

Small-Molecule Type III Secretion System Inhibitors Block Assembly of the *Shigella* Type III Secretion^{∇†}

Andreas K. J. Veenendaal,^{1‡} Charlotta Sundin,³ and Ariel J. Blocker^{1,2*}

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, United Kingdom,¹Departments of Cellular and Molecular Medicine and Biochemistry, School of Medical Sciences, University of Bristol, Bristol, BS8 1TD, United Kingdom,² and Innate Pharmaceuticals AB, Umestan Företagspark, SE-90347, Umeå, Sweden³

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Type III secretion systems (T3SSs) are essential virulence devices for many gram-negative bacteria that are pathogenic for plants, animals, and humans. They serve to translocate virulence effector proteins directly into eukaryotic host cells. T3SSs are composed of a large cytoplasmic bulb and a transmembrane region into which a needle is embedded, protruding above the bacterial surface. The emerging antibiotic resistance of bacterial pathogens urges the development of novel strategies to fight bacterial infections. Therapeutics that rather than kill bacteria only attenuate their virulence may reduce the frequency or progress of resistance emergence. Recently, a group of salicylidene acylhydrazides were identified as inhibitors of T3SSs in *Yersinia*, *Chlamydia*, and *Salmonella* species. Here we show that these are also effective on the T3SS of *Shigella flexneri*, where they block all related forms of protein secretion so far known, as well as the epithelial cell invasion and induction of macrophage apoptosis usually demonstrated by this bacterium. Furthermore, we show the first evidence for the detrimental effect of these compounds on T3SS needle assembly, as demonstrated by increased numbers of T3S apparatuses without needles or with shorter needles. Therefore, the compounds generate a phenocopy of T3SS export apparatus mutants but with incomplete penetrance. We discuss why this would be sufficient to almost completely block the later secretion of effector proteins and how this begins to narrow the search for the molecular target of these compounds.

Type III secretion systems (T3SSs) are key determinants of virulence in many gram-negative bacteria (10) that are pathogenic for plants, animals, and humans. These systems mediate a complex membrane transport process that directly delivers bacterial proteins across three biological membranes into host cells to enable bacterial infection. Major human pathogens that employ T3SSs for infection include *Shigella*, *Salmonella*, *Chlamydia*, *Yersinia*, and *Pseudomonas* species and enteropathogenic *Escherichia coli*.

We study the pathogenesis of *Shigella flexneri*. *Shigella* causes bacillary dysentery in humans, a disease characterized by invasion of, massive inflammation in, and destruction of the colonic mucosa. The genes required for invasion are clustered on a 31-kb region of a large virulence plasmid. There, the *mxi* and *spa* operons encode the T3SS apparatus and the *ipa* and *ipg* operons encode proteins known as effectors, five of which, IpaA to -D and IpgD, are abundantly secreted early in invasion (33). The T3SS apparatus comprises three distinct substructures: a cytoplasmic bulb, a region spanning both bacterial membranes and a hollow “needle” protruding from the bacterial surface. These last two portions can be purified together and are termed the needle complex (NC) (5, 21, 44). The

central 25-Å channel of the NC (9) is thought to serve as a conduit for effector protein secretion.

T3SSs are activated only upon direct physical contact with host cells. A protein complex at the tip of the needle, consisting of IpaB and IpaD, is directly involved in sensing host cells and regulating secretion (12, 31, 36, 42). Upon activation of the T3SS, IpaC is recruited to the needle tip, and this leads to insertion of the IpaBC translocon, which is required for translocation of the other early effectors into the plasma membrane of the host cell (42). The *Shigella* T3SS displays three different functional states of protein secretion: (i) a burst of protein secretion, known as induction, upon host cell contact (25) or addition of an artificial inducer, the small amphipathic dye molecule Congo red (CR) (2); (ii) a low rate of protein secretion, termed leakage, prior to host cell contact (23); and (iii) “constitutive secretion,” which represents an unregulated and higher rate of secretion than leakage that occurs in some needle mutants (19) and IpaB or IpaD deletion strains (6, 34, 35, 42).

To battle the increasing antibiotic resistance of pathogenic bacteria, it will be crucial to develop new antimicrobial agents. Strategies that rely on existing drugs and targets, which affect the viability of bacteria, all face the same problem, i.e., that bacteria quickly develop drug resistance under the huge selective pressure applied. The generation of drugs that specifically target pathogenic properties without killing bacteria is a strategy that might decrease the chance of bacterial resistance emerging against these drugs (20). Previously, in a large screening assay, small molecules that inhibit the expression of a virulence factor of the *Yersinia* T3SS without affecting the viability of the bacterial cell were identified (18). In subsequent years, these and other related salicylidene acylhydrazides were

* Corresponding author. Mailing address: Departments of Cellular and Molecular Medicine and Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, United Kingdom. Phone: 44-1173-312-019. Fax: 44-1173-312-091. E-mail: ariel.blocker@bristol.ac.uk.

† Supplemental material for this article may be found at <http://jb.asm.org/>.

‡ Present address: Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands.

