruginosa.*

In our screen, CHO, J774a.1, or RAW cells in confluent monolayer were treated with 20 μg/ml quinolozone, phenylquinazolinamine, or 2-phenylbenzoxazole compounds and subsequently inoculated with bacteria that use the T3SS to mediate cell death. Cell death was measured by assaying activity of lactate dehydrogenase (LDH) released by lysed cells into the extracellular media. LDH activity was quantified directly in 384 well culture plates using the Roche Cytotoxicity Detection Kit^{plus} (Roche Applied Science, Cat. 04 744 934 001) and Molecular Devices SpectraMax M5 (Abs 492-650). Percent LDH release from cells protected by experimental compounds was determined relative to uninfected cells treated with DMSO carrier control alone, or cells infected with $T3SS^+$ or $T3SS^-$ *P. aeruginosa*, *Yersinia* spp, or *S. typhimurium*, using the equation 100*(sample-background)/(positive control-background), where "sample" is cells treated with experimental compound and infected with $T3SS^+$ bacteria, "background" is uninfected cells treated with DMSO alone, and "positive control" is cells treated with DMSO and infected with $T3SS^+$ bacteria. Uninfected cells treated with DMSO behaved the same as those in DMSO free media. As an additional control, 10 μl of 1% Triton X-100 in tissue culture media was used to lyse cells to determine maximum LDH release.

Pathogen/Host used in quinolozone screens

P. aeruginosa PAK Δ*mexAB att::pro-exoU-spcU* pMMB-*exsA*: Bacteria were added to CHO K1 cells at MOI 10. LDH assay was performed 2 hours post infection.

S. typhimurium SL1344 pJK17: Bacteria were added to CHO K1 cells at MOI 30 and culture plate was centrifuged for 2 minutes at 172 *g* upon inoculation. Infection was stopped 90 minutes later by the addition of 1.5 mg/ml gentamicin (for 333 μg/ml final concentration), and LDH levels were assayed 90 minutes after addition of antibiotics.

S. typhimurium SL1344: Bacteria were added to J774a.1 cells at MOI 10 and culture plate was centrifuged for 2 minutes at 172 *g* upon inoculation. Infection was stopped 2 hours later by the addition of 1.5 mg/ml gentamicin (for 333 μg/ml final concentration), and LDH levels were assayed 3 hours after addition of antibiotics.

Y. enterocolitica w22703 pJK17: Bacteria were added to CHO K1 cells at MOI 10. Infection was stopped 2 hours later by the addition of 1.5 mg/ml streptomycin (for 333 μg/ml final concentration), and LDH levels were assayed 2 hours after addition of antibiotics.

Y. pseudotuberculosis YPIII: Bacteria were added to J774a.1 cells at MOI 20. Infection was stopped 2 hours later by the addition of 1.5 mg/ml streptomycin (for 333 μg/ml final concentration), and LDH levels were assayed 4.5 hours after addition of antibiotics.

Pathogen/Host used in phenylquinazolinamine and 2-phenylbenzoxazole screens

The phenylquinazolinamine and 2-phenylbenzoxazole screens were performed using the same methodology as the quinolozone screens, with slight modifications to pathogen/host combinations and duration of infection. As stated above, CHO K1 and CHO pro5 cells were used interchangeably, as were J774a.1 and RAW cells.

P. aeruginosa PAK Δ*mexAB att::pro-exoU-spcU* pMMB-*exsA:* Bacteria were added to CHO pro5 cells at MOI 10. LDH assay was performed 75 minutes post infection.

P. aeruginosa PAK: Bacteria were added to CHO pro5 cells at MOI 10. LDH assay was performed 5 hours post infection.

S. typhimurium SL1344: Bacteria were added to RAW cells at MOI 30 and culture plate was centrifuged for 5 minutes at 172 *g* upon inoculation. LDH levels were assayed 2 hours post infection.

Y. pseudotuberculosis YPIII: Bacteria were added to RAW cells at MOI 20. Infection was stopped 3 hours later by the addition of 1.5 mg/ml streptomycin (for 333 μg/ml final concentration), and LDH levels were assayed 2 hours after addition of antibiotics.

Determining target of PA-T3SS inhibitors

The compound screen is biased to detect inhibition of T3SS mediated cell death, but it does not give information about the compound target. After identifying a particular inhibitor from the screen, we used the compound in assays designed to elucidate the molecular target of T3SS inhibition. The assays we used to identify the molecular targets of inhibition are listed below, organized by potential mechanism of inhibition

including inhibition of cytotoxicity, machine assembly, secretion, translocation, and effector activity.

Inhibition of cytotoxicity

These assays were used to confirm the results from the initial screens and to determine host range of the PA T3SS inhibitors.

Cell viability staining

CHO K1 cells were seeded for confluency on sterile cover slips in a 24 well plate. 3.5 hours prior to inoculation, 1µl of DiOC membrane stain (3,3′-dioctadecyloxacarbocyanine perchlorate) was added to the cells. Cells were infected with PAK Δ*mexAB att::pro-exoU-spcU* pMMB-*exsA* MOI 10 for 2 hours, washed, and incubated for 5 minutes in the dark with 0.2% 3.75 mM propidium iodide. Cells were washed and cover slips mounted on glass slides for microscopic analysis.

Hypersensitive Response

P. syringae DC3000 and isogenic T3SS mutant Δ*hrc* were grown overnight at room temperature on LB agar. Bacteria were scraped from the agar and resuspended in 100 mM sucrose and 10 mM $MgCl_2$. Resuspensions were diluted and mixed with DMSO +/- experimental compound immediately prior to infiltration of *Nicotiana benthamiana*. Leaves were observed for Hypersensitive Response (HR) at 48 hours post infection.

Inhibition of machine assembly

These assays were used to interrogate the basal body and secretin as molecular targets for PA T3SS inhibitors.

Swim assay

Overnight cultures of *P. aeruginosa* PAK and isogenic mutant Δ*fliC* (lacking the major flagellar structural protein) were spotted on 0.3% LB agar plates supplemented with DMSO +/- experimental compound. Plates were incubated for 16 hours at 30°C with humidity.

Twitch assay

Overnight cultures of *P. aeruginosa* PAK and isogenic mutant Δ*pilA* (lacking the major fimbriael structural subunit) were spotted on 1% LB agar plates supplemented with DMSO +/- experimental compound. Plates were incubated for 16 hours at 30°C with humidity.

Proteolysis assay

P. aeruginosa PAK and isogenic general secretion pathway mutant Δ*xcp* were struck for isolation on LB agar supplemented with 10% milk and DMSO +/- experimental compound. Plates were incubated for 16 hours at 37°C.

Inhibition of secretion

This assay was used to determine if the PA-T3SS inhibitors blocked substrate recognition, machine engagement, and/or passage of effectors through the needle complex.

Overnight cultures of *P. aeruginosa* PAK pVL712 and PAK Δ*pscC* pVL712 were subcultured 1:1000 in LB broth with 5 mM EGTA and incubated for 3 hours at 37°C with aeration. Overnight culture of *Y. enterocolitica* w22703 pMM83 and Kum1 pMM83 were subcultured 1:200 in LB broth with 5 mM EGTA and incubated for 3 hours 37°C with aeration. At 3 hours 50 µg/ml nitrocefin (3-(2,4-Dinitrostyryl)-(6R, 7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic Acid, E-isomer) was added to wells and absorbance (OD 486 and 600) was measured using Molecular Devices SpectraMax M5 in 15-minute intervals for 75 minutes. Results reported at 60 minutes.

Inhibition of translocation

This assay was used to determine if the PA T3SS inhibitors blocked assembly and function of the translocon or recognition of the target host cell.

Overnight cultures of *P. aeruginosa* PAK Δ*exoS* pVL712 and *P. aeruginosa* PAK Δ*exoS* pVL710 were subcultured 1:50 and grown to OD 0.40 prior to inoculation of CHO pro5 cells (MOI 10). Two hours post infection, 9 µl substrate-loading solution (GeneBlazer CCF2-AM loading kit, Invitrogen) with anion transport inhibitor "solution D" (alternate loading solution, Invitrogen) was added to wells. The plate was covered and incubated at room temperature for 60 minutes. Excitation (390) and emission (465) of coumarin, and excitation (390) and emission of fluorescein (520) were measured using Molecular Devices SpectraMax M5.

Overnight cultures of *Y. enterocolitica* w22703 pMM83 and *Y. enterocolitica* w22703 Kum1 pMM83 were subcultured and grown to OD 1.0 prior to inoculation of RAW

cells (MOI 20). After inoculation, plates were centrifuged for 2 minutes at 127 *g* and incubated for 1 hour. One hour post infection, 9 µl of substrate loading solution (GeneBlazer CCF2-AM loading kit, without solution D, Invitrogen) was added to wells and the plate was covered and incubated at room temperature for 60 minutes. Excitation (390) and emission (465) of coumarin, and excitation (390) and emission of fluorescein (520) were measured using Molecular Devices SpectraMax M5.

Inhibition of effector activity

These assays were used to interrogate species-specific T3SS and effector proteins as molecular targets of the PA-T3SS inhibitors.

Visualization of actin cytoskeleton

CHO K1 cells were seeded for confluency on sterile cover slips and inoculated with *Y. enterocolitica* w22703 or *Y. enterocolitica* Kum1, MOI 10 for 3.5 hours at 37°C in 5% CO_2. After infection, cells were washed, fixed with 3.7% paraformaldehyde for 20 minutes, and permeabilized with 01% Triton X-100 for 20 minutes at room temperature. Cells were then stained with 1 ng/ml phalloidin-TRITC (tetramethyl rhodamine iso-thiocyanate) and 2.5 µg/ml DAPI (4',6-diamidino-2-phenylindole, dilactate. (Invitrogen) for 40 and 5 minutes, respectively. Cells were then washed and cover slips mounted to glass slides for microscopic analysis.

Gentamicin protection assay

S. typhimurium SL1344 MOI 30 were added to CHO K1 cells, centrifuged for 10 minutes at 172 *g* and incubated at 37°C in 5% CO_2. After 30 minutes, 15 mg/ml gentamicin was added to each well (for 105 µg/ml final concentration), and infection

was allowed to proceed for an additional 30 minutes. Cells were then washed, lysed with ddH_20 for 30 minutes, and plated on LB agar for CFUs. This method was adapted from Hong and Miller, 1998.

