Published in final edited form as:

Biochemistry. 2018 December 18; 57(50): 6906–6916. doi:10.1021/acs.biochem.8b01077.

Shutting Down *Shigella* Secretion: Characterizing Small Molecule Type Three Secretion System ATPase Inhibitors

Heather B. Case¹, Dominic S. Mattock¹, and Nicholas E. Dickenson^{1,*}

¹Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322

Abstract

Many important human pathogens rely on one or more type three secretion systems (T3SS) to inject bacterial effector proteins directly into the host cell cytoplasm. Protein secretion through the needle-like type three secretion apparatus (T3SA) is essential for pathogen virulence and relies on a highly-conserved ATPase at the base of the apparatus, making it an attractive target for anti-infective therapeutics. Here, we leveraged the ability to purify an active oligomeric *Shigella* T3SS ATPase to provide kinetic analyses of three T3SS ATPase inhibitors of Spa47. In agreement with *in silico* docking simulations, the inhibitors displayed non-competitive inhibition profiles and efficiently reduced Spa47 ATPase activity with IC₅₀s as low as $52 \pm 3 \mu\text{M}$. Two of the inhibitors functioned well *in vivo*, nearly abolishing effector protein secretion without significantly affecting *Shigella* growth phenotype or HeLa cell viability. Furthermore, characterization of Spa47 complexes *in vitro* and *Shigella* T3SA formation *in vivo* showed that the inhibitors do not function through disruption of Spa47 oligomers or by preventing T3SA formation. Together, these findings suggest that inhibitors targeting Spa47 may be an effective means of combating *Shigella* infection by shutting down type three secretion without preventing presentation of the highly antigenic T3SA tip proteins that aid in clearing the infection and developing pan-*Shigella* immunological memory. In summary, this is the first report of *Shigella* T3SS ATPase inhibitors and one of only a small number of studies characterizing T3SS ATPase inhibition in general. The work presented here provides much-needed insight into T3SS ATPase inhibition mechanisms and provides a strong platform for developing and evaluating non-antibiotic therapeutics targeting Spa47 and other T3SS ATPases.

Graphical Abstract

*To whom correspondence should be addressed: Nicholas Dickenson, Department of Chemistry and Biochemistry, Utah State University, 0300 Old Main Hill, Logan, UT 84321, Tel. 435-797-0982, Fax. 435-797-3390 nick.dickenson@usu.edu.

The authors declare no competing financial interest.

The X–Y data sets for Figure 2A–D and Figure 5B are indexed in OpenAIRE (<https://doi.org/10.5281/zenodo.1447212>)

Keywords

Shigella; Spa47; ATPase; Inhibitor; T3SS; Type three secretion system; Antibiotic resistance

Introduction:

Antibiotic therapy has been the treatment of choice for bacterial infections since the 1940's; however, the emergence of antibiotic and multi-antibiotic resistance has significantly reduced the breadth of antibiotics effective against many human pathogens.^{3,4} The Centers for Disease Control and Prevention (CDC) report more than 2 million annual antibiotic resistant bacterial infections in the United States alone, resulting in more than 23,000 deaths per year.⁶ In response to the global threat of antibiotic resistance, the World Health Organization (WHO) recently released a list of twelve antibiotic resistant “priority pathogens” that pose the greatest risk to human health and urgently require new antibiotics and treatment strategies to combat them.⁷ *Pseudomonas*, *Salmonella*, and *Shigella* are three of these priority pathogens and while the modes of transmission and the diseases they cause are distinct, each are members of a class of human pathogens that rely on one or more type three secretion systems (T3SSs) to inject effector proteins directly into the host cell cytoplasm.^{8–10} Additionally, each T3SS encodes a structurally conserved type three secretion apparatus (T3SA) that supports effector protein secretion and is essential to the virulence of the pathogen, providing an attractive target for novel anti-infective therapeutics.^{1,11–13}

The T3SA is a complex nano-machine that resembles a nano-hypodermic needle and syringe comprised of four main sections: the basal body spans the inner and outer bacterial membranes, the 2.5 nm inner diameter needle extends from the basal body past the extracellular lipopolysaccharide (LPS) layer of the bacteria, and an associated protein tip complex serves as an environmental sensor, regulator of protein secretion through the apparatus, and as an anchor that penetrates the host cell membrane.^{14–18} The cytoplasmic bulb includes several additional soluble protein components, including an essential T3SS ATPase, that assemble within the bacterial cytoplasm at the base of the apparatus and are believed to be responsible for substrate recognition,^{19,20} unfolding,¹³ and secretion through the narrow T3SA needle.^{21–23} Disruption of any of these processes or failure to assemble a complete apparatus eliminates the ability of many pathogens to cause infection and has fueled studies targeting the T3SS as a non-antibiotic means of treating infections.^{24–26} While antibiotics target critical cellular processes such as cell wall biosynthesis, DNA

replication, RNA transcription, and protein expression that result in strong selective pressure for survival,²⁷ targeting the T3SS would not place a direct selective pressure on the pathogen for viability when in the environment or during stages of infection when the T3SS is not required, but would prevent the pathogens' ability to infect host cells and evade host immune responses. The host immune system would then be free to clear the T3SS-compromised bacteria while the host gains the additional benefit of developing immunological memory of the pathogen and reducing the risk of the bacteria developing resistance against the drug.

One of the earliest discoveries of small molecule T3SS inhibitors came from a small molecule screen identifying three compounds that significantly reduced protein secretion through the T3SA of *Yersinia pseudotuberculosis*, though like many identified small molecule inhibitors, the precise mechanism of inhibition remains somewhat unclear.^{28,29} Similar high-throughput screens have continued to identify compounds that inhibit T3SS function and while the precise targets for these small molecules often remain enigmatic as well, those that have been elucidated are generally linked to downregulating transcription of essential T3SS genes.^{30–32} A notable exception, however, is the potent and stereoselective class of phenoxyacetamide T3SS inhibitors that specifically target the *Pseudomonas aeruginosa* T3SS needle protein PscF, preventing effector protein secretion through the apparatus.^{33,34} As an alternative to cell-based screening, a specific T3SS target can be first identified and small molecule ligands can either be designed or screened against that target protein. One of the most attractive T3SS targets for this approach is the highly-conserved ATPase located at the base of the T3SA. While the specific role(s) of the T3SS ATPases are not fully understood, it is clear that they are critical to both T3SA formation and effector protein secretion through the apparatus in many T3SSs^{1,35,36} and that their ability to form oligomeric complexes and hydrolyze ATP are intimately involved in these processes.^{1,37,38} Despite the attraction of T3SS ATPases as a target for small molecule therapeutics, limited structural and biochemical information about T3SS ATPases and challenges in expressing and purifying native, active species have limited the development of compounds targeting these critical enzymes. In fact, only three such studies have been published to date, identifying T3SS ATPase inhibitors against YscN (*Yersinia*), EscN (*E. coli*), and SctN (*Chlamydia*), with each identifying compounds that reduce effector protein secretion levels through the T3SA.^{39–41} With T3SS protein effector secretion strongly correlated to pathogen virulence, these pioneering studies provide promise for T3SS ATPases as viable targets for anti-infective therapeutics, making it more important than ever to fully understand the mechanism(s) through which T3SS ATPases are activated and regulated *in vivo* and to specifically dissect the means by which identified small molecule T3SS ATPase inhibitors function.

To provide insight into the specifics of T3SS ATPase inhibition mechanism(s) and the potential of T3SS inhibitors as cross-pathogen therapeutics, we examined the effects of three previously identified YscN inhibitors³⁹ on the activity of the related T3SS ATPase, Spa47, from *Shigella flexneri*. We additionally tested the effect of each inhibitor on *Shigella* protein secretion profiles, T3SA formation, and overall pathogen viability. While each of the tested compounds inhibit Spa47 activity *in vitro*, only two of the three compounds inhibited effector protein secretion through the T3SA. Furthermore, docking simulations to a 2.4 Å crystal structure of Spa47 together with detailed inhibition kinetic analyses support an

unexpected non-competitive inhibition mechanism of Spa47 that may explain the specificity of the compounds toward the T3SS ATPase as the inhibitors had no marked effect on *Shigella* growth profiles and as little as 6% toxicity toward cultured mammalian epithelial cells. Together, these findings provide detailed kinetic analysis of a T3SS ATPase with small molecule T3SS inhibitors, describe the effects of the Spa47 inhibitors on *Shigella* type three secretion profiles, and support the feasibility of targeting T3SS ATPases for the development of novel anti-infective therapeutics.