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TABLE 1. Overview of inhibitory effects of compounds in studied T3SS-carrying pathogens

Compound	Alternative name(s) (reference)	Effect on (reference) ^a :		Other effects (reference)
		T3SS protein secretion/expression	In vitro virulence	
INP0007	Compound 2 (18), compound 1 (30)	<i>Yersinia</i> , yes (18, 30); <i>Salmonella</i> , yes (15, 29)	<i>Yersinia</i> , yes (30); <i>Salmonella</i> , yes (15, 29)	<i>Yersinia</i> motility (18)
INP0010	Compound 8 (30)	<i>Yersinia</i> , yes (30); <i>Salmonella</i> , yes (29); <i>Chlamydia</i> , yes (3)	<i>Yersinia</i> , yes (3); <i>Salmonella</i> , yes (29); <i>Chlamydia</i> , yes (3)	
INP0400		<i>Salmonella</i> , yes (29); <i>Shigella</i> , yes (this study)	<i>Yersinia</i> , yes (3); <i>Salmonella</i> , yes (29); <i>Chlamydia</i> , yes (28); <i>Shigella</i> , yes (this study)	<i>Shigella</i> T3SS needle assembly (this study)
INP0401	Compound 10 (30)	<i>Yersinia</i> , yes (30); <i>Salmonella</i> , yes (29); <i>Shigella</i> , yes (this study)	<i>Salmonella</i> , yes (29); <i>Shigella</i> , no (this study)	<i>Shigella</i> T3SS needle assembly (this study)
INP0402	Compound 15 (30)	<i>Yersinia</i> , yes (30); <i>Salmonella</i> , yes (29); <i>Shigella</i> , yes (this study)	<i>Salmonella</i> , yes (29); <i>Shigella</i> , yes (this study)	<i>Shigella</i> T3SS needle assembly (this study)
INP0403	Compound 11 (30)	<i>Yersinia</i> , yes (30); <i>Salmonella</i> , yes (15, 29)	<i>Salmonella</i> , yes (15, 29)	<i>Salmonella</i> induced in vivo inflammatory response (15)
INP0404	Compound 20 (30)	<i>Yersinia</i> , yes (30); <i>Salmonella</i> , yes (29); <i>Shigella</i> , yes (this study)	<i>Salmonella</i> , yes (29); <i>Shigella</i> , yes (this study)	<i>Salmonella</i> motility and flagellin expression (29), <i>Shigella</i> T3SS needle assembly (this study)
INP0405	Compound 9 (30)	<i>Yersinia</i> , yes (30); <i>Salmonella</i> , yes (29); <i>Shigella</i> , yes (this study)	<i>Salmonella</i> , yes (29); <i>Shigella</i> , yes (this study)	<i>Salmonella</i> motility and flagellin expression (29)
INP0406		<i>Yersinia</i> , no (C. Sundin, unpublished data); <i>Salmonella</i> , yes (29); <i>Shigella</i> , no (this study)	<i>Yersinia</i> , no (3); <i>Salmonella</i> , yes (29); <i>Chlamydia</i> , no (3); <i>Shigella</i> , no (this study)	

^a T3SS-mediated protein secretion or expression (the latter only for reference 31) and in vitro virulence represent key assays for studying T3SS activity.

found to inhibit secretion of T3SS-related proteins and to affect host cell infection by *Yersinia*, *Chlamydia*, and *Salmonella* (3, 15, 28–30, 43). An overview of the T3SS-related inhibitory effects on studied pathogens for the compounds used in this work is given in Table 1. Results are shown for T3SS-mediated protein secretion and in vitro virulence (pathogen replication, host cell infection, and macrophage survival), which represent key assays to study T3SS activity. These T3SS inhibitors were also found to reduce the motility of *Yersinia* and *Salmonella* (18, 29) and to inhibit secretion and surface localization of flagellin in *Salmonella* (29). Taken together, these findings indicate that the inhibitors act on a conserved target, since they interfere with both the T3SS virulence determinant and the genetically related bacterial flagellar export system that mediates motility.

In this study we have investigated the effects of the small-molecule inhibitors on the *Shigella flexneri* T3SS. The extensive assays to analyze the *Shigella* T3SS-related protein secretion and virulence and the ultrastructure of its apparatus should be of great value in better understanding the general mode of action of these T3SS inhibitors. In addition, knowledge on how the inhibitors act on the T3SS should help us increase our understanding of the molecular mechanism of this complex biological system. Our results show that the T3SS inhibitors affect T3SS protein secretion and consequently *Shigella* virulence. Analysis of the secretin structure reveals that the inhibitors act on secretin assembly, leading to a twofold increase in needleless secretions and to a population of secretions with significantly shorter needles. MxiH, the major component of the *Shigella* T3SS needle, is itself one of the first T3SS substrates secreted during secretin biogenesis. This suggests that the molecular target of these T3SS-inhibitory molecules lies

within the highly conserved cytoplasmic or inner membrane export apparatus.

MATERIALS AND METHODS

Compounds. All small-molecule compounds used in this study were provided by Innate Pharmaceuticals AB (Umeå, Sweden). While some compounds have been used in previous studies under different names, they are now designated according to a new naming convention (INPxxxx). The same set of compounds was used and their structural formulas shown in a study performed by Negrea and coworkers (29); the formulas are reprinted in Fig. S1 in the supplemental material. Stock solutions (20 mM) of the compounds were prepared in dimethyl sulfoxide (DMSO), stored in the dark, and kept for no more than 4 weeks. The compounds were added at 50 μ M except when indicated otherwise. DMSO was used as a control, and the same volume as for the stock solutions was added.

Bacterial strains and growth conditions. The *S. flexneri* strains used in this study are listed in Table 2. Bacteria were grown in TCSB medium as previously described (4), except when indicated otherwise. To assess the effect of the compounds on *Shigella* growth in culture, overnight cultures were diluted in fresh TCSB medium to an optical density at 600 nm (OD₆₀₀) of 0.1, and then DMSO or small-molecule compounds were added. The cultures were incubated on a shaker at 37°C for 8 h. The growing cultures were sampled at 2-h intervals to determine their cell densities (OD₆₀₀).