ExoS mediated apoptosis

P. aeruginosa PAK was added to CHO pro5 cells at MOI 10 and incubated at 37°C in 5% CO_2. LDH assay performed 5 hours post infection. Cell death was measured by assaying activity of lactate dehydrogenase (LDH) released into the extracellular media. LDH activity was quantified directly in 384 well culture plates using the Roche Cytotoxicity Detection Kit^{plus} (Roche Applied Science, Cat. 04 744 934 001) and Molecular Devices SpectraMax M5 (Abs 492-650). Percent apoptosis of cells protected by experimental compounds was determined relative to DMSO carrier control using the equation 100*(sample-background)/(positive control-background), where "sample" is cells treated with experimental compound and infected with $T3SS^+$ bacteria, "background" is uninfected cells treated with DMSO alone, and "positive control" is cells treated with DMSO and infected with $T3SS^+$ bacteria. Uninfected cells treated with DMSO behaved the same as those in DMSO free media. As an additional control, 10 µl of 1% Triton X-100 in tissue culture media was used to lyse cells to determine maximum LDH release.

Release of ^{3}H-arachidonic acid

CHO pro5 cells seeded in a sterile 24 well plate (8.3×10^4 cells/well) in MEMα with ribonucleosides and deoxyribonucleosides, supplemented with 1% glutamine and 10% FBS supplemented with 4 µCI of 37 MBq/mL ^{3}H-arachidonic acid, and grown

for 40 hours at 37°C in 5% CO_2. Cells were inoculated with either *P. aeruginosa* PAK Δ*mexAB att::pro-exoU-spcU* pMMB-*exsA* (MOI 10), *S. typhimurium* SL1344 pJK17 (MOI 30), or *Y. enterocolitica* w22703 pVL127 (MOI 10). After infection for 2.5, 3.75, and 3 hours respectively, cell culture media was collected. Cells were then treated with ddH_2O for 5 minutes, and media was collected. Finally, cells were treated for 5 minutes with 0.25% Triton X-100 in ddH_2O and media was collected. All samples were analyzed by scintillation counting.

Chapter 3: Quinolozone Compounds

Results

Quinolozone compounds PA T3SS 1-10 inhibit ExoU mediated cytotoxicity of CHO cells

Using the screen adapted from Lee et al, 2007, we employed *P. aeruginosa* strain PAK Δ*mexAB* att::*pro-exoU-spcU* pMMB-*exsA* (modified to inject ExoU for infection of CHO K1) to screen a class of chemicals called quinolozones for inhibition of cell death. ExoU is a *P. aeruginosa* T3SS substrate with phospholipase activity that causes rapid and acute cytotoxicity of host cells (Rabin 2003, Sato 2004). This strain was chosen because deletion of the *mexAB* genes that encode the efflux pump allows us to eliminate potential expulsion of compounds from bacterial cells, while the T3SS transcriptional promoter *exsA* on an IPTG inducible plasmid allows for the induction and overexpression of the T3SS.

Ten quinolozones (PA T3SS 1-10) showed a range of protection from ExoU mediated cytotoxicity with the most potent inhibitors reducing cell death by 65% to 95% relative to cells treated with DMSO alone (Figure 1a), and cell death was T3SS dependent and failed to occur during infection with *P. aeruginosa* T3SS mutant Δ*pscC*. To determine if inhibition was species dependent, *S. typhimurium* and *Y. enterocolitica* were engineered to secrete ExoU through their own heterologous T3SS, and subsequently used as the infectious agents in the screen. The compounds were noticeably less effective against *S. typhimurium*-ExoU mediated cell death

(Figure 1b), possibly because of differences in the *S. typhimurium* SPI-1 T3SS machinery (reviewed in Cornelis 2006), though the compounds offered protection similar to that with *P. aeruginosa* from *Y. enterocolitica*-ExoU mediated cell death (Figure 1c). We then confirmed compound protection of CHO cells from ExoU mediated cell death by propidium iodide staining. CHO K1 cells incubated with PA T3SS 1-10 prior to infection with *P. aeruginosa* PAK Δ*mexAB* att::*pro-exoU-spcU* pMMB-*exsA* were stained with propidium iodide nuclear and DiOC membrane stains for visualization of membrane permeability. Membrane permeabilization of CHO cells clearly resulted in cells treated with only the DMSO carrier control (Figure 2, Panel L) and failed to occur when cells were protected by ExoU inhibitor pseudolipasin A (Lee 2007) (Figure 2, Panel K), suggesting that the rapid cell death observed in the screen was both T3SS and ExoU mediated. Inhibition of ExoU mediated membrane permeability and cell death by the quinolozone panel was confirmed (Figure 2) with the exception of compound PA T3SS-5 (Figure 2, Panel E). Notably, the staining revealed that although compound PA T3SS-5 reduced cytotoxicity by 70% in the initial screen (Figure 1), it did not prevent permeabilization of CHO cells (Figure 2, Panel E). Structurally, all compounds are derived from a PA T3SS-5 backbone, suggesting inhibitory activity is conferred by side group substitutions within the PA T3SS-5 family. Compounds PA T3SS-1, PA T3SS-2, PA T3SS-3, PA T3SS-4, PA T3SS-6, PA T3SS-7, PA T3SS-8, PA T3SS-9, and PA T3SS-10 reduced ExoU mediated cytotoxicity of CHO cells by 80-95% *in vitro*. Since the compounds lack antibacterial activity *in vitro* (data not shown) we

next asked if the inhibition was targeted to ExoU or structural/regulatory elements of the T3SS.

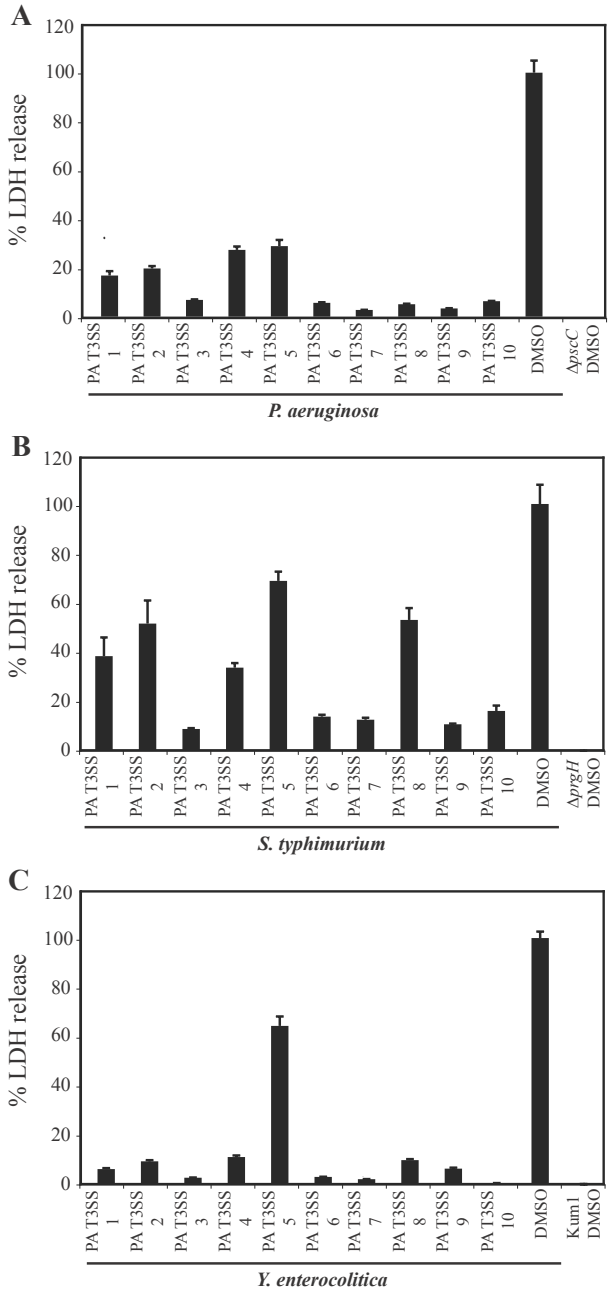


Figure 1. Quinolozone compounds PA T3SS 1-10 protection of CHO cells from ExoU mediated cell death. CHO K1 cells were treated with DMSO +/- 20 µg/mL compound 30 minutes prior to infection by *P. aeruginosa* PAK Δ*mexAB* att::*pro-exoU-spcU* pMMB-*exsA* or isogenic T3SS mutant Δ*pscC*. LDH release by cells treated with DMSO and infected with *P. aeruginosa* represents 60% of maximum LDH release (A), *S. typhimurium* SL1344 pJK17 or isogenic T3SS mutant Δ*prgH*. LDH release by cells treated with DMSO and infected with

S. typhimurium represents 90% of maximum LDH release (B), or *Y. enterocolitica* w22703 pJK17 or *Y. enterocolitica* Kum1 pVL127. LDH release by cells treated with DMSO and infected with *Y. enterocolitica* represents 52% of maximum LDH release (C). Cell death was reported as release of LDH measured using the Roche Cytotoxicity Detection Kit^{+}. The screen was done in duplicate on different days; data shown is the average of 8 wells from one assay plate and is representative of both days.