Experimental Procedures:

Materials.

Wild-type *Shigella flexneri* corresponds to the serotype 2a 2457T strain originally isolated in 1954.⁴² The *S. flexneri spa47* null strain was engineered by Abdelmounaaim Allaoui as described by Jouihri et al.³⁵ The Superdex 200 Increase 16/600 and Superdex 200 Increase 5/150 size exclusion columns and 5 mL HiTrap Q FF columns were purchased from GE Healthcare (Pittsburgh, PA). Chitin resin was from New England Biolabs (Ipswich, MA). ATP and Congo Red were from Sigma-Aldrich (St. Louis, MO), and α -³²P ATP was from PerkinElmer (Boston, MA). Dithiothreitol (DTT), and ampicillin were from Gold Biotechnology (St. Louis, MO). The malachite green assay kit was purchased from BioAssay Systems (Hayward, CA). Rabbit polyclonal antibodies against IpaC were a generous gift from Wendy and William Picking (University of Kansas). The conjugated monoclonal anti-GAPDH antibody was from Thermo Scientific (Waltham, MA) and the Alexa 647 goat anti-rabbit secondary antibody was from Life Technologies (Carlsbad, CA). The Spa47 inhibitors were from Enamine (Monmouth Jct., NJ). The MTT cytotoxicity assay kit was purchased from Biotium (Fremont, CA). All other solutions and chemicals were of reagent grade. The UniProtKB accession number for Spa47 is P0A1C1.

Methods:

Molecular Docking Studies.

The recently solved 2.4 Å monomeric structure of Spa47 (PDB code 5SWJ)¹ was aligned to each of the protomers of the 2.8 Å hetero-hexameric F1 $\alpha_3\beta_3$ ATP synthase structure (PDB code 1BMF)⁵ using PyMol,⁴³ as described previously.¹ The resulting Spa47 homo-hexamer model was energy minimized using Discovery Studio ProViewer. Two adjacent Spa47 subunits were extracted from the energy minimized hexameric model to provide a single interface and complete active site between Spa47 protomers for the downstream docking simulations. The PyRx Virtual Screening tool⁴⁴ and Auto Dock Vina⁴⁵ were used to perform docking simulations for each of the tested Spa47 inhibitors. The Spa47 protein was modeled as a single rigid-body molecule while the inhibitors were flexible around torsional angles. This provided both visual models and corresponding binding energies for each of the top nine highest affinity interactions for each inhibitor.

Protein Expression and Purification.

The *spa47* gene was cloned into the expression plasmid pTYB21 for expression in *E. coli* and pWPsf4 for expression in *Shigella* as described previously.^{1,37} Spa47 encoded in

pTYB21 was transformed into *E. coli* Tuner (DE3) cells and was expressed and purified as previously described.^{1,37} Briefly, the expression strain was grown to mid log phase in Terrific Broth (TB) medium containing 0.1 mg/ml ampicillin at 37 °C, 200 rpm. The culture was then cooled to 17 °C before induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for ~20 h (17 °C, 200 rpm). All subsequent steps were carried out at 4 °C unless otherwise stated. The cells were pelleted by centrifugation, resuspended in binding buffer (20 mM Tris, 500 mM NaCl, 48 mg/L 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), pH 7.9), and lysed by sonication. The sonicated product was then centrifuged, and the supernatant was run over a chitin affinity column to capture the chitin binding domain (CBD)-intein-Spa47 fusion complex. Spa47 was eluted from the column by intein cleavage in binding buffer containing 50 mM DTT. Several Spa47 elutions were collected and analyzed for protein by SDS-PAGE. The elution fractions containing Spa47 were pooled and diluted using 20 mM Tris buffer to reduce the final NaCl concentration to 100 mM and the final DTT concentration to 10 mM. The Spa47 was further purified by negative selection over a 5-ml Q Sepharose FF anion exchange column. The purified Spa47 in the anion exchange flow-through was concentrated using an ultra centrifugal filter unit with a 30-kDa molecular mass cutoff and further purified/characterized using a Superdex 200 16/600 size exclusion column equilibrated with 20 mM Tris, 100 mM NaCl, 5 mM DTT, pH 7.9. Because Spa47 does not contain any tryptophan residues, all Spa47 concentrations were determined using in-gel densitometry of Coomassie-stained protein with bovine serum albumin (BSA) as a standard (as previously described).³⁷ All Spa47 concentrations are reported in monomer concentration units for consistency and clarity.

Kinetic Analyses of Spa47.

Spa47 ATPase activity was measured using a malachite green assay kit according to manufacturer guidelines. The effect of the inhibitors on the rate of ATP hydrolysis was examined on SEC-isolated Spa47 oligomers. The concentration of Spa47 was held constant at 0.05 μM and exposed to increasing concentrations of each of the tested small molecule inhibitors (50 μM, 100 μM, 200 μM, 350 μM, and 500 μM). The inhibitors were initially dissolved in DMSO to a concentration of 20 mM and the amount of DMSO in each reaction was maintained at 10% as this concentration of DMSO did not adversely affect the activity of the Spa47 oligomers. Kinetic analyses were additionally performed on Spa47 in the presence of varying inhibitor and substrate (ATP) concentrations. The Spa47 and DMSO concentrations were held constant at 0.05 μM and 10%, respectively, while the inhibitor concentrations tested included 0 μM, 50 μM, 225 μM, and 500 μM. Initial velocities were determined for each of the inhibitor concentrations described above in the presence of 0.025 mM, 0.075 mM, 0.15 mM, 0.3 mM, and 0.6 mM ATP and were plotted as a function of ATP concentration. SigmaPlot 12 was used to fit each data set to the Michaelis–Menten equation (Eq. 1):

$$v = \frac{V_{\max}[S]}{K_M + [S]} \quad \text{Equation 1}$$

where v is the initial reaction velocity of the reaction, $[S]$ is the ATP concentration, K_M is the Michaelis constant, and V_{max} is the maximum velocity of the enzyme.

Effect of Spa47 Inhibitors on *Shigella* Growth.

Wild-type *S. flexneri* (2457T) was grown overnight on a tryptic soy agar (TSA)-Congo red plate, and a small number of isolated colonies were used to inoculate 10 mL of tryptic soy broth (TSB). The inoculated culture was grown to OD₆₀₀ 0.05 and a small amount was diluted and plated onto a TSA-Congo red plate as time point zero in the collected growth curves. The parent culture was then split into 1 mL cultures containing 5% DMSO and 500 μ M of the appropriate inhibitor as 5% DMSO was determined to have little effect on *Shigella* culture growth rates. In addition to the inhibitor conditions, a control flask containing no inhibitor, but 5% DMSO was included. Because the inhibitors interfered with optical density readings of the cultures at 600 nm, a sample was taken from each flask, diluted, and spread on TSA-Congo red plates every hour for eight hours. The plates were incubated overnight at 37 °C and the colonies were counted to generate growth curves for the control culture and the cultures containing each inhibitor.

Effect of Spa47 Inhibitors on HeLa Cell Viability.