TABLE 2. *Shigella* strains used in this study

Strain	Description	Reference or source
M90T	Wild type, serotype 5a	37
M90T-AfaE	M90T with plasmid p1018	41
CCUG 29416	Wild type, serotype 2a	CCUG, Sweden
<i>mxiG</i> /pSZ3 mutant	Carries pBAD::His ₆ - <i>mxiG</i>	44
SF622	Δ <i>ipaD</i>	26
SH116 (<i>mxiH</i> /D73A mutant)	Carries pRKMxiH D73A	19

Secretion assays. The analyses of secretion of T3SS effectors were performed as described previously (19), except for the following modifications. For leakage and constitutive secretion assays, overnight cultures were diluted in fresh TCSB medium to an OD₆₀₀ of 0.1, and then small-molecule compounds were added. The cultures were incubated on a shaker at 30°C for 30 min and then further grown on a shaker at 37°C for 8 h. The culture supernatants were analyzed for protein content. For CR induction, an overnight culture of wild-type *Shigella* was diluted 1:100 in fresh TCSB medium and grown on a shaker at 37°C to an OD₆₀₀ of 0.7. The small-molecule compounds were added and growth of the cultures was continued for another 30 min, after which cells pellets were harvested and resuspended in phosphate-buffered saline (PBS) to an OD₆₀₀ of 5. Small-molecule compounds were added again at 100 μM to compensate for the higher cell density, and the CR induction assay was performed. Fast constitutive secretion of the *ΔipaD* strain (42) was assayed as in the CR induction protocol but with the CR dye left out.

HeLa cell invasion. HeLa cell invasion by *Shigella* strain M90T-AfaE was monitored using a modified gentamicin protection assay (1). In short, HeLa cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin-streptomycin in a 24-well plate. The small-molecule compounds were added at the start of exponential growth of *Shigella* cultures. Bacterial cultures were grown to an OD₆₀₀ of 0.8 and spun down, and cell pellets were resuspended in PBS. HeLa cells were washed twice with PBS and then incubated in DMEM with 20 mM HEPES (pH 7.4) just before infection. *Shigella* cells were added to the HeLa cells to a multiplicity of infection of 2 and the plate centrifuged for 10 min at 900 × *g* and 20°C. *Shigella* M90T-AfaE expresses the *E. coli* afimbrial adhesin AfaE (22). This results in faster and more efficient binding of *Shigella* to eukaryotic cells and allows a reduction of the incubation time for HeLa cell infection from the usual 30 min to 5 min (8) at 37°C. The latter is important to prevent further *Shigella* growth during the incubation time and thus the formation of bacterial cells in the absence of the small-molecule compounds. After infection, the medium was aspirated, and HeLa cells were washed four times with cold PBS to remove free bacterial cells and incubated with DMEM supplemented with 100 μg/ml gentamicin for 45 min at 37°C to kill noninvading bacteria. The gentamicin solution was aspirated, and 0.1% Triton X-100 in PBS was added and incubated for 10 min at room temperature to lyse the HeLa cells. Finally, the lysate mix was plated out in serial dilutions, and CFU were determined on the following day.

Macrophage infection. A single red colony and a single white colony of *Shigella flexneri* strain CCUG 29416 were picked from an LB plate containing 0.01% CR and incubated overnight in LB on a shaker at 37°C. The white colony was obtained by repeated growth in liquid culture and plating on a CR plate. The white color of the colony reflects the loss of the virulence plasmid (24), and this was checked by the absence of a PCR product for *ipaC* and the absence of IpaC and IpaB proteins on a Western blot. The macrophage cell line, J774A.1, was seeded into a 96-well plate at a density of 7 × 10⁴ cells/well in DMEM (100 μl/well) plus 10% fetal calf serum and gentamicin (3 μg/ml) and grown for 16 h at 37°C in 5% CO₂. After 16 h the cells were washed once with PBS, and fresh DMEM (50 μl) containing the different compounds at 100 μM and bacteria (50 μl; OD₆₀₀ = 0.002) were added to give a final OD₆₀₀ of 0.001 and a compound concentration of 50 μM. Prior to infection, the overnight culture of *Shigella* was diluted 10-fold in DMEM and incubated on a shaker for 1.5 h at 37°C. After 5 h of infection, the medium was removed and 100 μl fresh PBS was added to the wells, and then a stock solution containing CalceinAM (Invitrogen) and Sytox Orange (Invitrogen) in PBS (20 μl; final concentrations of 1 μM and 0.05 μM for CalceinAM and Sytox Orange, respectively) was added and the plate incubated for 40 min at 37°C in a CO₂ incubator. The fluorescence was then read in a microplate reader (TECAN GENios; Calcein at 485/535 nm and Sytox Orange at 535/595 nm), and the results were confirmed by fluorescence microscopy.

Western blotting for secretion component level determination. To analyze whole-cell levels of secretion components, overnight cultures were diluted into fresh TCSB medium to an OD₆₀₀ of 0.1 and then small-molecule compounds were added. The cultures were incubated on a shaker at 30°C for 30 min and then further grown on a shaker at 37°C to an OD₆₀₀ of 0.8. The cell pellets were harvested and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting and immunodetection using mouse monoclonal and rabbit polyclonal antibodies directed against MxiJ (44) and MxiH (23), respectively.