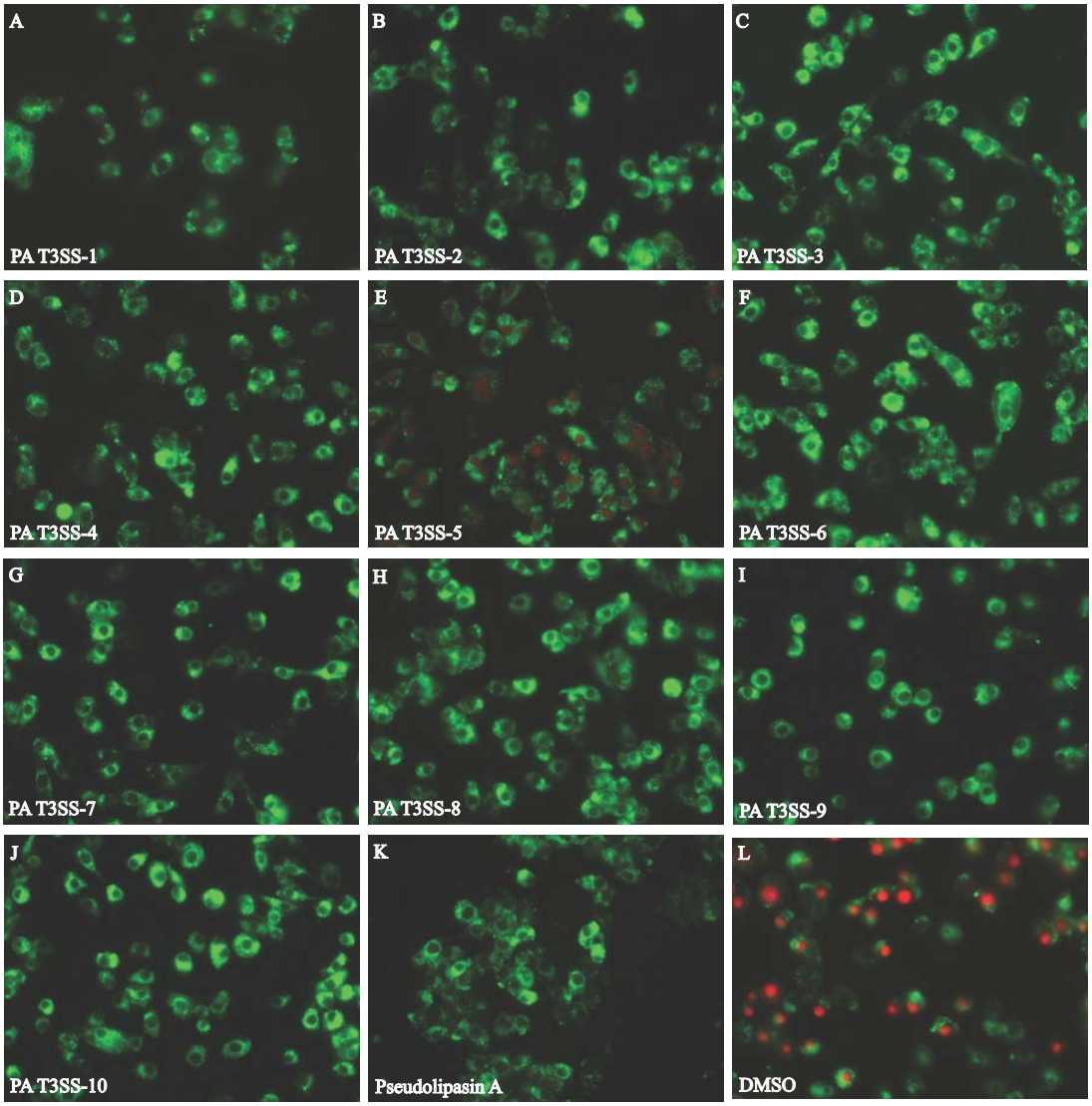


Figure 2. PA T3SS 1-10 protect CHO K1 cells from *P. aeruginosa* ExoU mediated cytotoxicity. CHO cells were treated with DMSO +/- 20 µg/mL quinolozone compound or pseudolipasin A, a known ExoU inhibitor, 30 minutes prior to infection by *P. aeruginosa* PAK Δ*mexAB* att::*pro-exoU-spcU* pMMB-*exsA* for 2 hours. CHO cells were stained with propidium iodide and DiOC prior to visualization. ExoU-mediated cytotoxicity is demonstrated by increased propidium iodide staining. The assay was done in duplicate on sequential days; data shown is representative of both days.

Quinolozone PA T3SS compounds do not target the heterologous T3SS of *S. typhimurium* or *Y. enterocolitica*

Nine quinolozone compounds strongly inhibit ExoU mediated cytotoxicity. To determine if the compounds target a common T3SS structural element, they were assayed for inhibition of *S. typhimurium* and *Y. enterocolitica* T3SS mediated virulence.

S. typhimurium uses a T3SS located on pathogenicity island 1 (SPI-1) to mediate invasion into intestinal epithelium. Expression of SPI-1 is upregulated in conditions of low oxygen; and upon host cell contact, effector proteins SopE, SopE2, SopB, SipA (Collazo 1997, Bakshi 2000, Friebel 2001) are translocated into the host where they mediate cytoskeletal changes promoting invasion and formation of a *Salmonella*-containing vacuole (SCV) (Raffatellu 2005, reviewed in Patel 2005). We asked if the quinolozone compounds inhibit the T3SS by measuring the percent invasion of *S. typhimurium* SL1344 into compound treated CHO K1 cells relative to invasion into cells treated with DMSO carrier control, using an assay adapted from Hong and Miller (1998). In brief, CHO cells were incubated with *S. typhimurium* and subsequently treated with gentamicin to kill the remaining extracellular bacteria. Cells were then lysed and the lysate was plated to calculate intracellular CFUs. Although we observed a slight reduction of *S. typhimurium* invasion in the presence

of compounds PA T3SS-1, PA T3SS-6, PA T3SS-8 and PA T3SS-9 (Figure 3), it was in a background where invasion in the presence of the DMSO carrier control alone was < 3% (Figure 3). In the context of such modest *S. typhimurium* invasion of CHO cells, we conclude that PA T3SS 1-10 failed to significantly inhibit invasion and do not target T3SS elements conserved between *P. aeruginosa* and *S. typhimurium*.

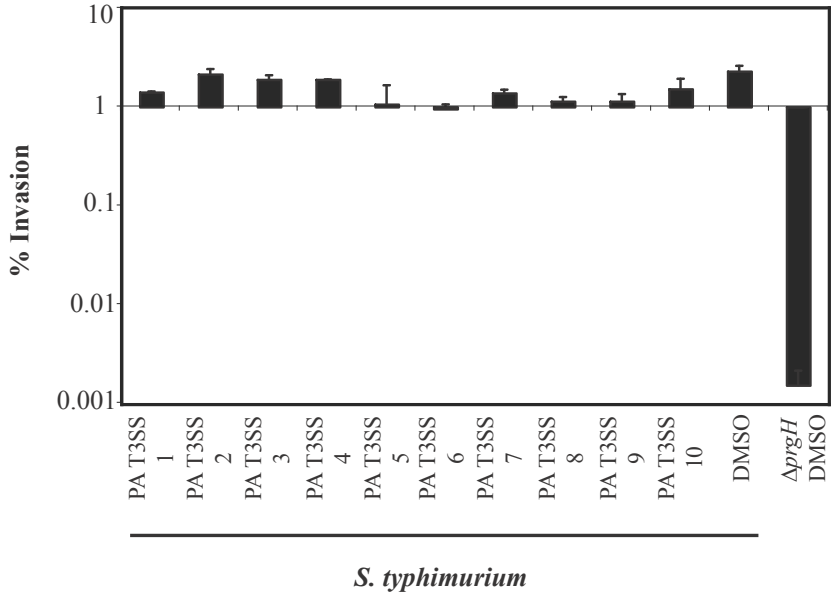


Figure 3. PA T3SS 1-10 inhibition of *S. typhimurium* T3SS mediated invasion. CHO K1 cells were treated with quinolozone compounds PA T3SS 1-10 30 minutes prior to infection by *S. typhimurium* SL1344 or isogenic T3SS mutant Δ*prgH* for 30 minutes. Following treatment of extracellular bacteria by gentamicin, CHO cells were lysed and lysate was plated for CFUs. The screen was done in duplicate on different days; data shown is the average of 2 dilutions from one well and is representative of both days.

As an additional confirmation, we tested PA T3SS 1-10 for inhibition of *S. typhimurium* T3SS dependent cell death. *S. typhimurium* is thought to mediate pyroptotic cell death in macrophages via interaction of translocated effector protein SipB with host caspase-1 (Hersh 1999, Brennan 2000, Fink 2006). Using the LDH

cytotoxicity assay, we tested the compounds for inhibition of *S. typhimurium* T3SS mediated cell death of J774a.1 murine macrophage-like cells. PA T3SS 1-10 failed to inhibit *S. typhimurium* mediated death of murine macrophage-like cells relative to infection in the presence of DMSO carrier control (Figure 4). These data support observation that the molecular target of inhibition is not conserved among species. Cell death was T3SS dependent, and failed to occur during infection with T3SS mutant Δ*prgH* (Figure 4).

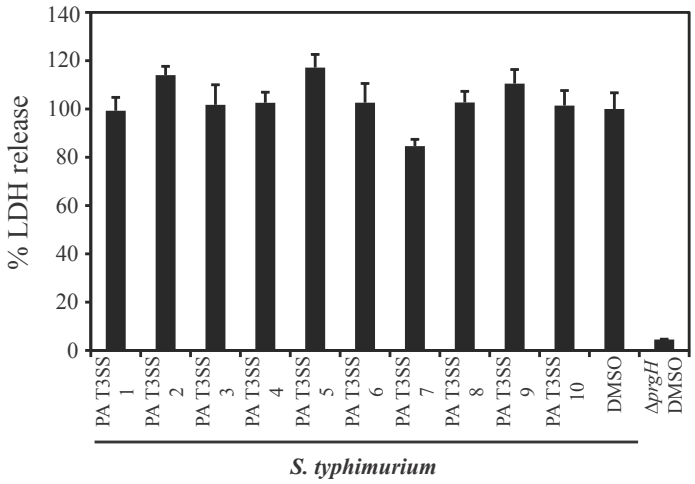


Figure 4. PA T3SS 1-10 fail to protect against *S. typhimurium* T3SS mediated cell death. J774a.1 murine macrophage-like cells were treated with DMSO +/- 20µg/mL compound 30 minutes prior to infection by *S. typhimurium* SL1344 or isogenic T3SS mutant Δ*prgH*. LDH release by cells treated with DMSO and infected with *S. typhimurium* represents 50% of maximum LDH release. Cell death reported as release of LDH, measured using the Roche Cytotoxicity Detection Kit^{+}. The screen was done in duplicate on different days; data shown is the average of 8 wells from one assay plate and is representative of both days.

Y. enterocolitica uses a T3SS to block phagocytosis by host macrophages; upon contact, effectors YopE, YopT, YopH and YpkA (Yop O) are translocated into the

macrophage cytosol and rearrange the actin cytoskeleton to block phagocytosis (reviewed in Cornelis 2002). We asked if the compounds inhibit injection of effectors by observing cell rounding in fluorescently labeled CHO K1 cells after infection by *Y. enterocolitica*. Cellular actin and nuclei were labeled with Phalloidin-TRITC and DAPI, respectively, prior to visualization. Treatment with the quinolozone PA T3SS compounds failed to prevent cell rounding as compared to cells treated with DMSO alone (Figure 5, Panel K), and rounding was T3SS dependent as cells infected with T3SS mutant Kum1 remained in confluent monolayer (Figure 5, Panel L). These data indicate that PA T3SS 1-10 do not inhibit *Y. enterocolitica* T3SS and taken with the *S. typhimurium* data, confirm that PA T3SS 1-10 do not target a T3SS structure conserved between *P. aeruginosa*, *S. typhimurium*, and *Y. enterocolitica*. Furthermore, since translocation of native effectors was allowed to proceed, inhibition of the *P. aeruginosa* T3SS is likely downstream, at or beyond effector activity.