An MTT colorimetric assay was used to quantify the cytotoxic effects the small molecule T3SS ATPase inhibitors had on HeLa cells.⁴⁶ HeLa cells purchased from the American Type Culture Collection (ATCC) were passaged according to protocol and were seeded in a sterile 96 well plate. The cells were incubated overnight in DMEM supplemented with 10% fetal calf serum and a penicillin/streptomycin antibiotic cocktail at 37 °C, 100% humidity, and 5% CO₂. The cells were then incubated under identical environmental conditions (37 °C, 100% humidity, and 5% CO₂) for 30 minutes with 100 μ M inhibitor and 1% DMSO prior to the addition of MTT. The cells were then incubated for an additional 4 hours with 100 μ M inhibitor, 1% DMSO, and MTT at 37 °C prior to the addition of 200 μ L DMSO to fully dissolve the produced formazan salt. The formazan levels produced by viable cells in each well were then compared to control conditions where the cells were not exposed to the inhibitors by measuring the absorbance of each condition at 570 nm. The cytotoxicity results were obtained from three independent measurements and are reported as the mean viability $\pm$ standard deviation with respect to the control condition lacking inhibitor.

Quantitation of *S. flexneri* T3SS Translocator Secretion.

The small diazo dye Congo red effectively induces active secretion of translocator proteins through the *Shigella* T3SS by mimicking the natural trigger resulting from host cell membrane interaction.⁴⁷ Thus, Congo red exposure serves as a valuable tool that allows for artificial activation of *Shigella* type three secretion. The protocol has been described in detail elsewhere.⁴⁸ Briefly, a *S. flexneri* strain lacking the gene for Spa47 and a strain expressing wild-type Spa47 were grown overnight on TSA-Congo red plates, and a small number of isolated colonies were used to inoculate 15 mL of TSB containing appropriate antibiotics. Cultures were grown at 37 °C to OD₆₀₀ 0.05 and the wild-type *Shigella* strain was split into 1 mL cultures containing increasing concentrations of the tested inhibitor (0, 25 μ M, 75 μ M, 150 μ M, 250 μ M, and 500 μ M) and a final DMSO concentration of 5%. One milliliter control growths of wild-type *Shigella* lacking DMSO and *spa47* null *Shigella flexneri* $\pm$

DMSO were also prepared. The cultures were grown until the wild-type *Shigella* culture lacking inhibitor reached an OD₆₀₀ of 1.0. The cultures were centrifuged and rinsed to separate the bacteria from the culture supernatant and any proteins that had been secreted up to that point. The cells were then resuspended in sodium phosphate buffer containing 0.28 mg/ml Congo red, the appropriate inhibitor and DMSO concentration, and were incubated at 37 °C for 1 hour to promote active T3SS secretion. Cultures were then chilled on ice for 5 min to limit further secretion and the bacteria were separated from the protein-containing supernatant by centrifugation at 13,000 × g for 15 min at 4 °C. The secreted proteins within the supernatant from each strain were separated using SDS-PAGE, transferred to PVDF membranes by western blot, and probed using anti-IpaC rabbit polyclonal antibodies and Alexa 647 goat anti-rabbit secondary antibodies. Secreted IpaC levels were compared using a Bio-Rad ChemiDoc imaging system and the associated Image Lab analysis software. As validated previously,⁴⁹ a monoclonal antibody against the cytoplasmic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a cytoplasmic control in the western blot to ensure that the IpaC detected in the supernatant was only secreted from the bacteria and was not the result of cell lysis.

Effect of Spa47 ATPase Inhibitors on Spa47 Oligomer State.

To determine if the inhibitors reduced Spa47 activity by disrupting active Spa47 oligomers, size exclusion chromatography (SEC) was performed on isolated Spa47 oligomers in both the absence and presence of each of the inhibitors. Specifically, Spa47 was first purified as described above and the active oligomer isolated via SEC. Thirty-three micromolar Spa47 was then incubated in 250 μM inhibitor and 10% DMSO for 1 hour at room temperature before it was analyzed using a Superdex 200 increase 5/150 size exclusion column equilibrated with the tested inhibitor and DMSO concentrations and run at 0.1 mL/min. Control conditions included isolated monomeric and oligomeric Spa47 in the absence of inhibitors, but in the presence of 10% DMSO, which itself did not affect the distribution of Spa47 oligomers observed previously.^{1,37,38}

Inhibitor Effect on Type Three Secretion Apparatus Formation.

The effect of the inhibitors on *Shigella* T3SA formation was investigated using flow cytometry coupled with fluorescence detection using a slightly modified version of a protocol published previously.¹ *Shigella* strains lacking the gene for Spa47 (Spa47 null) and expressing wild-type Spa47 were streaked onto TSA-Congo red plates and grown overnight. A small number of isolated colonies were used to inoculate 15 ml of TSB containing appropriate antibiotics. Cultures were grown at 37 °C to an OD₆₀₀ of 0.05 and the wild-type Spa47 *Shigella* culture was split into 1 mL cultures containing 500 μM of inhibitor 3624 or 9652 and a final DMSO concentration of 5%. Control growths were additionally prepared in the presence of 5% DMSO but no inhibitor. After the control culture reached an OD₆₀₀ of 0.6, bacteria were collected by centrifugation at 4 °C and gently rinsed with PBS. The bacteria were then chemically fixed for 15 min in 4% formaldehyde in PBS at room temperature (20–22 °C). The fixed cells were rinsed and treated with rabbit polyclonal antibodies against IpaD and Alexa 647 goat anti-rabbit secondary antibodies to fluorescently label surface exposed IpaD as described previously.¹ The fluorescently-labeled *Shigella* cells were analyzed using a BD Accuri C6 flow cytometer, collecting 100,000 events per

condition. The resulting data sets were analyzed using De Novo FCS Express 5 flow cytometry software to generate fluorescence intensity histograms and determine whether the inhibitors affected the ability of *Shigella* to form the external needle and tip components of the T3SA.

Results:

T3SS inhibitors are predicted to bind multiple sites at the interface between adjacent protomers in the activated Spa47 complex.

Three compounds previously shown to be effective T3SS inhibitors targeting the *Yersinia* T3SS ATPase YscN (2834, 3624, and 9652) were investigated as potential cross-pathogen inhibitors targeting the *Shigella* T3SS ATPase, Spa47. These inhibitors were each originally predicted to bind the active site of YscN based on docking simulations against a structural model of YscN.³⁹ Here, the ligand docking software, AutoDock Vina, predicted interactions between the compounds and a recently solved 2.4 Å crystal structure of Spa47. Because Spa47 ATPase activation requires homo-oligomerization and the activated complex is predicted to be a homo-hexamer,¹⁴ inhibitor docking simulations were performed against an energy-minimized Spa47 homo-hexamer modeled after the structure of F1 ATP synthase⁵ (Figure 1). The nine highest affinity interactions between each of the inhibitors and Spa47 localized to two distinct binding sites in Spa47; one within the active site (Site 1) and the second located approximately 25 Å away from the active site (Site 2). Both predicted binding sites are located at the interface of Spa47 protomers within the hexamer model and the highest affinity interaction for each inhibitor has been highlighted in a darker shade of the inhibitors' respective color. The most favored interactions for compounds 3624 and 9652 were located within Site 2, while the most favorable interaction for compound 2834 is in Site 1.

Small Molecule T3SS Inhibitors are Effective against the *Shigella* T3SS ATPase Spa47.

The small molecule inhibitors 2834, 3624, and 9652 were tested for their effect on Spa47 ATPase activity using a colorimetric malachite green activity assay. Because the limited solubility of each of the potential inhibitors required dissolving them in DMSO, the effect of DMSO on Spa47 activity was first characterized. DMSO concentrations as high as 12.5% had no detrimental effect on the activity of the enzyme (data not shown) so each of the tested reaction conditions contained 10% DMSO to maximize inhibitor solubility. Figure 2A is a plot of inhibitor concentration versus initial reaction velocity for 50 nM Spa47 oligomer. Each of the inhibitors slightly reduce Spa47 activity at concentrations as low as 50 μM, with 88%, 81%, and 99% inhibition at 500 μM inhibitor 2834, 3624, and 9652, respectively. The data were fit to 4-parameter logistic sigmoidal dose response curves that all resulted in R² values greater than 0.99 with IC₅₀s of 69 ± 9 μM, 52 ± 3 μM, and 123 ± 17 μM for 2834, 3624, and 9652, respectively.