***Shigella* osmotic shock treatment.** Overnight cultures were diluted into fresh TCSB medium to an OD₆₀₀ of 0.1, and then the compounds were added. The cultures were incubated on a shaker at 30°C for 30 min and then further grown on a shaker at 37°C to an OD₆₀₀ of 1. Ghost cells were prepared by osmotic shock treatment of bacterial cultures using glass beads as described previously (19).

NC purification. NCs were purified as described previously (44). Overnight cultures were diluted in fresh TCSB medium to an OD₆₀₀ of 0.1, small-molecule compounds were added, and then the cultures were grown on a shaker at 37°C to an OD₆₀₀ of 1. The compounds were maintained until bacterial lysis, which preceded the affinity purification step. This results in a total time for incubation with the compounds of approximately 5 h. In the control sample, a culture was grown in the absence of any compounds and INP0400 was added just before cell lysis.

Electron microscopy. Samples containing *Shigella* ghost cells or purified NCs were deposited onto 300-mesh Formvar and carbon-coated glow-discharged copper grids and subsequently stained for 1 min with either 1% (ghost cells) or 2% (NCs) phosphotungstic acid (pH 7.0). Electron microscopy images were taken on a Philips Tecnai12 or Zeiss Leo 912 transmission electron microscope at 80 kV. Micrographs were recorded at a magnification of ×20,000 on ProScan charge-coupled device cameras using the SIS software. Quantification of complete secretions and of bases was performed by hand on enlarged prints of the micrographs. Only secretions and bases at the edges of osmotically shocked cells, where the presence or absence of needles can be unambiguously discerned, were used. Needle lengths in NC preparations were measured in the same way. The measured needle length was converted to actual length using the thickness of the needle (7 nm) as a scale. The significance of the differences in the distribution of the needle length frequencies was assessed using a *G* test. The expected frequencies were set at 11% below 35 nm, 79% within 35 to 63 nm, 9% within 63 to 91 nm, and 1% within 91 to 112 nm using the average of the mean percentages obtained for the INP0400 control, INP0406, DMSO, and no addition, the mean frequencies and standard deviations of which were comparable (see Table 3). The statistical analysis was performed using the data sets pooled from either two or three independent experiments.

RESULTS

The small-molecule compounds inhibit secretion of *Shigella* T3SS proteins. The compounds were analyzed for their effect on protein secretion by the *Shigella* T3SS. First, their effect on the viability of *Shigella* cultures was tested. The compounds were added at 50 μM at the start of the exponential growth of wild-type *Shigella* cultures. An equivalent amount of DMSO was added as a control and showed no effect on *Shigella* culture growth (data not shown). INP0401, INP0402, INP0405, and INP0406 had no significant effect on cell growth, while INP0400 and INP0404 had only a mild effect (Fig. 1). The presence of INP0007, INP0010, and INP0403 led to severe growth defects (Fig. 1) and often led to cell lysis as observed by SDS-PAGE analysis of culture supernatants (data not shown). Similar effects of the compounds on culture growth were observed for the *mxiH/D73A* and *ΔipaD* mutant strains (data not shown). In light of these results, INP0007, INP0010, and INP0403 were excluded from subsequent experiments. The effects of the remaining compounds on the different type III secretion phenotypes were assessed. For the leakage and constitutive secretion assays, all drugs were added at the beginning of culture growth. For the fast constitutive secretion and CR induction assays, the drugs were added 30 min before the end of culture growth and maintained throughout the secretion assay. INP0400, and to a lesser extent INP0401 and INP0402, inhibited T3SS “leakage” by wild-type *Shigella* (Fig. 2A). INP0400, as well as INP0404, also inhibited constitutive secretion of the *mxiH/D73A* (Fig. 2B) and *ΔipaD* (data not shown) mutant strains. Similarly, both INP0400 and INP0405 inhibited the fast constitutive secretion phenotype (42) of the *ΔipaD* strain (Fig. 2C). INP0400 is the only compound that effectively inhibited CR-induced secretion (Fig. 3). Of note, no inhibitory effect on CR-induced secretion was observed when the compounds were added only after the cells were resuspended in PBS buffer (data not shown). In conclusion, while the variety of

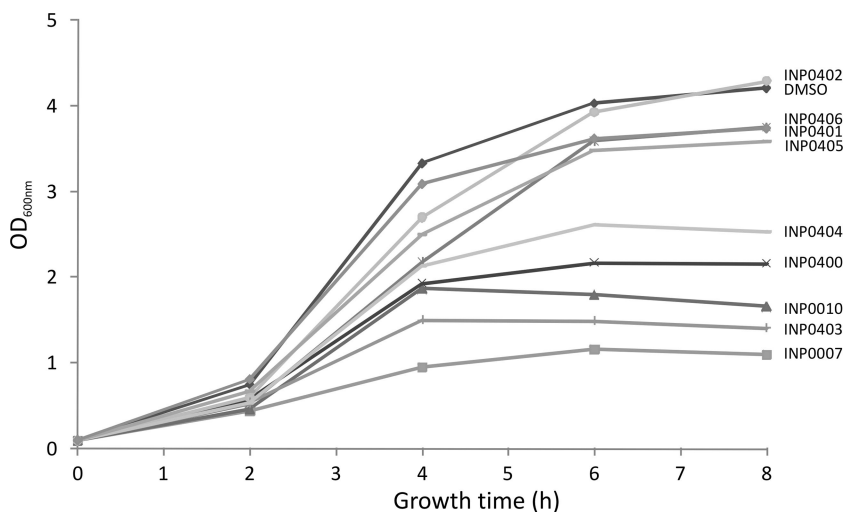


FIG. 1. Effect of the compounds on *Shigella* growth in broth culture. An overnight culture of wild-type *Shigella* was diluted to an OD_{600} of 0.1 and then grown in the presence of DMSO or 50 μ M of the compounds for 8 h. The cultures were sampled at 2-h intervals for cell density measurements at 600 nm.

secretion phenotypes that the *Shigella* T3SS displays were affected by different compounds, INP0400 was the strongest and the only consistent inhibitor, while INP0406 showed no effect in any of the secretion assays. INP0406 was therefore used as a control throughout all experiments.