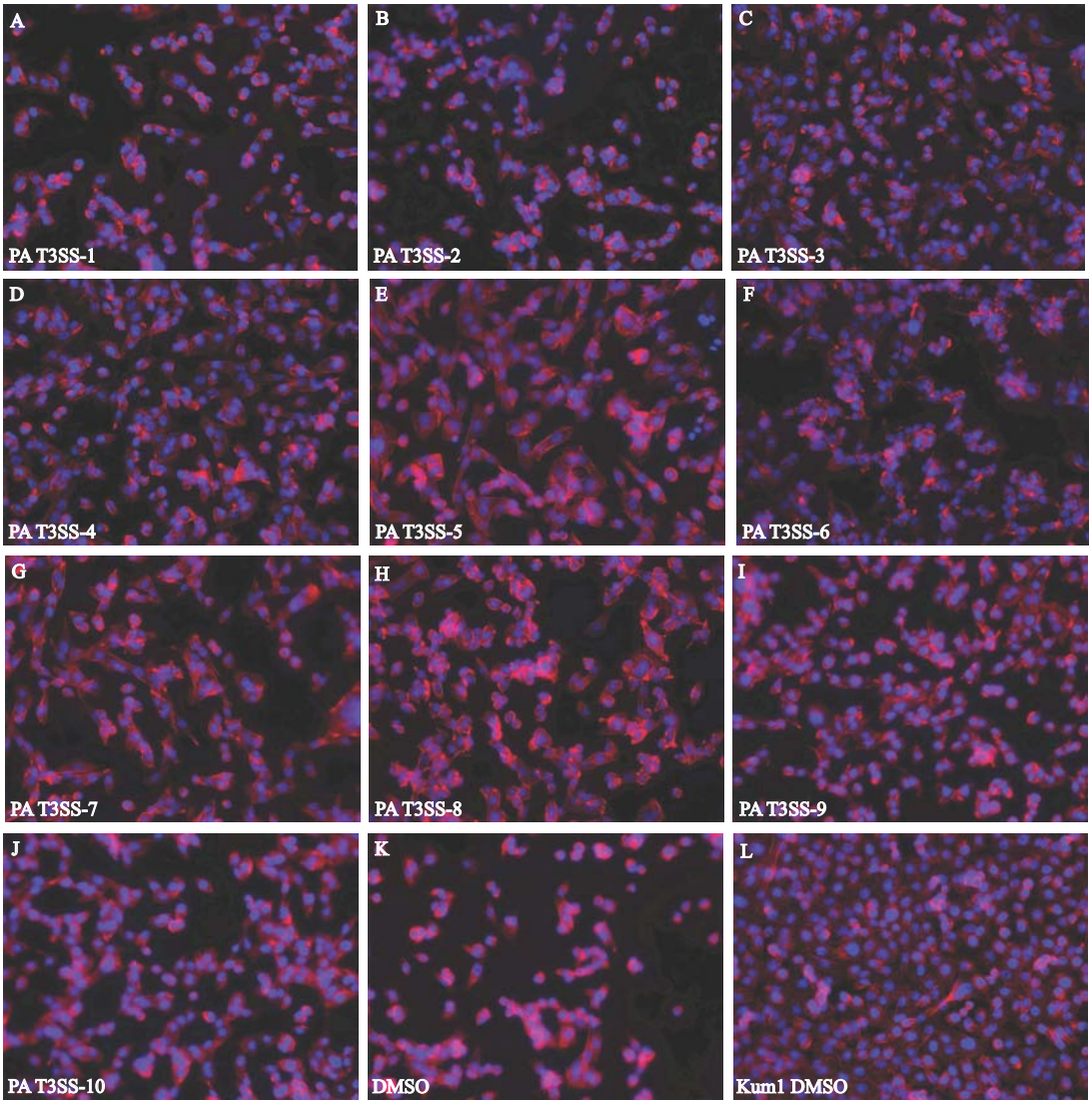


Figure 5. PA T3SS 1-10 do not inhibit *Y. enterocolitica* T3SS mediated actin remodeling. CHO K1 cells were treated with DMSO +/- compound 30 minutes prior to infection by *Y. enterocolitica* w22703 or *Y. enterocolitica* Kum1 for 3.5 hours. CHO cells were fixed, permeabilized and stained with phalloidin-TRITC and DAPI prior to visualization. The screen was done in duplicate on different days; data shown is representative of both days.

Although the data shows that PA T3SS 1-10 do not inhibit the *Y. enterocolitica* T3SS, as a confirmation, we tested PA T3SS 1-10 for inhibition of *Y. pseudotuberculosis* T3SS dependent cell death. *Y. pseudotuberculosis* initiates apoptosis in macrophages by blocking phosphorylation of MAPKK6 via activity of translocated effector YopJ (Mukherjee 2006). Using the LDH cytotoxicity assay, we tested the compounds for inhibition of *Y. pseudotuberculosis* T3SS mediated cell death of J774a.1 murine macrophage-like cells. In the LDH screen, compounds PA T3SS-6, PA T3SS-7, and PA T3SS-10 showed modest inhibition (~50%) of *Y. pseudotuberculosis* mediated cell death of J774a.1 cells relative to DMSO carrier control (Figure 6). Despite the modest inhibition, overall the compounds lacked the same efficacy seen in the ExoU screen (Figure 1). T3SS J774a.1 cell death was T3SS dependent, and failed to occur during infection with T3SS mutant Δ*yopHEJMOB* (Figure 6). Considered separately from the morphological data (Figure 5), it is possible that PA T3SS-6, PA T3SS-7, and PA T3SS-10 target a conserved element of the *P. aeruginosa* and *Yersinia* spp, but given the different strains and cell types used in each experiment, more work must be done to conclude that definitively. As it stands, our interpretation remains that the compounds are acting at, or downstream of, effector activity, possibly in a *P. aeruginosa* ExoU dependent manner.

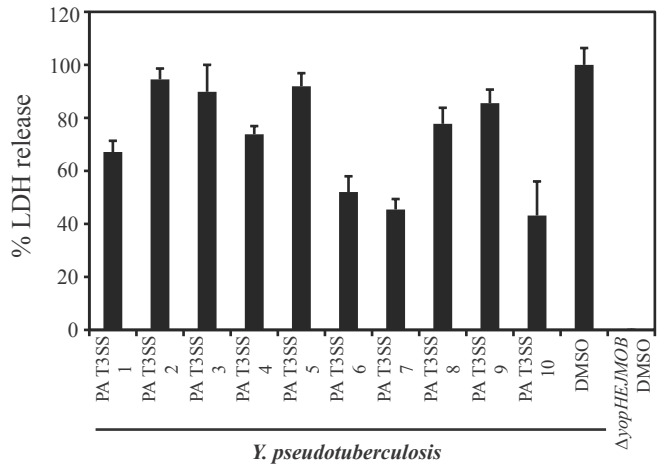


Figure 6. PA T3SS 1-10 do not protect against *Y. pseudotuberculosis* T3SS mediated cell death. J774a.1 cells were treated with DMSO +/- 20 μg/mL compound 30 minutes prior to infection by *Y. pseudotuberculosis* YPIII and isogenic T3SS mutant Δ*yopHEJMOB*. LDH release by cells treated with DMSO and infected with *Y. pseudotuberculosis* represents 50% of maximum LDH release. Cell death reported as release of LDH measured using the Roche Cytotoxicity Detection Kit^{+}. The screen was done in duplicate on different days; data shown is the average of 8 wells from one assay plate and is representative of both days.

Compound PA T3SS-9 blocks ExoU mediated cytotoxicity without altering enzyme function

Our observations support the hypothesis that the PA T3SS 1-10 are acting downstream of ExoU translocation. However, previous unpublished data demonstrated that the quinolozones failed to inhibit ExoU enzymatic activity (Vince Lee, spoken communication). ExoU is a phospholipase A_2 (Sato 2003) that cleaves the SN-2 acyl bond, releasing lysophospholipids and arachidonic acid (Saliba 2005), a potent intracellular signaling molecule and precursor of eicosanoids, cyclooxygenase and lipoxygenase (Dennis 1997, Sitkiewicz 2007).

It is believed that ExoU mediates necrotic cell death by cleavage of the cell membrane (Sato 2004), and this hypothesis is supported by work from Phillips et al,

showing that eicosanoid inhibitors fail to block ExoU mediated cell death *in vitro* (Phillips 2003). However, Saliba et al demonstrated the ability of cyclooxygenase (COX) inhibitors to reduce ExoU mediated inflammation *in vivo*, suggesting a possible role for eicosanoids in clinical infection (Saliba 2005). In light of this, we asked if PA T3SS 1-10 were acting downstream of effector activity by targeting a product of ExoU cleavage of membrane phospholipids.

To test this, we measured release of labeled arachidonic acid after infection of CHO cells in the presence of compound. CHO pro5 cells were allowed to incorporate ^{3}H-arachidonic acid (^{3}H-AA) into the plasma membrane prior to infection by *P. aeruginosa* PAK Δ*mexAB* att::*pro-exoU-spcU* pMMB-*exsA*. Post infection, ^{3}H-AA in cellular and media fractions was quantified by scintillation counting and percent ^{3}H-AA release was calculated. Compounds PA T3SS-1, PA T3SS-2, PA T3SS-3, and PA T3SS-4 reduced free ^{3}H-AA by about 38% while PA T3SS-6, PA T3SS-7, PA T3SS-8, and PA T3SS-10 reduced free ^{3}H-AA by up to 95% (Figure 7a). These results were similar to ExoU inhibitor pseudolipasin A, suggesting that inhibition of cell death by these compounds is mediated through interactions with ExoU. Only compound PA T3SS-9 appeared to minimally affect the overall release of arachidonic acid, showing ^{3}H-AA release levels similar to DMSO control and cytotoxic compound PA T3SS-5, suggesting that compound PA T3SS-9 is acting downstream of phospholipase mediated arachidonic release to inhibit cell death.

We repeated the experiment with *S. typhimurium* SL1344 pJK17, and *Y. enterocolitica* w22703 pVL127 but failed to duplicate the *P. aeruginosa* results; in the *S. typhimurium* infection model, compound PA T3SS-9 allowed as much ^{3}H-AA

release as compounds PA T3SS-1, PA T3SS-2, PA T3SS-3, and PA T3SS-4, with ^{3}H-AA release in the presence of PA T3SS-6, PA T3SS-7, PA T3SS-8, and PA T3SS-10 elevated as well, relative to ^{3}H-AA release during infection by *P. aeruginosa* (Figure 7b). When inoculated with *Y. enterocolitica* w22703 pJK17, all compounds, including ExoU inhibitor pseudolipasin A, allowed levels of ^{3}H-AA close to that of DMSO carrier control (Figure 7c). This result could be due, however, to inadequate compound concentration relative to hyper-secretion of ExoU by *Y. enterocolitica.* It should also be noted that in order to detect ^{3}H-AA release by cells infected by *S. typhimurium* pJK17 and *Y. enterocolitica* pVL127, infection had to proceed longer than in the original LDH screen. As a result, these data do not reflect arachidonic acid release at the time of inhibition of cytotoxicity seen in Figure 1 and should be considered accordingly. As it stands, these data suggest that compound PA T3SS-9 may block ExoU mediated cell death in a *P. aeruginosa* specific manner, unique from the other quinolozone PA T3SS compounds. However, more work must be done to identify the compound targets and mechanisms of inhibition.