Kinetic Analyses Uncover Non-Competitive Inhibition of Spa47.

Having shown that each of the tested small molecules efficiently inhibit Spa47 ATPase activity, full inhibition kinetic analyses were performed on each inhibitor. Specifically, substrate concentration-dependent Spa47 activity profiles were collected at 0 μM, 50 μM,

225 μM , and 500 μM inhibitor concentrations (Figure 2B–2D). Each of the data sets were fit to the Michaelis Menten equation to solve for the apparent V_{max} and apparent K_{M} values under the same conditions (Table 1). The baseline V_{max} and K_{M} values determined at 0 μM inhibitor concentration are consistent with our previously published values for Spa47 oligomer.³⁷ Titrating in each of the inhibitors significantly reduced the apparent V_{max} values with 500 μM inhibitor 2834 and 3624 both reducing apparent V_{max} to $0.08 \pm 0.01 \mu\text{M}/\text{sec}$ and inhibitor 9652 abolishing Spa47 activity completely. The apparent K_{M} values were not affected by any of the inhibitors, meaning that none of the inhibitors alter Spa47's sensitivity to ATP concentrations. The lack of effect on the apparent K_{M} together with the observed decrease in apparent V_{max} is consistent with a non-competitive inhibition mechanism where the inhibitor binds the enzyme outside of the active site and does not directly compete for enzyme binding with the native substrate. The inhibition kinetics data for each inhibitor were additionally fit to multiple potential inhibition profiles using the software package VisualEnzymics, finding that each inhibitor profile did indeed fit best to a non-competitive inhibition model. While we were certainly not expecting to identify non-competitive inhibition by the tested inhibitors, the findings are supported by the AutoDock modeling results identifying a second binding site approximately 25 Å from the ATP binding site in the oligomeric Spa47 model (Figure 1).

Identified V_{max} and K_{M} data were used to calculate apparent k_{cat} values and specificity constants ($k_{\text{cat}}/K_{\text{M}}$) for each of the tested inhibitor conditions (Table 1). As expected, the apparent k_{cat} values trended identically to the observed decrease in apparent V_{max} for each inhibitor as the total enzyme concentration was held constant at 0.05 μM for all reaction conditions. Likewise, because the K_{M} values did not change significantly with inhibitor concentration, the apparent specificity constants also followed a similar descending trend as a function of inhibitor concentration.

Inhibitors do not Disrupt Spa47 Oligomers.

Spa47 ATPase activity relies on the formation of a completed active site that results from amino acid side chain contributions from at least two adjacent Spa47 protomers within the homo-oligomeric complex.¹ Because of this reliance on stable oligomeric complexes for optimal activity, we tested whether the inhibitors characterized above reduce/eliminate Spa47 activity through disruption of the isolated Spa47 oligomers. Initial purification of Spa47 includes separation of the stable oligomeric and monomeric species via size exclusion chromatography, allowing the isolated Spa47 oligomer to be incubated with 500 μM inhibitor prior to analysis on a Superdex 200 increase 5/150 size exclusion column preequilibrated with the same inhibitor condition. The resulting size exclusion chromatograms clearly show that oligomeric Spa47 incubated with any of the three inhibitors continues to elute at ~1.3 mL, consistent with the oligomeric Spa47 control condition and much earlier than the monomeric Spa47 control that elutes at ~2.1 mL (Figure 3). These findings clearly show that the inhibitors tested in this study do not function through disrupting Spa47 oligomers.

Spa47 ATPase Inhibitors do not Affect *Shigella* Growth Profiles and Minimally Impact Mammalian Cell Viability.

While the tested inhibitors clearly reduce/eliminate the ATPase activity of Spa47 *in vitro* (Figure 2) (Table 1), the effect of the inhibitors on *Shigella* growth curves was tested to ensure that the inhibitors do not reduce the overall metabolic activity of the bacteria by targeting additional ATPases beyond Spa47. Because the inhibitors require DMSO to maintain solubility in the culture media, the effect of DMSO concentration on *Shigella* growth rates was tested first. DMSO concentrations as high as 5% had minimal effect on *Shigella* growth rates/curves, however exceeding 5% DMSO was detrimental to *Shigella* growth (data not shown). Growth curves of the *Shigella flexneri* clinical isolate 2457T were recorded for cultures containing 5% DMSO and 500 μ M of each inhibitor and were compared to a control growth containing 5% DMSO and no inhibitor (Figure 4). The growth curves of *Shigella* cultures containing each of the inhibitors are very similar to that of the control culture, confirming that none of the inhibitors significantly affect *Shigella* metabolism and suggesting that they do not target additional *Shigella* ATPases beyond Spa47.

An MTT cytotoxicity assay was additionally performed on cultured HeLa cells to test the effect of the inhibitors on mammalian cell viability. Consistent with previously published findings, the assay first determined that the HeLa cells are significantly more susceptible to DMSO exposure than the more robust *Shigella* cells,⁵⁰ limiting the assay to a final concentration of 1% DMSO and 100 μ M inhibitor concentrations. A 4.5 hour exposure of each tested inhibitor to HeLa cells resulted in $26 \pm 6\%$, $14 \pm 5\%$, and $6 \pm 3\%$ toxicity for compounds 2834, 3624, and 9652, respectively.

Spa47 Inhibitors Prevent Secretion of Critical *Shigella* T3SS Effectors.

The *Shigella* type three secretion apparatus (T3SA) is a complex nano-machine with a basal body that requires Spa47 catalyzed ATP hydrolysis to support protein secretion through the associated needle and tip complex.^{1,37} The mechanism(s) that initiate the secretion of effector proteins into the host cell remain largely unclear, but likely require environmental cues such as interaction of the apparatus tip proteins with bile salts and eukaryotic cell membrane components.^{49,51–53} Alternatively, the small molecule Congo red can mimic *in vivo* activation of the T3SS⁴⁷ to determine the effect of the Spa47 inhibitors on the ability of the *Shigella* T3SS to actively secrete effector proteins through the T3SA. Specifically, *Shigella* cultures were grown in media containing 5% DMSO and increasing concentrations of each of the tested inhibitors, ranging from 0 μ M to 500 μ M inhibitor. The cultures were activated with Congo red as described in the Experimental Procedures section and the levels of the secreted effector protein, IpaC, were quantified via western blot analysis (Figure 5). Inhibitors 3624 and 9652 reduced IpaC secretion levels to $2.5 \pm 1.5\%$ and $3.7 \pm 3.1\%$ of the wild-type control, respectively. Inhibitor 3624 displayed an EC₅₀ of 134 ± 40 μ M and 9652 an EC₅₀ of 377 ± 184 μ M. Inhibitor 2834 had no effect on IpaC secretion levels despite a clear inhibitory impact on Spa47 ATPase activity *in vitro* (Figure 2 and Table 1). While the calculated EC₅₀ values for 3624 and 9652 fit well to a 4-parameter logistic sigmoidal dose response with R² values > 0.98, the standard errors for the EC₅₀ values are large and make direct comparison to the IC₅₀s determined *in vitro* for Spa47 inhibition difficult. Empirical

comparison of the responses does, however, suggest that T3SS protein secretion is less sensitive to low concentrations of inhibitor than *in vitro* Spa47 ATPase activity inhibition is (ie EC50 > IC50). The specific reason for this is not yet clear, but may be the result of difficulties of the inhibitors crossing the bacterial membranes and accessing the cytoplasm or an efficient efflux mechanism that actively removes the inhibitors from the bacterial cytoplasm and limits the inhibitor concentrations exposed to Spa47. Additionally, it is possible that the tested inhibitors interact/act differently (less efficiently) on Spa47 that is incorporated into the T3SA sorting platform compared to the isolated Spa47 homo-oligomers tested in Figure 2A. Either way, these studies provide the first description of Spa47 inhibition by small molecule inhibitors and give promise for broad spectrum T3SS ATPase-specific small molecule therapeutics.

Spa47 inhibitors do not significantly disrupt construction of the *Shigella* T3SA.