The secretion inhibitors affect host cell infection. The compounds were tested for their ability to inhibit invasion of HeLa cells and killing of macrophages by *Shigella*. For the HeLa cell invasion assay, the selection of compounds was limited to the inhibitors INP0400 and INP0402, while INP0406 was used as a negative control. Although INP0400 had a significant impact on the ability of *Shigella* to invade HeLa cells, INP0402 appeared to be the strongest inhibitor in this assay (invasion, relative to the result for the DMSO control set to 100%, was $40\% \pm 13\%$, $17\% \pm 11\%$, and $81\% \pm 13\%$ [means \pm standard deviations from duplicate experiments] for INP0400, INP0402, and INP0406, respectively). No inhibitory effect was observed when the compounds were added just 30 min before the end instead of at the start of cell growth (data not shown).

The inhibitors affect the induction of macrophage apoptosis. To further investigate the effect of the compounds on *Shigella flexneri*, an ex vivo assay in which the effect of the compounds was measured during cell infection was developed. In these experiments the mouse macrophage cell line J774 was infected with the virulent and plasmid-cured *Shigella flexneri* strain CCUG 29416. After 5 h of infection, the fluorescent dyes CalceinAM and Sytox Orange were added, at which point CalceinAM is taken up by the cells. If the cells are alive, an enzyme converts nonfluorescent CalceinAM to intensely green fluorescent Calcein (3). If the membranes of the eukaryotic cells have been damaged, Sytox Orange is taken up and will bind DNA, which increases the red fluorescence of Sytox Orange 400 times. The wild-type *Shigella* strain caused cell death by injection of effector proteins into the macrophage cytosol via the T3SS (45), and a reduced green fluorescence signal (Fig. 4) and an increased red fluorescence signal were observed. In contrast, the virulence plasmid-cured strain had no or little effect on macrophage viability compared to uninfected

cells (not shown). The Sytox Orange results are not shown since they display similar but opposite trends. However, they are a fast indicator of toxicity of the compounds for macrophages and show that the compounds were not toxic to macrophages (reference 3 and data not shown). INP0404 and INP0405 showed the strongest inhibitory effect on the T3SS-mediated macrophage killing, and they restored the green fluorescence signal to the same level as for uninfected cells at 20 μ M (not shown) and 50 μ M (Fig. 4), respectively. INP0400, INP0401, and INP0402 had less of an effect on inhibiting cell death, while the negative control, INP0406, had little inhibiting effect (Fig. 4). This assay provides information on virulence inhibition and toxicity and shows that this class of compounds clearly inhibits T3SS-mediated virulence and helps the macrophages clear the infection.

The secretion inhibitors affect assembly of the T3SS needle. The results described so far show that the compounds have inhibitory effects on *Shigella* T3SS activity. This could result from an effect of the inhibitors on the assembly of the T3SS machinery. To investigate whether the drugs prevented expression of or destabilized T3S components, we examined the levels of MxiJ, an inner membrane protein of the NC, and MxiH, the needle subunit, in whole-cell extracts of bacteria exposed to the compounds. Western blotting showed that the levels of these proteins remained generally similar in all drug-treated samples and in particular compared to control compound INP0406 (Fig. 5) or a DMSO control. However, the possibility remained that the compounds affected assembly of the T3SS without an apparent effect on the levels of its protein components. To test this hypothesis, the macromolecular T3SS structure was analyzed by electron microscopy. *Shigella* cells from cultures grown in the presence of the inhibitors were osmotically shocked to produce ghost cells. In these cells, the cytoplasm is washed out to allow the visualization of the secretions at the cell periphery. The number of secretions per cell was decreased by 30 to 40% in all compound-treated samples, although the sample with the control compound INP0406 was much less affected (Fig. 6). The DMSO and INP0406 controls