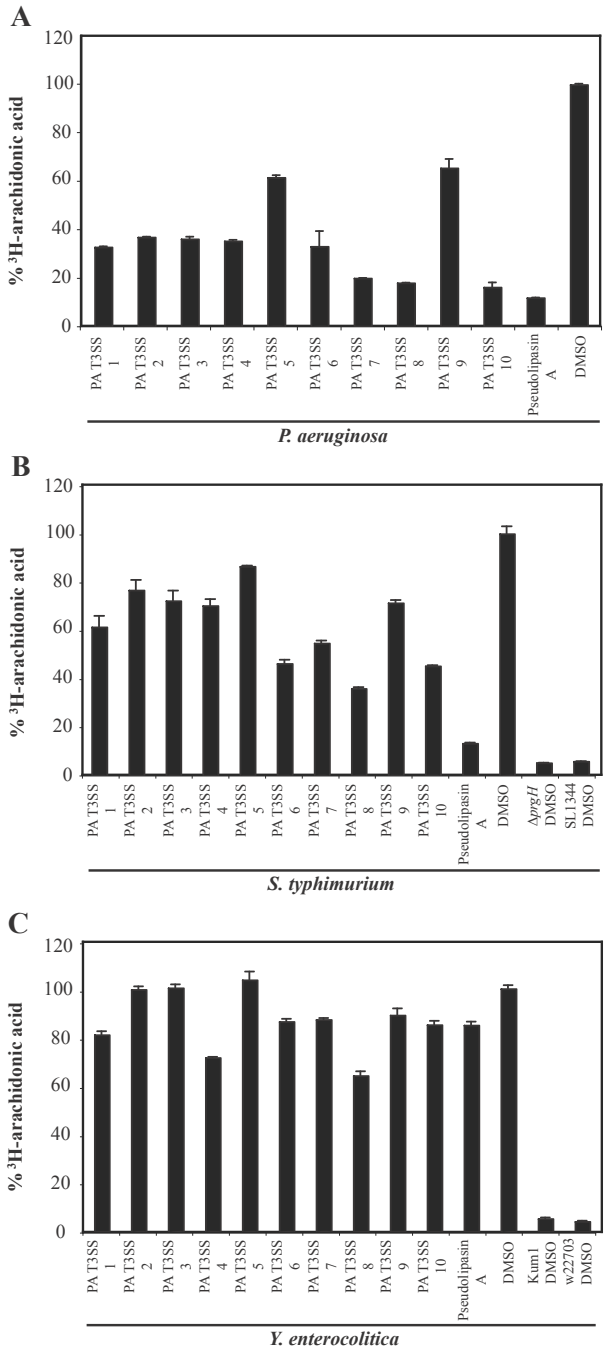


Figure 7. PA T3SS-9 does not inhibit ExoU mediated ^{3}H-arachidonic acid release. CHO pro5 cells were grown in media containing 4µCi of 37 MBq/mL ^{3}H-arachidonic acid 40 hours prior to infection by PAK Δ*mexAB* att::*pro-exoU-spcU* pMMB-*exsA* or isogenic T3SS mutant *ΔpscC (*A*), S. typhimurium* SL1344 pJK17 or isogenic T3SS mutant Δ*prgH* pJK17 or wt strain *S. typhimurium* SL1344(B), and *Y. enterocolitica* w22703 pJK17 or *Y. enterocolitica* KUM1 pVL127 or wt strain *Y. enterocolitica* w22703 (C). Post infection, culture media and cell lysates were collected and measured by scintillation counting. The screen was done in duplicate on different days; data shown is the average of 8 wells from one assay plate and is representative of both days.

Discussion

Using the screen adapted from Lee et al, 2007, we identified a class of compounds called quinolozones that inhibit ExoU mediated cell death *in vitro*. Quinolozones PA T3SS 1-10 did not block T3SS mediated invasion of CHO cells or T3SS mediated cell death of J774a.1 murine macrophage-like cells by *S. typhimurium*. PA T3SS 1-10 did not inhibit T3SS mediated actin cytoskeleton rearrangement by *Y. enterocolitica*, but compounds PA T3SS-6, PA T3SS-7, AND PA T3SS-10 showed modest inhibition of *Y. pseudotuberculosis* T3SS mediated cell death of J774a.1 murine macrophage-like cells. These data indicate that the molecular target of inhibition is not conserved between *P. aeruginosa*, *S. typhimurium*, or *Yersinia* spp., and that the mechanism of inhibition is ExoU specific. Of the 10 compounds, PA T3SS-9 stands out as inhibiting cell death without significantly reducing production of ExoU phospholipase cleavage products in a *P. aeruginosa* infection model (Figure 7a). This observation led us to hypothesize that rather than causing necrotic cell death by disruption of the lipid bilayer, ExoU phospholipase activity generates a lipid intermediate that initiates a programmed cell death cascade, and it is upstream or within that cascade that compound PA T3SS-9 is working. We tried several approaches to test this hypothesis. To identify the putative signaling molecule, we collected cellular fractions post *P. aeruginosa* infection of CHO cells with DMSO +/- compound PA T3SS-9 and analyzed them using mass spectrometry, but were unable to identify differences between the samples. To confirm the existence of the putative signaling molecule, we took filtered lysates of CHO cells post infection with *P. aeruginosa* and syringe loaded them into untreated CHO cells to trigger "lipid intermediate" mediated cell death, but the background of cell death from the technique was very high which made the experiment difficult to control for.
As a result, we were unable to characterize the mechanism of inhibition by the compounds. Nevertheless, our data reveal that PA T3SS-9 is a good candidate for

further study as both an inhibitor of ExoU mediated cell death as well as a tool to study how ExoU causes acute cytotoxicity of host cells.

Chapter 4: Phenylquinazolinamine and 2-phenylbenzoxazole Compounds

Results

A cell-based screen identifies phenylquinazolinamine compound PA T3SS-11 and 2-phenylbenzoxazole compound PA T3SS-19 as inhibitors of *P. aeruginosa*, *S. typhimurium*, and *Y. pseudotuberculosis* T3SS mediated cell death

Using the same initial screen employed to test the quinolozone compounds, we tested previously identified phenylquinazolinamine and 2-phenylbenzoxazole inhibitors of PAK Δ*mexAB* att::*pro-exoU-spcU* pMMB-*exsA* ExoU mediated cytotoxicity of CHO pro5 cells for inhibition of T3SS mediated cell death of RAW murine macrophage-like cells by *S. typhimurium* SL1344 and *Y. pseudotuberculosis* YPIII, respectively. We identified two compounds, PA T3SS-11 and PA T3SS-19, that inhibited T3SS mediated cell death by ≥ 80% in all three screens, at 20 μg/mL (Figure 8). However, the structural analogs of compounds PA T3SS-11 and PA T3SS-19 did not perform consistently against the three tested species. Of the PA T3SS-11 analogs (PA T3SS-12, 13, and 14) compounds PA T3SS-12 and PA T3SS-14 inhibited ExoU mediated death of CHO cells by 60% and PA T3SS-13 inhibited by 40% (Figure 8a), whereas compounds PA T3SS-13 and PA T3SS-14 protected RAW cells from *S. typhimurium* induced cell death by 60% but PA T3SS-12 only protected by 20% (Figure 8b). None of the PA T3SS-11 structural analogs protected RAW cells from *Y. pseudotuberculosis* mediated cell death (Figure 8c). Among the PA T3SS-19 structural analogs, PA T3SS-15, 16, 17, 18, and 20, compound PA T3SS-16 inhibited CHO cell death by 40%, but only PA T3SS-18 protected CHO cells as well as PA

T3SS-19 did (80% inhibition, Figure 8d). Compounds PA T3SS-16 and PA T3SS-18 protected RAW cells from *S. typhimurium* mediated cell death by 60%, but only when measured at 60 minutes, suggesting that these analogs were metabolized by the host, came out of solution, or lost activity in some other way (Figure 8e). Finally, all of the PA T3SS-19 analogs inhibited *Y. pseudotuberculosis* mediated cell death to varying degrees, but none as well as PA T3SS-20, which blocked cell death by 90% (Figure 8f). The changes made to PA T3SS-11 and PA T3SS-19 to create the structural analogs affected their ability to protect against broad range T3SS mediated cell death, but in some cases conferred protection against individual species. This could reflect the existence of a moiety on the compounds that is essential for multi-species targeting, and the structure of the analogs could have changed the overall character of the compound, hence the different phenotypes observed.