It has been shown previously that Spa47-catalyzed ATP hydrolysis is essential for proper protein effector secretion through the T3SA and for the formation of the external needle and tip complex components of the apparatus.¹ Because we showed that each of the tested inhibitors reduced Spa47 ATPase activity *in vitro* and that inhibitors 3624 and 9652 significantly reduced IpaC secretion levels through the apparatus, we explored the effect of inhibitors 3624 and 9652 on the ability of *Shigella* to assemble a T3SA. As a highly antigenic, surface exposed protein at the tip of the T3SA, IpaD is a sensitive probe for determining whether the Spa47 inhibitors affect apparatus formation.^{1,54} Flow cytometry detection of fluorescently-labeled IpaD showed that the *Shigella* strain expressing wild-type Spa47 resulted in a significant positive shift in the fluorescence intensity histogram compared to the Spa47 null strain (Figure 6), consistent with our previous results showing that Spa47 null *Shigella* do not form the external portion of the T3SA.¹ Growing the wild-type *Shigella* strain in the same conditions that strongly affected IpaC protein secretion levels (5% DMSO and 500 μ M inhibitor 3624 and 9652), however, only modestly reduced the levels of IpaD surface localization observed by flow cytometry. This suggests that while the inhibitors 3624 and 9652 nearly eliminate secretion of the effector protein IpaC, they provide a much smaller impact on T3SA formation, perhaps because T3SA formation requires minimal levels of Spa47 activity compared to those required for detectable effector protein secretion.

Discussion:

Type three secretion systems are complex nano-machines that deliver effector proteins directly into the cytoplasm of eukaryotic host cells to support infection and evade host immune responses. Many human pathogens including *Shigella*, *Yersinia*, *Burkholderia*, enteropathogenic and enterohemorrhagic *Escherichia coli*, *Pseudomonas*, *Chlamydia*, and *Salmonella* rely on one or more type three secretion systems as primary virulence factors required to initiate and sustain host infection.¹¹ The secreted effector proteins themselves are uniquely tailored to support the infection profiles and replicative niches of the specific pathogens that express them; however, all virulence associated type three secretion systems must secrete their effectors through the needle-like T3SA and into the host cell cytoplasm. The T3SA is comprised of 20–25 distinct protein components with multiple copies of each

protein generally included in the apparatus.^{14,17} High-resolution structures and biochemical characterization of individual apparatus proteins have been instrumental in uncovering the functions of many T3SS proteins as well as determining how they interact with one another to support overall apparatus function. Not surprisingly, the roles of the T3SA proteins vary greatly and include critical responsibilities such as regulating needle length during apparatus formation, environmental sensing and signaling by the apparatus, host membrane interaction at the tip of the apparatus, recognition and partial unfolding of protein substrates prior to secretion, and providing the energetic driving force to support protein secretion through the narrow apparatus needle.^{13,18–20,37,55}

Disruption of any of these key events has significant impact on protein secretion through the apparatus and often renders the pathogen expressing the handicapped T3SS avirulent, making the T3SS an attractive target for anti-infective agents against a broad class of human pathogens.^{24–26} High-throughput screening for small molecules that decrease T3SS protein expression and secretion levels has identified several structurally diverse classes of T3SS inhibitors that appear to generally act through down-regulating expression of key T3SS proteins.^{30–32} An alternate approach to cell-based high-throughput screening involves first identifying a protein target within the T3SS and then performing an experimental or computational screen for potential ligands/inhibitors against the target protein. To date, three groups have performed *in silico* screening against the T3SS ATPase proteins YscN, EscN, and SctN from *Yersinia*, *E. coli*, and *Chlamydia*, respectively.^{39–41} Given the clear correlation between T3SS ATPase activity, effector protein secretion through the apparatus, and pathogen virulence,^{11,56} it is not surprising that each of the screens identified compounds able to reduce protein secretion levels through what is assumed to be specific targeting of T3SS ATPase activity.

In the work presented here, we expanded upon these foundational studies by testing the effect of three compounds originally identified as *Yersinia* T3SS ATPase inhibitors³⁹ on the *Shigella* T3SS ATPase Spa47. Challenges expressing and purifying native T3SS ATPases has been a longstanding hurdle to not only developing T3SS ATPase inhibitors, but to characterizing the specific mode/mechanism of inhibition by identified inhibitors. Here, we leveraged our ability to express and purify full length soluble Spa47 as stable and active homo-oligomers to provide a model of the T3SS ATPase complex for testing kinetic inhibition profiles. It was not surprising to find that each of the tested compounds inhibited Spa47 activity *in vitro*, but it is interesting to note the differences in inhibition efficiencies observed between Spa47 and YscN. Specifically, we determined the IC₅₀ values with respect to Spa47 ATPase activity to be $69 \pm 9 \mu\text{M}$, $52 \pm 3 \mu\text{M}$, and $123 \pm 17 \mu\text{M}$ for 2834, 3624, and 9652, respectively (Figure 2A). An opposite trend that was observed with YscN, where Swietnicki and colleagues found an IC₅₀ of 50 μM for 9652 and compound 2834 only achieved a maximum mean inhibition of approximately 30% at 100 μM inhibitor concentration.⁴⁷ These initial findings provided hope that ATPase inhibitors may act as cross-pathogen T3SS inhibitors, though the differences in inhibition efficiency between Spa47 and YscN suggest that even though the two enzymes share 42% sequence identity,³⁷ maximal ATPase inhibition efficiencies will require tailoring inhibitors to individual T3SS ATPases. Furthermore, it is important to recognize that while the specific inhibitors tested in this study provided a valuable comparison of *in vitro* and *in vivo* effects of the inhibitors on

Yersinia and *Shigella* T3SS ATPases and helped to uncover much needed details about Spa47 inhibition mechanisms, they suffer from limited solubility and modest IC₅₀ values against both YscN and Spa47. These practical limitations will likely prevent them from being useful therapeutics in their current form, however, they continue to serve as valuable probes in defining the role of T3SS ATPases in T3SS function with efforts to modify the inhibitors to enhance solubility and efficacy currently underway.

Full kinetic analyses of the inhibitors with respect to Spa47 activity showed a reduction in apparent V_{max} and no effect on the apparent K_M, consistent with a non-competitive inhibition mechanism (Figure 2 and Table 1) and our docking calculations predicting that each of the three inhibitors can bind locations outside the Spa47 active site (Figure 1). We were originally quite surprised that the inhibitors acted non-competitively against Spa47, given that the tested compounds were identified based on *in silico* docking to the YscN active site.³⁹ With our models suggesting that the inhibitors could bind multiple sites at the protomer interface of the activated Spa47 oligomer, we considered that perhaps the inhibitors are capable of binding the active site of Spa47 competitively, but that binding disrupts the activated oligomers to generate inactive inhibitor-bound monomeric Spa47 and provide a false sense of non-competitive inhibition. We directly tested this hypothesis using SEC to monitor Spa47 oligomer state in the absence and presence of each of the inhibitors. The results clearly show that none of the inhibitors disrupted Spa47 oligomers (Figure 3), supporting the idea that the inhibitors do in fact bind outside the Spa47 active site, reducing ATPase activity in a classic example on non-competitive inhibition. While identifying the inhibitors as non-competitive was somewhat surprising, it provides the benefit of avoiding interaction with the highly conserved ATPase active site, perhaps providing the level of specificity necessary to target T3SS ATPases and avoid off-target ATPase inhibition. In fact, none of the three tested inhibitors appear to affect *Shigella* or mammalian cell ATPases involved in metabolism as none had a significant effect on *Shigella* growth phenotype at concentrations as high as 500 μM (Figure 4) and only reduced HeLa cell viability by 26 ± 6%, 14 ± 5%, and 6 ± 3% for compounds 2834, 3624, and 9652, respectively. Spa47 activity was still inhibited *in vivo*, however, as compounds 3624 and 9652 both reduced T3SS protein effector secretion levels to essentially zero following incubation with 500 μM inhibitor (Figure 5). Despite potent inhibition of Spa47 activity *in vitro*, compound 2834 had no effect on IpaC secretion levels. The mechanism behind this lack of secretion inhibition is not clear, but the disconnect between *in vitro* and *in vivo* inhibition by compound 2834 suggests that interactions within the context of the T3SA may prevent inhibitor binding, mitigate the effect of the inhibitor, that 2834 is unable to efficiently cross the bacterial membranes and access the bacterial cytoplasm, or that it is perhaps actively secreted from the *Shigella* cytoplasm via efflux pumps that do not act on the other tested inhibitors. Interestingly, comparing these *Shigella* secretion phenotype data to *Yersinia* YopE secretion profiles again finds strikingly different responses to the inhibitors by the two pathogens. While concentrations as high as 500 μM inhibitor 2834 had no effect on IpaC secretion by *Shigella*, the same inhibitor reduced YopE secretion by *Yersinia* nearly 90% at only 100 μM inhibitor concentration. Additionally, while inhibitor 3624 provided the strongest secretion inhibition response in *Shigella* with an EC₅₀ of 134 ± 40 μM and greater than 97.5 ± 1.5% inhibition at 500 μM, it was even more effective in *Yersinia* with a 60% secretion reduction