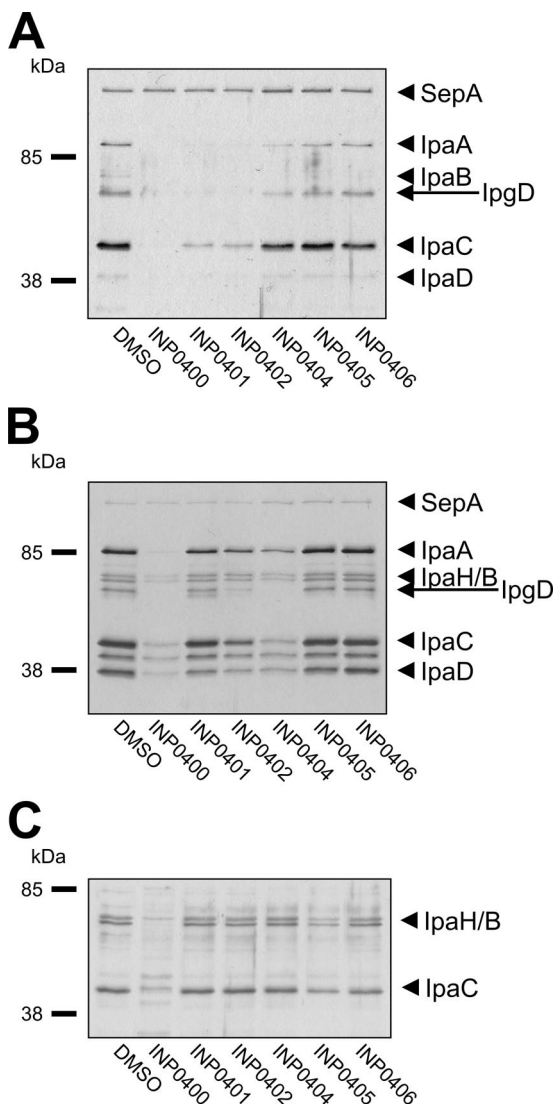


FIG. 2. Inhibition of leakage and constitutive secretion of T3SS proteins. *Shigella* cultures were grown in the presence of DMSO or compounds, and then culture supernatants were isolated. Samples were separated by SDS-PAGE, and the 12% gels were silver stained. The gels show “leakage” of wild-type *Shigella* (A), constitutive secretion of the *mxiH/D73A* mutant, (B) and fast constitutive secretion of the Δ *ipaD* strain (C). The positions of the abundant T3SS proteins are indicated on the right. The SepA protein, which is secreted by an autotransporter pathway, acted as a loading control.

showed that 20% of secretons in each cell lacked a detectable needle. We term these needleless secretons “bases.” About half of these can be attributed to assembly of immature and thus incomplete secretons (A. J. Blocker, unpublished data), while the rest are likely caused by the mechanical force (vortexing with glass beads) applied during the preparation of ghost cells. More importantly, the percentage of secretons without a detectable needle (percentage of bases/secreton) increased significantly for inhibitors INP0400 to INP0404 (two-fold increase) (Fig. 6). This increase in needleless secretons is probably an underestimate because bases are harder to identify with certainty. Indeed, in view of the fact that the levels of

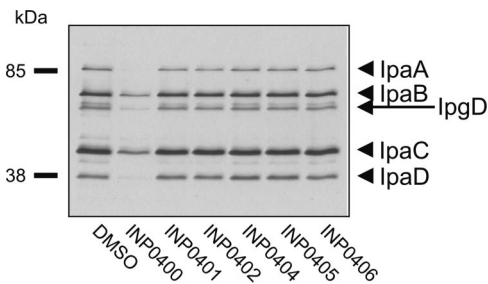


FIG. 3. Inhibition of CR-induced secretion. Wild-type *Shigella* cultures were grown in the presence of DMSO or compounds, and then culture supernatants were isolated. Samples were separated by SDS-PAGE, and the 12% gel was silver stained. The positions of the major T3SS proteins are indicated on the right.

bacterial MxiJ and MxiH remain constant, nondetection of bases may also explain the apparent decrease in total secreton numbers in the samples treated with the active drugs.

To assess the effect of the inhibitors on the assembly of the T3SS needle in greater detail, NCs were purified from *Shigella* cultures grown in the presence of DMSO, INP0400, or INP0406 (see “NC purification” in Materials and Methods). For this, the *mxiG/pSZ3 Shigella* strain, which produces secretons with a His-tagged MxiG inner membrane component that allows affinity purification of NCs (44), was used. The proportion of NCs that lacked the needle structure was markedly increased in these samples (data not shown) relative to the percentage of needleless bases observed in osmotically shocked cells. As this proportion did not change in the different samples, it was assumed to result primarily from mechanical damage occurring to NCs during isolation. Therefore, in

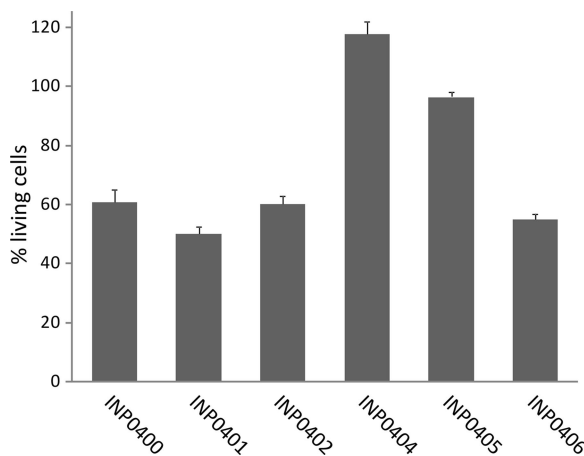


FIG. 4. Survival of *Shigella*-infected macrophages. Cells of macrophage cell line J774 were infected with wild-type and plasmid-cured *Shigella flexneri* strain CCUG 29416 grown in the presence of the compounds. The fluorescence intensity was measured in a microplate reader (Calcein, 485/535 nm; Sytox Orange, 535/595 nm), and the relative amounts of living cells and cells with the cell membrane destroyed, respectively, were calculated. The bars show the mean values from three samples, and the standard deviations were calculated according to a Gauss formula suitable for percentage values. Cells infected by wild-type *Shigella* without addition showed a value of around 40% living cells. The value for uninfected cells was set to 100% living cells.