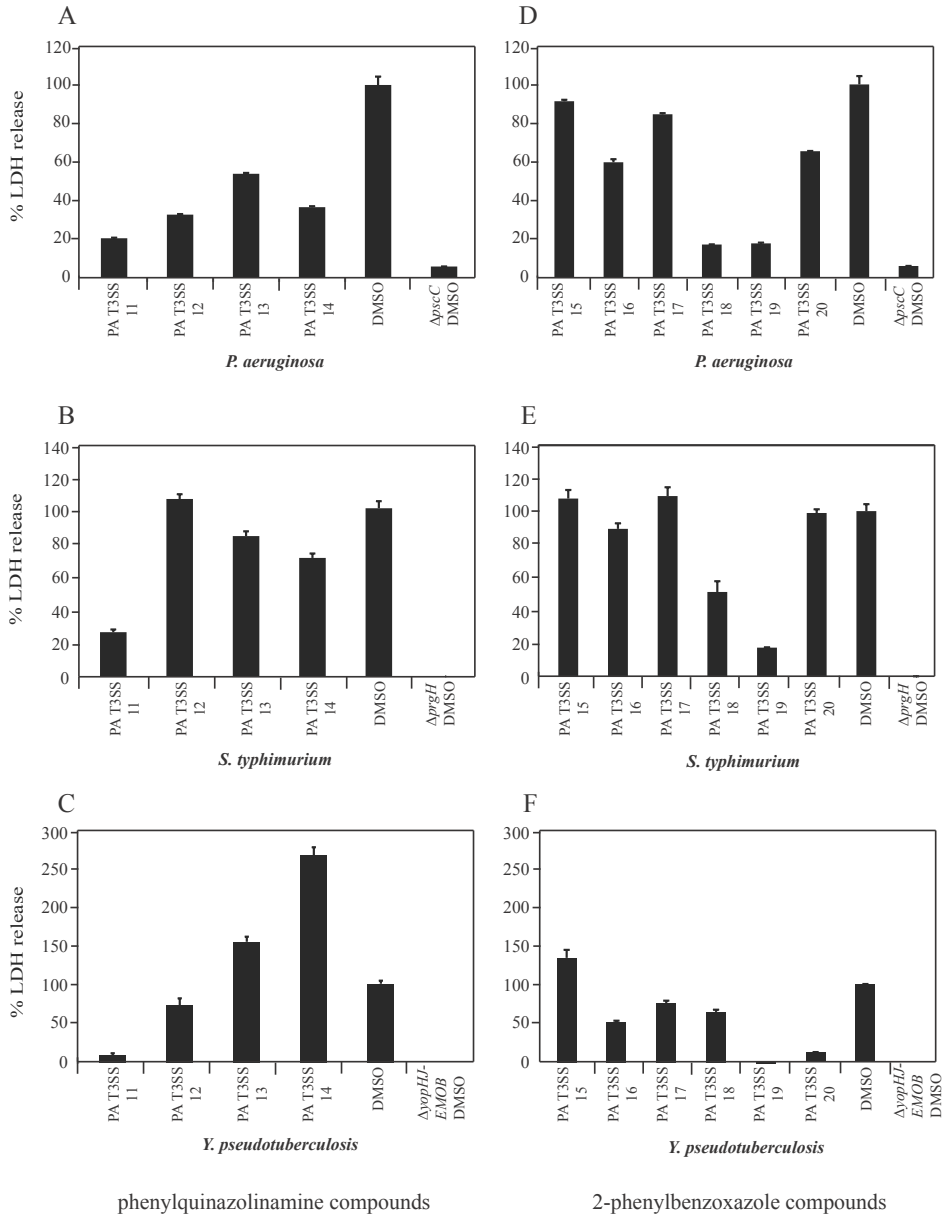


Figure 8. Phenylquinazolinamine and 2-phenylbenzoxazole compounds protect against T3SS mediated cell death. CHO cells were treated with DMSO +/- 20 µg/mL compound 30 minutes prior to infection by *P. aeruginosa* PAK Δ*mexAB* att::*pro-exoU-spcU* pMMB-*exsA* or isogenic T3SS mutant Δ*pscC* (A, D). RAW cells were treated with DMSO +/- 20 µg/mL compound 30 minutes prior to infection by *S. typhimurium* SL1344 or isogenic T3SS mutant Δ*prgH* (B, E), and *Y. pseudotuberculosis* YPIII or isogenic T3SS mutant Δ*yopHEJMOB* (C, F). In the phenylquinazolinamine screens, LDH release by cells treated with DMSO and infected with *P. aeruginosa*, *S. typhimurium*, and *Y. pseudotuberculosis* represents 100%, 82%, and 31% of maximum LDH release, respectively. In the 2-phenylbenzoxazole screens, LDH release by cells treated with DMSO and infected with *P. aeruginosa*, *S. typhimurium*, and *Y. pseudotuberculosis* represents 85%, 66%, and 44% of maximum LDH release, respectively. Cell death reported as release of LDH measured using the Roche Cytotoxicity

Detection Kit^{+}. The screen was done in duplicate on different days; data shown is the average of 8 wells from one assay plate and is representative of both days.

As only two broad range inhibitors the T3SS have been identified to date (Kauppi 2003, Bailey 2007, Negrea 2007, Felise 2009), we decided to focus on characterizing compounds PA T3SS-11 and PA T3SS-19. Although *P. aeruginosa, S. typhimurium* and *Y. pseudotuberculosis* all utilize a T3SS for effector delivery, the effectors themselves are diverse and modulate host cell structure and function in unique ways, and thus a single inhibitor of T3SS across species is probably acting on a conserved structure, rather than on genetic regulation of the T3SS or activity of the effectors. To assess the broad range ability of compounds PA T3SS-11 and PA T3SS-19, we tested them for protection of *Nicotiana benthamiana* from plant pathogen *P. syringae* T3SS mediated cell death. *P. syringae* uses a T3SS to colonize the apoplast of a pathovar specific host and cause necrotic disease. However, when infiltrating a non-specific host, *P. syringae* attempts to colonize but the T3SS instead elicits a host immune "Hypersensitive Response" (HR), characterized by controlled apoptosis around the site of infiltration that changes the leaf texture from smooth, green, and waxy to dry, rough and grey (reviewed in Lindeberg 2006). Using a protocol adapted from Felise et al, 2009, we inoculated leaves with $5x10^5$ cfu of *P. syringae* in the presence of compound PA T3SS-11 or PA T3SS-19 but failed to see any inhibition of HR relative to DMSO carrier control (Figure 9a). HR is T3SS dependent, and did not occur when leaves were infiltrated with T3SS mutant *P. syringae* Δ*hrc*. Failure to observe inhibition could be due to the concentration of *P. syringae,* or to lack of the appropriate compound target, so we repeated the experiment with inoculum diluted

twofold. Despite the diluted infiltrate, the compounds did not inhibit the T3SS dependent HR (Figure 9b). The structure of the *P. syringae* T3SS machine is less related to those from the animal pathogens (reviewed in Cornelis 2006), and since compounds PA T3SS-11 and PA T3SS-19 failed to inhibit T3SS mediated HR at either concentration, we conclude that the compound target is not conserved between animal pathogens and *P. syringae* pv. tomato.

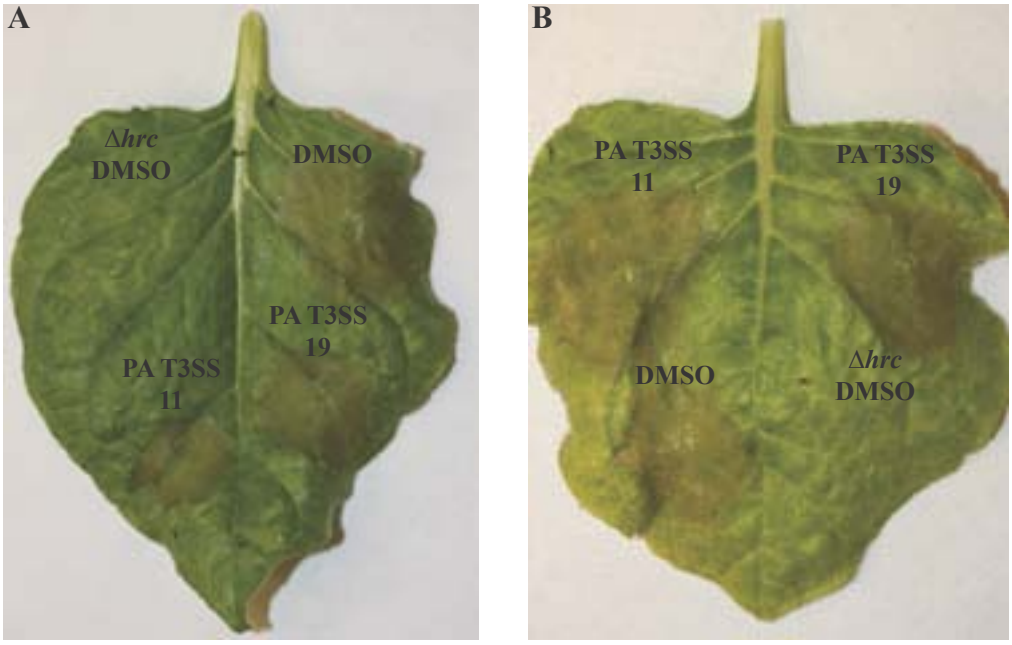


Figure 9. PA T3SS-11 and PA T3SS-19 do not inhibit *P. syringae* T3SS dependent Hypersensitive Response. $5x10^5$ cfu *P. syringae* DC 3000 and DC3000 Δ*hrc* were mixed with DMSO +/- 20 µg/mL compound immediately prior to infiltration of *N. benthamiana* (A). $2.5x10^5$ cfu *P. syringae* DC 3000 and DC3000 Δ*hrc* were mixed with DMSO +/- 20 µg/mL compound immediately prior to infiltration of *N. benthamiana* (B). Photos taken at 48 hours post infection. The screen was done in duplicate on different days; data shown is representative of both days.

PA T3SS-11 and PA T3SS-19 do not target structures homologous to the T3SS basal body or secretin

The T3SS shares homology with MS ring and basal body proteins, core structural proteins of the bacterial flagella that mediate anchoring within the inner membrane (reviewed in Blocker 2003). To test if compounds PA T3SS-11 and PA T3SS-19 are acting on those homologous regions, we used flagella mediated swimming as a metric for molecular targeting of conserved proteins by PA T3SS-11 and PA T3SS-19. Briefly, *P. aeruginosa* PAK inoculated on 0.3% agar with DMSO +/- compound use their flagella to move away from the inoculation site in search of additional nutrients, thus forming a hazy swim zone radiating from the inoculation site. Compounds PA T3SS-11 and PA T3SS-19 do not significantly reduce swim zones mediated by *P. aeruginosa* PAK relative to DMSO carrier control, or the negative control strain PAK Δ*fliC*, an amotile genetic mutant lacking the main flagellar structural protein (Figure 10). This suggests that the T3SS inhibition phenotype is not MS ring or basal body dependent and that PA T3SS-11 and PA T3SS-19 act elsewhere to inhibit T3SS mediated virulence.

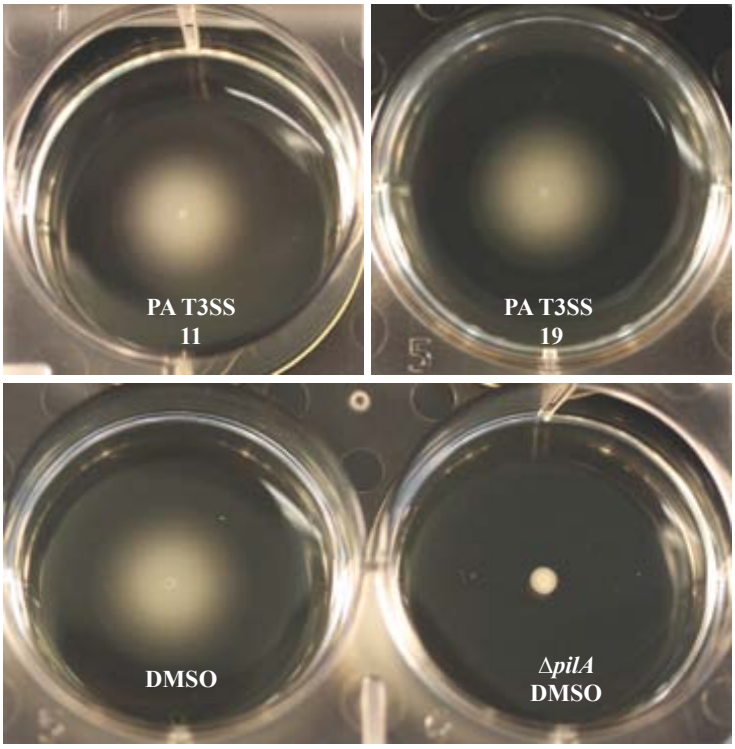


Figure 10. PA T3SS-11 and PA T3SS-19 do not inhibit flagella mediated swimming. *P. aeruginosa* PAK and PAK Δ*fliC* were spotted on 0.3% LB agar supplemented with DMSO +/- 20 μg/mL compound and incubated for 16 hours. Swim zones indicated by halo.