at only 10 μ M inhibitor. It seems unlikely that these differences are driven by drastically different abilities of the inhibitors to cross the bacterial membranes of these related gram negative pathogens and more likely that the inhibitors are actively removed from the bacteria with varying efficiencies or that they act on different T3SS ATPases with differential potency and perhaps different inhibition mechanisms all together. More importantly, we have also shown that specific characterization of inhibitor impact on pathogen type three secretion phenotype is essential as *in vitro* T3SS ATPase inhibition does not necessarily correlate to secretion reduction *in vivo*. Despite these challenges, however, studies such as this one are identifying and characterizing T3SS ATPase inhibitors effective against multiple bacterial species and are expanding our understanding of the relationship between T3SS ATPase inhibition and pathogen phenotype. High-resolution structures of inhibitor-bound T3SS ATPases, inhibitor structure optimization, and proof of concept experiments in animal infection models will all play critical roles in the near future as T3SS ATPase inhibitors are developed as much needed alternatives to antibiotic treatments.

Acknowledgments

Funding Source Statement:

This work was supported in part by a National Institutes of Health Grant 1R15AI124108-01A1 and R. Gaurth Hansen endowment funds to N. E. D.

Abbreviations:

AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
ATCC	American Type Culture Collection
BSA	bovine serum albumin
CBD	chitin binding domain
CDC	Centers for Disease Control and Prevention
DTT	dithiothreitol
DMSO	dimethyl sulfoxide
EC50	concentration of drug that provokes a response halfway between the baseline and maximum response
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IC50	half maximal inhibitory concentration
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LPS	lipopolysaccharide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD₆₀₀	optical density at 600 nm

PDVF	polyvinylidene fluoride
PBS	phosphate buffered saline
SEC	size exclusion chromatography
T3SA	type three secretion apparatus
T3SS	type three secretion system
TB	terrific broth
TSB	tryptic soy broth
TSA	tryptic soy agar
WHO	World Health Organization

References:

1. Burgess JL, Burgess RA, Morales Y, Bouvang JM, Johnson SJ, and Dickenson NE (2016) Structural and Biochemical Characterization of Spa47 Provides Mechanistic Insight into Type III Secretion System ATPase Activation and Shigella Virulence Regulation. *J Biol Chem* 291, 25837–25852. [PubMed: 27770024]
2. Gao X, Mu Z, Yu X, Qin B, Wojdyla J, Wang M, and Cui S (2018) Structural Insight Into Conformational Changes Induced by ATP Binding in a Type III Secretion-Associated ATPase From *Shigella flexneri*. *Front Microbiol* 9, 1468. [PubMed: 30013545]
3. Aminov RI (2010) A brief history of the antibiotic era: lessons learned and challenges for the future. *Front Microbiol* 1, 134. [PubMed: 21687759]
4. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, and Piddock LJ (2015) Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 13, 42–51. [PubMed: 25435309]
5. Abrahams JP, Leslie AG, Lutter R, and Walker JE (1994) Structure at 2.8 Å resolution of F1-ATPase from bovine heart mitochondria. *Nature* 370, 621–628. [PubMed: 8065448]
6. Centers for Disease Control and Prevention (CDC). Antibiotic Resistance Threats in the United States. CDC: Atlanta, Georgia, 2013 Available online: <https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf> (accessed 6 October 2018).
7. World Health Organization (WHO). Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics. WHO: Geneva, Switzerland, 2017 Available online: http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf (accessed 6 October 2018).
8. Carayol N, and Tran Van Nhieu G (2013) The inside story of *Shigella* invasion of intestinal epithelial cells. *Cold Spring Harb Perspect Med* 3, a016717.
9. Diepold A, and Armitage JP (2015) Type III secretion systems: the bacterial flagellum and the injectisome. *Philos Trans R Soc Lond B Biol Sci* 370
10. Notti RQ, and Stebbins CE (2016) The Structure and Function of Type III Secretion Systems. *Microbiol Spectr* 4
11. Deng W, Marshall NC, Rowland JL, McCoy JM, Worrall LJ, Santos AS, Strynadka NCJ, and Finlay BB (2017) Assembly, structure, function and regulation of type III secretion systems. *Nat Rev Microbiol* 15, 323–337. [PubMed: 28392566]
12. Zarivach R, Vuckovic M, Deng W, Finlay BB, and Strynadka NC (2007) Structural analysis of a prototypical ATPase from the type III secretion system. *Nat Struct Mol Biol* 14, 131–137. [PubMed: 17237797]
13. Aakeda Y, and Galan JE (2004) Genetic analysis of the *Salmonella enterica* type III secretion-associated ATPase InvC defines discrete functional domains. *J Bacteriol* 186, 2402–2412. [PubMed: 15060043]