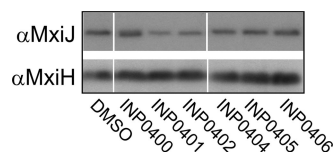


FIG. 5. Whole-cell levels of T3SS component proteins. Wild-type *Shigella* cultures were grown in the presence of DMSO or compounds, and then whole-cell extracts were isolated. Samples were separated by SDS-PAGE and analyzed by Western blotting. The antibodies used for the blots are indicated on the left. Lanes were removed for consistency of display of the samples. For each blot, the same exposure was used to make the composite image, and the remaining lanes are shown in their initial loading order.

this assay we have discounted the needleless structures and instead focused on those that retained the needle. The needle lengths of the latter NCs were measured from electron microscopy images and the length distribution determined. The needles were assigned to four different classes of needle length, i.e., less than 35, 35 to 63, 63 to 91, and 91 to 112 nm (Table 3). For all samples, the majority of needles were in the 35- to 63-nm range. However, NCs purified from cultures treated with the inhibitor INP0400 had significantly increased numbers of needles shorter than 35 nm (and consequently decreased numbers of needles in the 35- to 63-nm class) than the DMSO and INP0406 controls. To exclude the possibility of a destabilization or depolymerization effect of INP0400 on the needles, NCs were purified from a *Shigella* culture grown in the absence of any compound and INP0400 was added after the spheroplasting step, just before cell lysis. These NCs displayed a needle length distribution similar to that of the DMSO and INP0406 controls (Table 3, INP0400C). When the statistical significance of these findings was tested, the distribution of needle lengths in the INP0400 sample was found to differ from

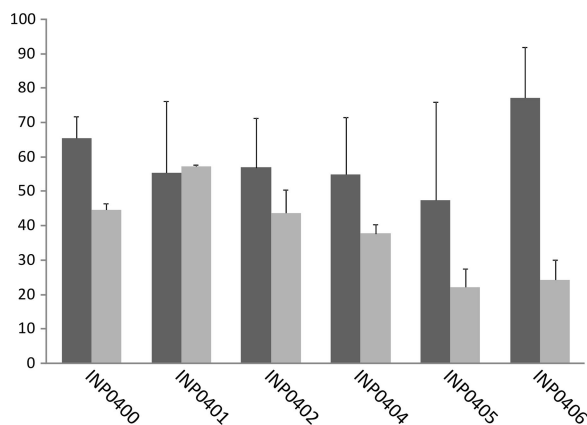


FIG. 6. Analysis of secretion assembly. Quantification of complete secretions and needleless bases from electron micrographs of ghost cells prepared from wild-type *Shigella* cultures grown in the presence of DMSO or compounds is shown. Data are derived from experiments in duplicate or triplicate ($n = 10$ to 13 images per sample per experiment). Micrographs of the DMSO control showed ~ 5 secretions/image and $19\% \pm 5\%$ bases/secretions (not shown). Percentages of secretions (dark gray bars) are relative to the DMSO control (set to 100%). Percentages of bases/secretions (light gray bars) are normalized to the DMSO control for each experiment separately. The errors given are standard deviations.

TABLE 3. Effects of inhibitors on secretion needle length

Needle length (nm)	% of needles (mean \pm SD) ^a after the following addition:				
	INP0400	INP0400C ^b	INP0406	DMSO	None
<35	18 \pm 4	11 \pm 7	9 \pm 4	11 \pm 1	13 \pm 1
35–63	66 \pm 10	73 \pm 4	79 \pm 5	81 \pm 9	81 \pm 6
63–91	15 \pm 13	15 \pm 8	10 \pm 8	8 \pm 10	5 \pm 4
91–112	1 \pm 1	1 \pm 1	1 \pm 0	0 \pm 0	1 \pm 1

^a Data derived are from either two or three independent experiments.

^b Control in which *Shigella* cultures were grown in the absence of compounds and INP0400 was added just prior to the cell lysis step.

those of all other samples at a P value of 7.6×10^{-9} , whereas the distributions of needle lengths in all other samples were similar to the expected frequencies in the control samples (P values were all above 0.02). Taken together, these results show that the inhibitors affect assembly of the needle.

DISCUSSION

We have shown that a variety of tested small-molecule compounds (INP0400 to INP0402, INP0404, and INP0405) inhibit all known forms of T3SS secretion in *Shigella*, as well as this bacterium's ability to invade HeLa cells and cause apoptosis of macrophages. We further showed that these compounds partially block the assembly of the *Shigella* T3SS. The block was found to occur at the level of export of the first substrate(s) in the secretion hierarchy, the needle protein component(s). This step is a highly conserved part of the T3SS/flagellar assembly pathway, as is further supported by a previous finding that these drugs also inhibit motility/flagellar protein surface presentation. In light of these observations, we propose that the inhibition of T3SS assembly is the primary effect of these drugs in gram-negative bacteria.