Recently, Felise et al described a compound that inhibits T3SS by targeting the T3SS outer membrane donut, or secretin, a structural component of the T3SS machine (Felise 2009). The T3SS secretin shares homology with the secretin of the type 4 pilus, a structure used by *P. aeruginosa* PAK to mediate twitching motility. Similar to the swim test, we used the twitch phenotype as a metric for inhibition. Briefly, *P. aeruginosa* PAK inoculated on 1% agar with DMSO +/- compound use type 4 pili to twitch away from the inoculation site in the zone in between the agar and the plastic plate. No reduction in the PA T3SS-11 or PA T3SS-19 twitch zones relative to the zones of PAK with DMSO compound control or genetic mutant Δ*pilA*, which lacks

the fimbriael structural subunit, were observed (Figure 11), suggesting that the compound target is not the secretin.

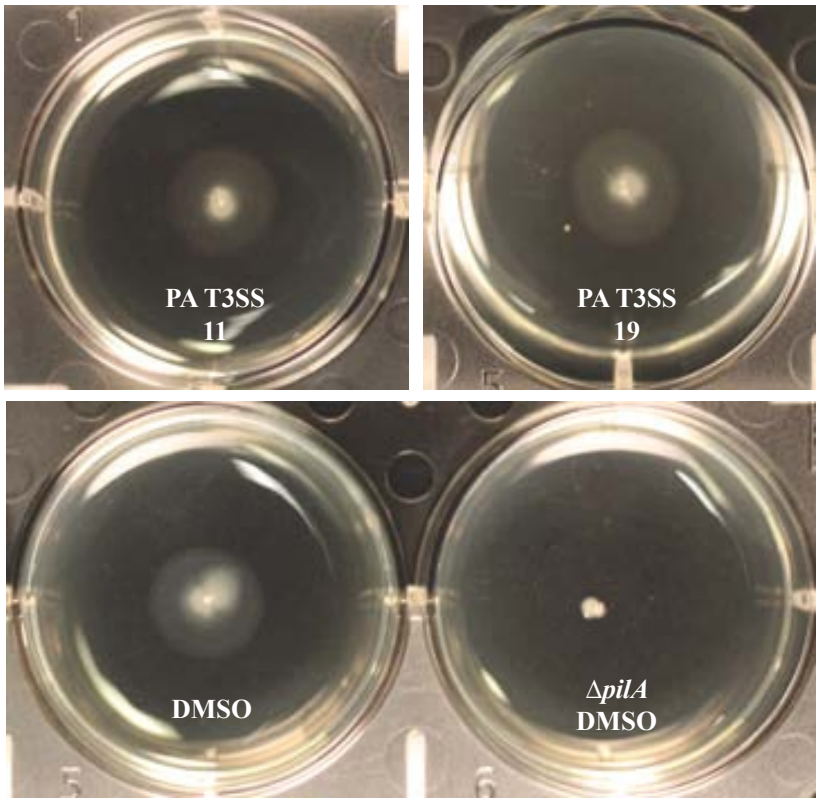


Figure 11. PA T3SS-11 and PA T3SS-19 do not inhibit pili mediated twitching. *P. aeruginosa* PAK and PAK Δ*pilA* were spotted on 1% LB agar supplemented with DMSO +/- 20 µg/mL compound and incubated for 16 hours at 30°C with humidity. Twitch zones indicated by halo.

For additional confirmation, we looked at the type two secretion system (T2SS). The T2SS secretin shares homology with the secretin of the T3SS. Taking advantage of this similarity, we used secretion of elastase as a metric for inhibition. *P. aeruginosa* uses the T2SS to secrete elastase into the extracellular milieu (reviewed in Filloux et

al, 1998). Elastase preferentially cleaves elastin, a structural component of mammalian tissue but also shows non-specific proteolysis of milk proteins like casein and whey. This elastase dependent proteolysis was assayed by streaking *P. aeruginosa* PAK on protease plates (2.5% milk) with DMSO +/- compound and observing zones of clearance surrounding the bacterial colonies. No inhibition of PAK T2SS elastase dependent cleavage of milk proteins in the presence of PA T3SS-11 or PA T3SS-19 was observed relative to DMSO carrier control (Figure 12) or a PAK Δ*xcp* T2SS mutant that lacks the general secretion pathway operon, confirming that compounds PA T3SS-11 and PA T3SS-19 are not targeting the secretin for T3SS inhibition.

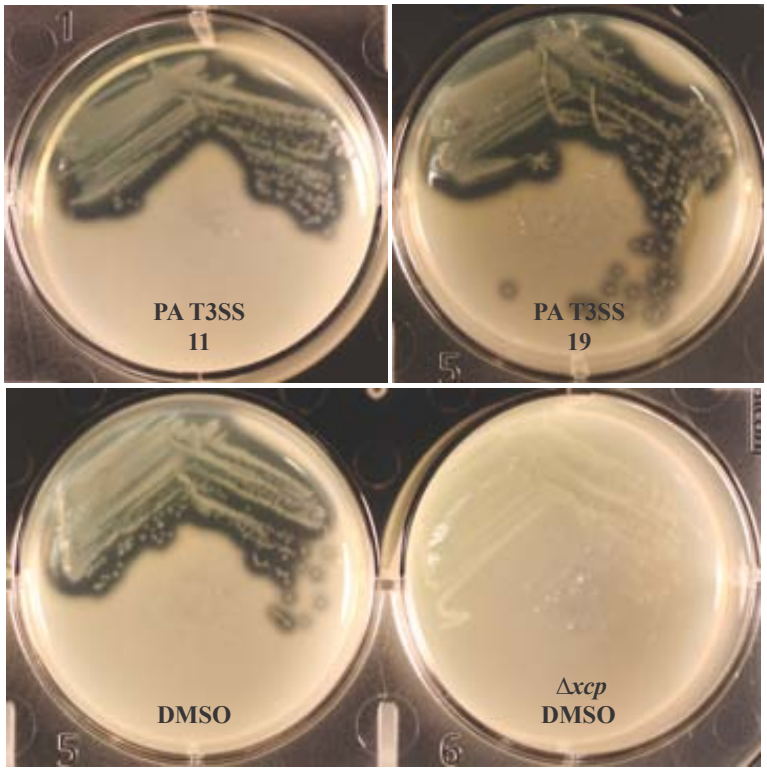


Figure 12. PA T3SS-11 and PA T3SS-19 do not inhibit T2SS secretion of elastase. *P. aeruginosa* PAK and PAK Δ*xcp* were spotted on 1% LB agar supplemented with 10% milk and DMSO +/- 20 µg/mL compound and incubated for 16 hours at 37°C. Proteolysis indicated by zones of clearing.

Compounds PA T3SS-11 AND PA T3SS-19 do not inhibit secretion of T3SS substrates

Secretion of T3SS substrates is a multi step and multi protein process that includes substrate recognition, entry, and passage through the needle (Lee 1999); this process is distinct from translocation of substrates into the host cell and can be assayed *in vitro* by measuring effectors in culture media. To test if the compounds were inhibiting effector secretion, we measured hydrolysis activity of an ExoU- β-lactamase fusion (pVL712) secreted through the PAK T3SS. The *exoU* gene with catalytic mutation S142A was fused to the N-terminus of β-lactamase, which hydrolyzes penicillin analog nitrocefin present in the culture media, allowing secretion to be measured as a function of nitrocefin hydrolysis. Compound PA T3SS-11 showed a modest, 15% decrease in secretion but neither PA T3SS-11 nor PA T3SS-19 significantly reduced secretion of ExoU S142A-β-lactamase (Figure 13a) relative to DMSO carrier control. To confirm these results, we repeated the experiment using *Y. enterocolitica* engineered to secrete a YopM-β-lactamase fusion (pMM83), and while both PA T3SS-11 and PA T3SS-19 reduced secretion by 16% and 20% respectively, relative to DMSO carrier control, the reduction was not significant (Figure 13b). In both cases, nitrocefin cleavage was dependent on the effector- β-lactamase fusion and was substantially reduced in the presence of *P. aeruginosa* Δ*pscC* and *Y. enterocolitica* Kum1 T3SS mutants. Despite the modest

reduction in secretion by compounds PA T3SS-11 and PA T3SS-19, these data suggest that the compounds do not inhibit substrate recognition or passage through the needle complex.

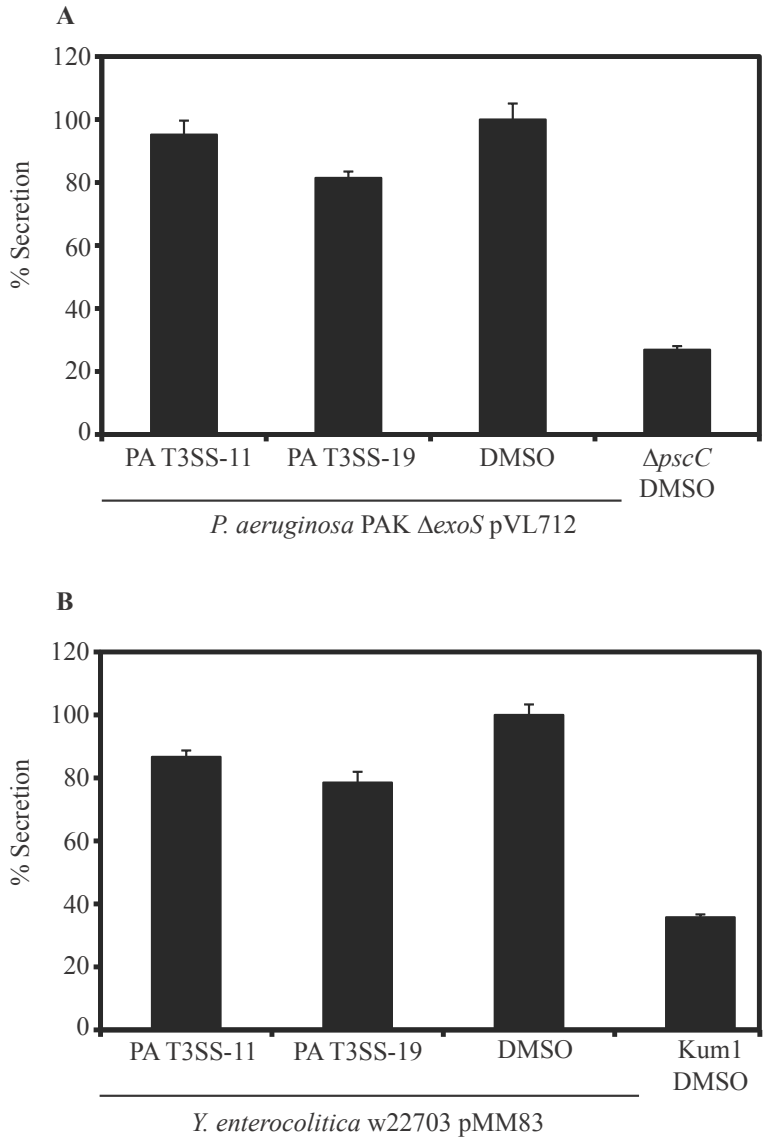


Figure 13. PA T3SS-11 and PA T3SS-19 do not inhibit secretion of effectors. *P. aeruginosa* PAK pVL712 or isogenic T3SS mutant Δ*pscC* (A) and *Y. enterocolitica* w22703 pMM83 or isogenic T3SS mutant Kum1 (B) were incubated with DMSO +/- 20 µg/mL compound. After incubation, 50 µg/ml nitrocefin was added to wells and absorbance (OD 486 and 600) was determined. Results reported at 60 minutes post addition of nitrocefin. The screen was done in duplicate on different days; data shown is the average of 8 wells from one assay plate and is representative of both days.