14. Hu B, Morado DR, Margolin W, Rohde JR, Arizmendi O, Picking WL, Picking WD, and Liu J (2015) Visualization of the type III secretion sorting platform of *Shigella flexneri*. *Proc Natl Acad Sci U S A* 112, 1047–1052. [PubMed: 25583506]
15. Portaliou AG, Tsolis KC, Loos MS, Zorzini V, and Economou A (2016) Type III Secretion: Building and Operating a Remarkable Nanomachine. *Trends Biochem Sci* 41, 175–189. [PubMed: 26520801]
16. Erhardt M, Namba K, and Hughes KT (2010) Bacterial nanomachines: the flagellum and type III injectisome. *Cold Spring Harb Perspect Biol* 2, a000299.
17. Wagner S, Grin I, Malmshiemer S, Singh N, Torres-Vargas CE, and Westerhausen S (2018) Bacterial type III secretion systems: a complex device for the delivery of bacterial effector proteins into eukaryotic host cells. *FEMS Microbiol Lett* 365
18. Park D, Lara-Tejero M, Waxham MN, Li W, Hu B, Galan JE, and Liu J (2018) Visualization of the type III secretion mediated *Salmonella*-host cell interface using cryo-electron tomography. *Elife* 7
19. Yoshida Y, Miki T, Ono S, Haneda T, Ito M, and Okada N (2014) Functional characterization of the type III secretion ATPase SsaN encoded by *Salmonella* pathogenicity island 2. *PLoS One* 9, e94347. [PubMed: 24722491]
20. Allison SE, Tuinema BR, Everson ES, Sugiman-Marangos S, Zhang K, Junop MS, and Coombes BK (2014) Identification of the docking site between a type III secretion system ATPase and a chaperone for effector cargo. *J Biol Chem* 289, 23734–23744. [PubMed: 25035427]
21. Chatterjee S, Zhong D, Nordhues BA, Battaile KP, Lovell S, and De Guzman RN (2011) The crystal structures of the *Salmonella* type III secretion system tip protein SipD in complex with deoxycholate and chenodeoxycholate. *Protein Sci* 20, 75–86. [PubMed: 21031487]
22. Schlumberger MC, Muller AJ, Ehrbar K, Winnen B, Duss I, Stecher B, and Hardt WD (2005) Real-time imaging of type III secretion: *Salmonella* SipA injection into host cells. *Proc Natl Acad Sci U S A* 102, 12548–12553. [PubMed: 16107539]
23. Enninga J, Mounier J, Sansonetti P, and Tran Van Nhieu G (2005) Secretion of type III effectors into host cells in real time. *Nat Methods* 2, 959–965. [PubMed: 16299482]
24. Izore T, Job V, and Dessen A (2011) Biogenesis, regulation, and targeting of the type III secretion system. *Structure* 19, 603–612. [PubMed: 21565695]
25. Duncan MC, Linington RG, and Auerbuch V (2012) Chemical inhibitors of the type three secretion system: disarming bacterial pathogens. *Antimicrob Agents Chemother* 56, 5433–5441. [PubMed: 22850518]
26. Keyser P, Elofsson M, Rosell S, and Wolf-Watz H (2008) Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria. *J Intern Med* 264, 17–29. [PubMed: 18393958]
27. Allen RC, Popat R, Diggle SP, and Brown SP (2014) Targeting virulence: can we make evolution-proof drugs? *Nat Rev Microbiol* 12, 300–308. [PubMed: 24625893]
28. Kauppi AM, Nordfelth R, Uvell H, Wolf-Watz H, and Elofsson M (2003) Targeting bacterial virulence: inhibitors of type III secretion in *Yersinia*. *Chem Biol* 10, 241–249. [PubMed: 12670538]
29. Nordfelth R, Kauppi AM, Norberg HA, Wolf-Watz H, and Elofsson M (2005) Small-molecule inhibitors specifically targeting type III secretion. *Infect Immun* 73, 3104–3114. [PubMed: 15845518]
30. Gauthier A, Robertson ML, Lowden M, Ibarra JA, Puente JL, and Finlay BB (2005) Transcriptional inhibitor of virulence factors in enteropathogenic *Escherichia coli*. *Antimicrob Agents Chemother* 49, 4101–4109. [PubMed: 16189086]
31. Zhang Y, Liu Y, Wang T, Deng X, and Chu X (2018) Natural compound sanguinarine chloride targets the type III secretion system of *Salmonella enterica* Serovar Typhimurium. *Biochem Biophys Rep* 14, 149–154. [PubMed: 29761161]
32. Li J, Lv C, Sun W, Li Z, Han X, Li Y, and Shen Y (2013) Cytosporone B, an inhibitor of the type III secretion system of *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* 57, 2191–2198. [PubMed: 23459474]
33. Bowlin NO, Williams JD, Knoten CA, Torhan MC, Tashjian TF, Li B, Aiello D, Mecsas J, Hauser AR, Peet NP, Bowlin TL, and Moir DT (2014) Mutations in the *Pseudomonas aeruginosa* needle

protein gene *pscF* confer resistance to phenoxyacetamide inhibitors of the type III secretion system. *Antimicrob Agents Chemother* 58, 2211–2220. [PubMed: 24468789]

34. Aiello D, Williams JD, Majgier-Baranowska H, Patel I, Peet NP, Huang J, Lory S, Bowlin TL, and Moir DT (2010) Discovery and characterization of inhibitors of *Pseudomonas aeruginosa* type III secretion. *Antimicrob Agents Chemother* 54, 1988–1999. [PubMed: 20176902]
35. Jouihri N, Sory MP, Page AL, Gounon P, Parsot C, and Allaoui A (2003) MxiK and MxiN interact with the Spa47 ATPase and are required for transit of the needle components MxiH and MxiI, but not of Ipa proteins, through the type III secretion apparatus of *Shigella flexneri*. *Mol Microbiol* 49, 755–767. [PubMed: 12864857]
36. Woestyn S, Allaoui A, Wattiau P, and Cornelis GR (1994) YscN, the putative energizer of the *Yersinia* Yop secretion machinery. *J Bacteriol* 176, 1561–1569. [PubMed: 8132449]
37. Burgess JL, Jones HB, Kumar P, Toth R. T. t., Middaugh CR, Antony E, and Dickenson NE (2016) Spa47 is an oligomerization-activated type three secretion system (T3SS) ATPase from *Shigella flexneri*. *Protein Sci* 25, 1037–1048. [PubMed: 26947936]
38. Case HB, and Dickenson NE (2018) MxiN Differentially Regulates Monomeric and Oligomeric Species of the *Shigella* Type Three Secretion System ATPase Spa47. *Biochemistry* 57, 2266–2277. [PubMed: 29595954]
39. Swietnicki W, Carmany D, Retford M, Guelta M, Dorsey R, Bozue J, Lee MS, and Olson MA (2011) Identification of small-molecule inhibitors of *Yersinia pestis* Type III secretion system YscN ATPase. *PLoS One* 6, e19716. [PubMed: 21611119]
40. Bzdion L, Krezel H, Wrzeszcz K, Grzegorek I, Nowinska K, Chodaczek G, and Swietnicki W (2017) Design of small molecule inhibitors of type III secretion system ATPase EscN from enteropathogenic *Escherichia coli*. *Acta Biochim Pol* 64, 49–63. [PubMed: 27864920]
41. Grishin AV, Luyksaar SI, Kapotina LN, Kirsanov DD, Zayakin ES, Karyagina AS, and Zigangirova NA (2018) Identification of chlamydial T3SS inhibitors through virtual screening against T3SS ATPase. *Chem Biol Drug Des* 91, 717–727. [PubMed: 29068165]
42. Formal SB, Dammin GJ, Labrec EH, and Schneider H (1958) Experimental *Shigella* infections: characteristics of a fatal infection produced in guinea pigs. *J Bacteriol* 75, 604–610. [PubMed: 13538931]
43. The PyMOL Molecular Graphics System, version 1.8, Schrödinger, LLC.
44. Dallakyan S, and Olson AJ (2015) Small-molecule library screening by docking with PyRx. *Methods Mol Biol* 1263, 243–250. [PubMed: 25618350]
45. Trott O, and Olson AJ (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 31, 455–461. [PubMed: 19499576]
46. Cory AH, Owen TC, Barltrop JA, and Cory JG (1991) Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* 3, 207–212. [PubMed: 1867954]
47. Parsot C, Menard R, Gounon P, and Sansonetti PJ (1995) Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures. *Mol Microbiol* 16, 291–300. [PubMed: 7565091]
48. Kenjale R, Wilson J, Zenk SF, Saurya S, Picking WL, Picking WD, and Blocker A (2005) The needle component of the type III secretion of *Shigella* regulates the activity of the secretion apparatus. *J Biol Chem* 280, 42929–42937. [PubMed: 16227202]
49. Bernard AR, Jessop TC, Kumar P, and Dickenson NE (2017) Deoxycholate-Enhanced *Shigella* Virulence Is Regulated by a Rare pi-Helix in the Type Three Secretion System Tip Protein IpaD. *Biochemistry* 56, 6503–6514. [PubMed: 29134812]
50. Forman S, Kas J, Fini F, Steinberg M, and Ruml T (1999) The effect of different solvents on the ATP/ADP content and growth properties of HeLa cells. *J Biochem Mol Toxicol* 13, 11–15. [PubMed: 9890443]
51. Epler CR, Dickenson NE, Olive AJ, Picking WL, and Picking WD (2009) Liposomes recruit IpaC to the *Shigella flexneri* type III secretion apparatus needle as a final step in secretion induction. *Infect Immun* 77, 2754–2761. [PubMed: 19433542]

52. Dickenson NE, Arizmendi O, Patil MK, Toth R. T. t., Middaugh CR, Picking WD, and Picking WL (2013) N-terminus of IpaB provides a potential anchor to the Shigella type III secretion system tip complex protein IpaD. *Biochemistry* 52, 8790–8799. [PubMed: 24236510]
53. Dickenson NE, Zhang L, Epler CR, Adam PR, Picking WL, and Picking WD (2011) Conformational changes in IpaD from *Shigella flexneri* upon binding bile salts provide insight into the second step of type III secretion. *Biochemistry* 50, 172–180. [PubMed: 21126091]
54. Epler CR, Dickenson NE, Bullitt E, and Picking WL (2012) Ultrastructural analysis of IpaD at the tip of the nascent MxiH type III secretion apparatus of *Shigella flexneri*. *J Mol Biol* 420, 29–39. [PubMed: 22480614]
55. Wee DH, and Hughes KT (2015) Molecular ruler determines needle length for the *Salmonella* Spi-1 injectisome. *Proc Natl Acad Sci U S A* 112, 4098–4103. [PubMed: 25775540]
56. Diepold A, and Wagner S (2014) Assembly of the bacterial type III secretion machinery. *FEMS Microbiol Rev* 38, 802–822. [PubMed: 24484471]

Figure 1. Docking calculations predict multiple inhibitor binding sites in Spa47.