The results from the variety of secretion assays performed show differences in the spectra of activity of the tested compounds. They may result from the different functional states of the T3SS and the different set of substrates under conditions of leakage and induced secretion in wild-type cells and (fast) constitutive secretion in T3SS mutants (2, 6, 11, 13, 23, 25, 27, 34, 35, 39, 42). Thus, we propose that the different T3SS activity states are differentially sensitive to the action of the drugs. Such differences, together with the divergence in T3SSs, may explain why the groups of compounds that are most effective against the tested species of T3SS-carrying bacteria differ (3, 15, 18, 28–30, 38, 43). There are also differences in the spectra of activity of the tested compounds in the HeLa cell invasion and macrophage cytotoxicity assays. In contrast to the case for the similarly set up protein secretion assays, the drugs were added at the start of bacterial growth in the HeLa cell invasion assay, while they were added just prior to infection in the macrophage cytotoxicity assay. Thus, the drugs can be taken up by the bacterial cell prior to HeLa cell invasion, while they have to cross the macrophage membrane first to reach the intracellular bacteria. Therefore, these differences may reflect differences in properties of uptake of the compounds across the bacterial and macrophage cell envelope. In addition, altered solubility of the compounds in the different media when added in these assays may play a role. However, the com-

pounds all belong to the same chemical class of hydrazones, and given their strong similarity in chemical structure, it is likely that the effective compounds have the same molecular target(s), albeit with expected different binding affinities. This is supported by the fact that INP0400 was found to be the most effective compound in all but the macrophage survival assay, which first requires drug uptake by eukaryotic cells.

While all forms of T3SS-mediated secretion in *Shigella* were inhibited, the strongest effect was observed when the compounds were added during growth. No inhibition of CR-induced secretion was seen when the compounds were added less than 30 min prior to cell harvesting (not shown). Invasion of cells was also not inhibited when the drugs were added only during incubation of *Shigella* with the HeLa cells instead of during bacterial growth (not shown). Our findings are consistent with a study by Nordfelth and coworkers (30) in which they observed a delay of 30 min in the effect of the inhibitors on the *Yersinia* T3SS *yopE* promoter activity. Furthermore, Hudson et al. (16) showed that *Salmonella*-induced intestinal secretory and inflammatory responses in calves can be inhibited by preincubation of the bacteria with structurally related compounds but not by treatment of the bacteria with these chemicals at the time of inoculation. Interestingly, drug pretreatment had no effect on *Chlamydia* elementary bodies, which are unable to grow in vitro, whereas addition of the compounds after elementary body infection of eukaryotic cells leads to strong inhibition of bacterial replication within intracellular vacuoles, a phenomenon known to involve a T3SS (38). After overnight growth, it is during renewed exponential growth at 37°C that *Shigella* secretions are assembled de novo (Blocker, unpublished data). Hence, these data are consistent with those we obtained by electron microscopy showing that the compounds affect needle elongation, a key step in secretin maturation. Indeed, it is unlikely that the effects of the drugs are due to destabilization of preassembled secretions or depolymerization of needle subunits because (i) they were ineffective when added after bacterial growth, (ii) the levels of MxiJ and MxiH in whole-cell extracts were unaffected by addition of any of the compounds, and (iii) addition of INP0400 prior to the NC purification step, rather than during growth, did not lead to significantly shorter needles.

Our electron microscopy data demonstrate that the T3SS assembly block is not complete. Nevertheless, the assembly defect generated by the drugs is very reminiscent of the phenotype of many mutants with mutations in the cytoplasmic or inner membrane T3S export apparatus (40). This is why we propose that the compounds generate a phenocopy of T3SS export apparatus mutants with incomplete penetrance. The incomplete penetrance is probably due to the weak binding affinity of these (early-generation) drugs for their target(s). That would explain why the drugs act efficaciously only when added during growth of bacteria or during long incubation times when the bacteria are within host cells (as in the macrophage killing assay). However, the drugs significantly slow or even stochastically abolish needle maturation. Needle assembly must be complete before the secretin becomes capable of host cell interaction and effector protein secretion (reviewed in reference 10). This would explain the strong inhibitory effect of the drugs in our T3SS-related secretion and virulence assays.

Drug removal would allow rapid completion of needle assembly and consequently the recovery of T3SS activity (30).

These conclusions are also largely consistent with the work of Negrea et al. (29), who showed that a partially overlapping set of salicylidene acylhydrazides compounds inhibit secretion of SipB (the IpaB homolog) by the *Salmonella* SPI1 T3SS, intracellular growth of this bacterium (which is mediated by the SPI2 T3SS), and flagellum-mediated motility in this organism. Of particular relevance, they showed that the compounds reduce surface presentation of flagellin. This would be expected if the compounds led to a partial block in hook assembly, which is the morphological equivalent of the T3SS needle, as there is a known transcriptional feedback mechanism that prevents filament biogenesis when the hook is incomplete (7). Negrea et al. (29) also showed that treatment with the compounds led to transcriptional silencing of the SPI1 region that would contribute to the effect of the drugs. That could imply the existence of a negative regulatory mechanism that leads to SPI1 transcriptional silencing following inhibition of T3S, even though such a mechanism has not been observed to date.

In summary, our data suggest that the primary effect of these compounds is on T3SS export apparatuses. Thus, their direct or indirect target(s) probably lies within the inner membrane or cytoplasmic export apparatus components of both flagella and the T3SS. This explains why an incomplete block in T3SS assembly results in a clear inhibition of T3SS activity. As these components are the most conserved ones between the two related families of protein secretion machines, it is not surprising that the compounds exhibit such a broad efficacy spectrum (3, 15, 18, 28–30, 38, 43) (Table 1).

Future work should focus on the elucidation of the exact molecular target(s) of these exciting compounds within T3SSs. This work is urgently needed, as target identification is the only path toward rational improvement of the affinity of these compounds for their target(s) and hence of their efficacy. Such an effort is justified by the fact that these compounds are presently the only type well-known to broadly inhibit T3SS assembly and hence function (although two other families of T3SS inhibitors have been identified recently) (14, 16, 17, 32), representing an entirely new therapeutic opportunity.

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