PA T3SS-11 AND PA T3SS-19 do not inhibit translocation of T3SS substrates

In *Pseudomonas* and *Yersinia*, translocation is mediated by a protein multimer, the "translocon", composed of PopB, PopD, and PcrV, or YopB, YopD, or LcrV, respectively (Sarker 1998, Schoehn 2003, Goure 2004). These secreted proteins are thought to form a pore in the host cell membrane that facilitates translocation of effectors into the host cytosol (Håkansson 1996). We tested for inhibition of translocon function by measuring effector translocation into the host.

Effector translocation was determined by Fluorescence Resonance Energy Transfer (FRET) of CCF2-AM when cleaved by the translocated ExoU S142A- β-lactamase fusion within the host cell. CHO pro5 cells were infected with PAK pVL712 (ExoU S142A-β-lactamase) in the presence of DMSO +/- compound, and translocation was reported as a ratio of coumarin emission (465) to fluorescin emission (535). Fluorescence ratios from PA T3SS-11 treated cells were similar to those of the DMSO carrier control, and in the case of PA T3SS-19 treated cells looked to exceed that of the control, suggesting that compounds PA T3SS-11 and PA T3SS-19 both failed to inhibit translocation of *P. aeruginosa* PAK ExoU S142A-β-lactamase into CHO pro5 cells (Figure 14a). We confirmed this result by testing translocation of *Y. enterocolitica* w22703 YopM- β-lactamase into RAW cells, and again fluorescence ratios from PA T3SS-11 and PA T3SS-19 treated cells are similar to DMSO carrier

control (Figure 14b). Together these data indicate the compounds are working downstream from injection to prevent T3SS mediated cell death. Additionally these data support our previous findings that the compounds are not inhibiting any of the steps upstream from translocation.

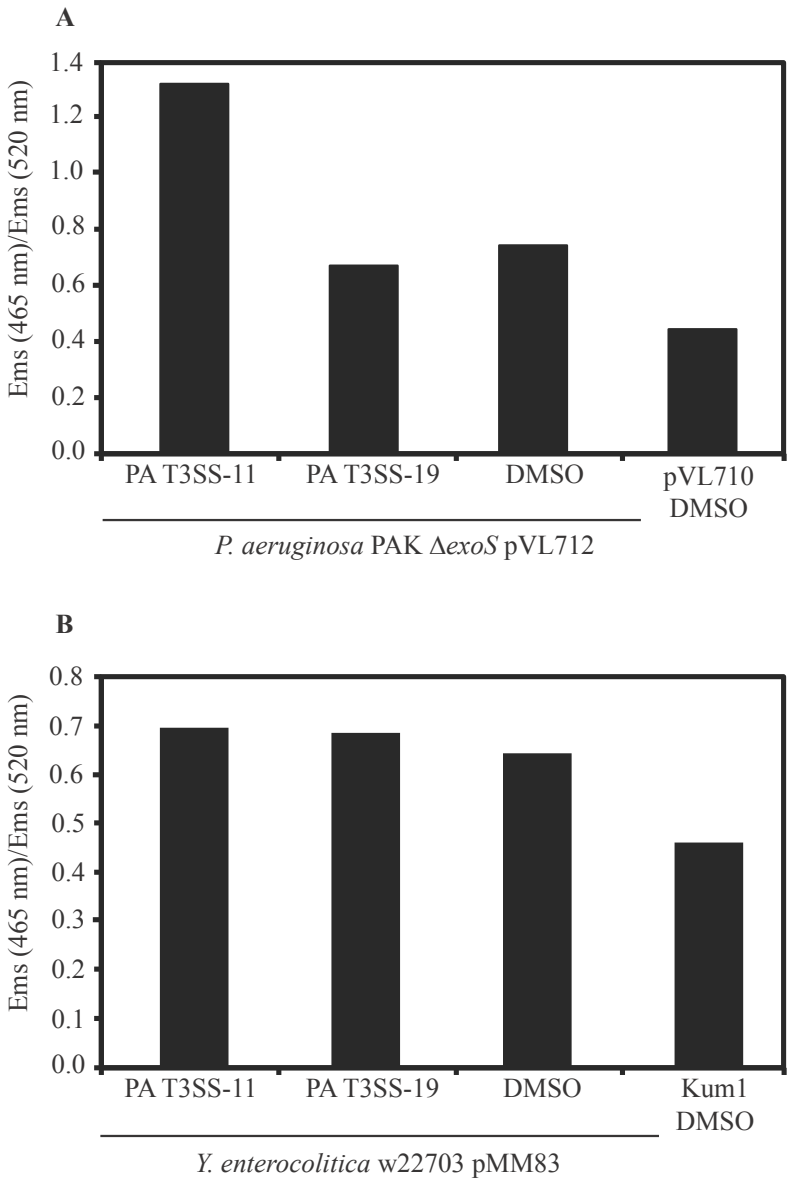


Figure 14. PA T3SS-11 and PA T3SS-19 do not inhibit translocation of effectors. CHO cells were treated with DMSO +/- 20 µg/mL compound 30 minutes prior to infection by *P.*

aeruginosa PAK pVL712 or PAK pVL710. (A). RAW cells were treated with DMSO +/- 20 µg/mL compound 30 minutes prior to infection by *Y. enterocolitica* w22703 pMM83 or isogenic T3SS mutant Kum1 (B). Translocation was determined by measuring FRET using the Invitrogen GeneBlazer loading kit, and FRET is reported as a ratio of coumarin to fluorescein fluorescence. The screen was done in duplicate on different days; data shown is representative of both days.

PA T3SS-19 inhibits ExoS mediated apoptosis

Our data indicates that compounds PA T3SS-11 and PA T3SS-19 are acting downstream from translocation to inhibit T3SS mediated cell death. Despite conservation of the T3SS machine, pathogens employ unique effector proteins to modify or subvert host cell function, so we tested PA T3SS-11 and PA T3SS-19 for inhibition of *P. aeruginosa* effector ExoS mediated cell death. ExoS is a bifunctional protein containing an N-terminal GAP domain and a C-term ADP ribosylating domain that cause cell rounding and apoptosis in CHO cells, respectively (Kaufman 2000, Lee 2005). To test for inhibition of ExoS mediated apoptosis, PAK was allowed to infect CHO pro5 cells in the presence of DMSO +/- compound for 5 hours prior to addition of LDH reagent. Compound PA T3SS-19 reduced ExoS mediated cell death by 60%, while PA-T3SS-19 appeared to increase cell death over that of cells treated with DMSO carrier control by 35% (Figure 15). These data allow the possibility that the compounds are acting on individual effector proteins rather than conserved T3SS motifs, but that hypothesis necessitates further investigation.

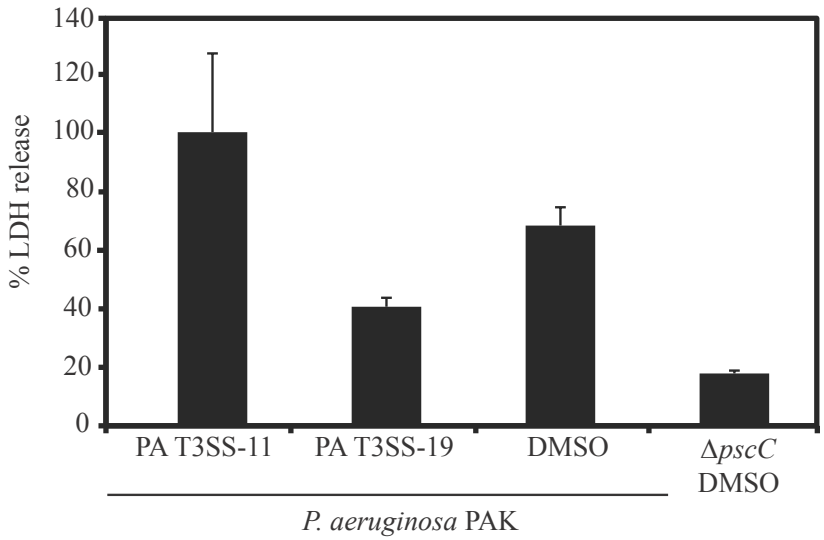


Figure 15. PA T3SS-19 protects CHO cells from ExoS mediated cell death. CHO cells were treated with DMSO +/- 20 μg/mL compound 30 minutes prior to infection by *P. aeruginosa* PAK or isogenic T3SS mutant Δ*pscC*. LDH release by cells treated with DMSO and infected with *P. aeruginosa* represented 42% of maximum LDH release. Cell death reported as release of LDH measured using the Roche Cytotoxicity Detection Kit^{+}. The screen was done in duplicate on different days; data shown is the average of 8 wells from one assay plate and is representative of both days.

Discussion

Using the screen adapted from Lee et al, 2007, we identified two compounds, PA T3SS-11 and PA T3SS-19, that are able to inhibit *P. aeruginosa* ExoU mediated cell death, *S. typhimurium* mediated cell death, and *Y. pseudotuberculosis* mediated cell death, but unable to protect *N. benthamiana* from *P. syringae* HR. The compounds' ability to protect epithelial and murine macrophage-like cells from multiple types of cell death suggested to us that they block conserved structures or functions of the T3SS, and we were able to rule out the basal body and secretin as well as secretion and translocation of effectors as targets. As mentioned earlier, effectors are species

specific and functionally diverse; they also require host cofactors (Sato 2006, Ottmann 2007) or substrates associated with specific cell types (Mukherjee 2006), so we initially overlooked effectors as compound targets. However, our results suggest that compounds PA T3SS-11 and PA T3SS-19 are working downstream from translocation to inhibit T3SS mediated cell death, so we tested for inhibition of apoptosis mediated by *P. aeruginosa* T3SS effector protein ExoS. PA T3SS-19 reduced ExoS mediated apoptosis by 35%, suggesting a molecular target of inhibition that is specific to secreted effectors. Alternatively, PA T3SS-19 could be working on the host side to inhibit cell death, but this is unlikely given the absence of common elements between *P. aeruginosa* ExoU and ExoS, *S. typhimurium* and *Y. pseudotuberculosis* mediated cell death. Ultimately, additional work is warranted to identify the molecular targets of PA T3SS-11 and PA T3SS-19 inhibition.

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