(A) Top view of the activated homo-hexameric Spa47 model described previously.¹ Each Spa47 protomer in the complex is colored independently and the central channel containing the active sites is clearly visible. (B) The model is rotated up 90 degrees and the resulting front three protomers are removed to view a single active site at the interface between two protomers (black rectangle). (C) A closer view of the interface between two Spa47 protomers with ATP- γ -S modeled into the active site based on alignment to the nucleotide-bound structure PDB 5ZT1.² The top nine predicted interactions between the inhibitors and Spa47 are displayed for (D) compound 2834, (E) compound 3624, and (F) compound 9652. The highest affinity interaction calculated for each compound is shown using a darker shade for each inhibitor. The Spa47 and F₁ ATP synthase structures used in generating the Spa47 hexamer model are PDB 5SWJ¹ and PDB 1BMF,⁵ respectively.

Figure 2. Kinetic analyses of Spa47 inhibition.

(A) Titration of the compounds 2834, 3624, and 9652 with Spa47 shows that each of the tested compounds inhibit Spa47 ATPase activity. Inhibition profiles for each compound were fit to a four parameter logistic sigmoidal dose response curve. The effect of each inhibitor on Spa47 substrate concentration dependence was tested by plotting initial reaction velocities as a function of ATP concentration. The kinetic analyses were performed in the presence of 0 μM , 50 μM , 225 μM , and 500 μM (B) inhibitor 2834, (C) inhibitor 3624, and (D) inhibitor 9652. Data are plotted as the mean $\pm$ standard deviation from three independent analyses and A-D were fit to the Michaelis–Menten equation.

Figure 3. Spa47 ATPase inhibitors do not disrupt Spa47 oligomers.

Isolated Spa47 oligomer was incubated with each of the three inhibitors and oligomer state analyzed by size exclusion chromatography. The Spa47 monomer control eluted at ~2.1 mL while the Spa47 oligomer control and Spa47 oligomer incubated with 500 μ M of each inhibitor eluted from the column at ~1.3 mL, consistent with previous analyses showing that the oligomer elutes substantially earlier than the monomer and after the void volume of the column (1.1 mL).

Figure 4. Spa47 ATPase inhibitors do not affect *Shigella* growth rates.

Wild-type *Shigella* 2457T cultures were grown at 37 °C in the presence of 500 μ M inhibitor and 5% DMSO. Dilution plating was performed hourly for each culture and colony forming units (CFU) are plotted as a function of time and fit to a 3 parameter sigmoidal function, demonstrating nearly identical growth rates and curve profiles for each of the inhibitor conditions and the control culture containing no inhibitor.

Figure 5. Spa47 inhibitors prevent secretion of the T3SS effector protein IpaC.

(A) Representative western blots of secreted IpaC following Congo red activation of *Shigella* cultures treated with the indicated concentrations of each Spa47 inhibitor and 5% DMSO. Control conditions include both a Spa47 null *S. flexneri* strain and an *S. flexneri* strain expressing wild-type Spa47 cultured in the presence and absence of 5% DMSO. The cytoplasmic enzyme GAPDH was observed in the whole cell extracts (WCE) but not in the supernatant containing the secreted IpaC protein. (B) The level of secreted IpaC was quantified and plotted relative to the strain expressing wild-type Spa47 cultured in 5% DMSO. The data for inhibitors 3624 and 9652 were fit to a 4-parameter logistic sigmoidal dose response curve. The plotted values represent the mean $\pm$ the standard deviation from three independent western blot analyses spanning two independent bacteria growths.

Figure 6. Spa47 inhibitors do not prevent type three secretion apparatus formation.

Flow cytometry fluorescence intensity histograms comparing surface localization of the T3SA tip protein IpaD for *Shigella flexneri* strains lacking the gene for Spa47 (light gray shading) and expressing wild-type Spa47 (black). Green, and blue traces represent surface exposed IpaD levels of the wild-type *Shigella* strain that had been grown in the presence of inhibitors 3624 and 9652, respectively. The histograms include 100,000 individual intensity measurements per condition.

Table 1.Effect of Inhibitors on Spa47 Enzyme Kinetics^a

Inhibitor	Concentration	K_M^b (μM)	$V_{\max}$ ($\mu\text{M sec}^{-1}$)	k_{cat}^c (sec^{-1})	k_{cat}/K_M ($\text{M}^{-1} \text{sec}^{-1}$)
2834	0 μM	126 $\pm$ 11	0.16 $\pm$ 0.01	3.2 $\pm$ 0.2	$2.5 \times 10^4 \pm 2.7 \times 10^3$
	50 μM	113 $\pm$ 13	0.13 $\pm$ 0.00	2.6 $\pm$ 0.0	$2.3 \times 10^4 \pm 2.6 \times 10^3$
	225 μM	138 $\pm$ 9	0.12 $\pm$ 0.00	2.4 $\pm$ 0.0	$1.7 \times 10^4 \pm 1.1 \times 10^3$
	500 μM	143 $\pm$ 39	0.08 $\pm$ 0.01	1.6 $\pm$ 0.2	$1.1 \times 10^4 \pm 3.4 \times 10^3$
3624	0 μM	177 $\pm$ 43	0.21 $\pm$ 0.02	4.2 $\pm$ 0.4	$2.4 \times 10^4 \pm 6.2 \times 10^3$
	50 μM	227 $\pm$ 32	0.15 $\pm$ 0.01	3.0 $\pm$ 0.2	$1.3 \times 10^4 \pm 2.1 \times 10^3$
	225 μM	158 $\pm$ 17	0.10 $\pm$ 0.00	2.0 $\pm$ 0.0	$1.3 \times 10^4 \pm 1.4 \times 10^3$
	500 μM	249 $\pm$ 48	0.08 $\pm$ 0.01	1.6 $\pm$ 0.2	$6.4 \times 10^3 \pm 1.5 \times 10^3$
9652	0 μM	211 $\pm$ 62	0.15 $\pm$ 0.02	3.0 $\pm$ 0.4	$1.4 \times 10^4 \pm 4.6 \times 10^3$
	50 μM	144 $\pm$ 22	0.08 $\pm$ 0.00	1.6 $\pm$ 0.0	$1.1 \times 10^4 \pm 1.7 \times 10^3$
	225 μM	173 $\pm$ 63	0.04 $\pm$ 0.01	0.8 $\pm$ 0.2	$4.6 \times 10^3 \pm 2.0 \times 10^3$
	500 μM	----- ^d	-----	-----	-----

^a Initial reaction velocities were measured as a function of substrate (ATP) and inhibitor concentration for isolated Spa47 oligomer.^b Apparent K_M and $V_{\max}$ values $\pm$ the standard error were determined by fitting the mean values from three independent experiments to the Michaelis Menten equation.^c Apparent k_{cat} and k_{cat}/K_M values $\pm$ the standard error were calculated using the apparent K_M and $V_{\max}$ values in the table.^d The 500 μM 9652 condition abolished Spa47 activity and the data could not be fit to the Michaelis Menten equation.