Inhibition of Type III Secretion in \textit{Salmonella enterica} Serovar Typhimurium by Small-Molecule Inhibitors$^\dagger$

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Type III secretion systems (T3SS) are conserved in many pathogenic gram-negative bacteria. Small molecules that specifically target T3SS in \textit{Yersinia} and \textit{Chlamydia} spp. have recently been identified. Here we show that two such compounds inhibit \textit{Salmonella} T3SS-1, preventing secretion of T3SS-1 effectors, invasion of cultured epithelial cells, and enteritis in vivo.

Salmonellae are important human and animal bacterial pathogens which cause a spectrum of diseases ranging from gastroenteritis to typhoid fever. Nontyphoidal salmonellosis involves acute intestinal inflammatory and secretory responses which are induced upon the interaction of intestinal epithelial cells with the pathogen (18). A type III secretion system (T3SS-1), encoded within the inv-spa-prg (SPI-1) chromosomal locus, is essential for the ability of \textit{Salmonella enterica} serovar Typhimurium to invade enterocytes and induce enteritis (19). Structural components of T3SS-1 form the secretion organelle known as the needle complex. This multiprotein structure spans both inner and outer bacterial membranes and functions to secrete \textit{Salmonella} virulence-associated proteins. The secreted proteins SipB, SipC, and SipD permit translocation of other secreted proteins, known as effectors, by forming a pore in the eukaryotic cell membrane through which effector proteins pass. The effector proteins are delivered into host cells and act in concert to elicit a variety of cellular responses for the benefit of the pathogen (for a review see reference 3). Muta-
tional inactivation of genes encoding the structural or regulatory components of T3SS-1, or in the three sip genes, completely abolishes effector protein translocation, \textit{Salmonella} invasion (7, 8), and enteropathogenesis (22). Inactivation of genes encoding individual effector proteins results in a partial attenuation (6) in a bovine ligated ileal loop model of infection.

T3SSs are conserved in a variety of gram-negative animal and plant pathogenic and symbiotic bacteria and deliver a set of effectors (unique for each bacterial pathogen) into host cells, which then subvert, inhibit, or activate cellular pathways. The conservation of structural components of T3SSs and their importance for virulence in many bacterial pathogens make them an attractive target for chemical inhibition (2, 15). Recently, a class of small-molecule inhibitors specifically targeting the T3SS of \textit{Yersinia} spp. has been identified. It has been shown that these compounds inhibit T3SS-dependent protein translocation and, thus, \textit{Yersinia} virulence (9, 13). Moreover, the same or similar compounds have also been shown to inhibit type III secretion (T3S) in enteropathogenic \textit{Escherichia coli} (4) and \textit{Chlamydia trachomatis} (12, 23). Here we report that two of these compounds, which are inhibitors of the \textit{Yersinia} T3SS (13), are also effective inhibitors of \textit{Salmonella} T3SS-1.

Small-molecule inhibitors of \textit{Yersinia} T3SS affected protein secretion via \textit{Salmonella} T3SS-1 but had no effect on bacterial growth. Several compounds identified as small-molecule inhibitors of the \textit{Yersinia pseudotuberculosis} T3SS (13) were tested for their ability to inhibit the T3SS-1 of \textit{Salmonella}. The compounds used in this study were provided by In innate Pharmaceuticals and were prepared according to published procedures (1). All compounds were >95% pure according to $^3$H nuclear magnetic resonance spectroscopy. Initially the ability of these compounds to impair T3SS-1-mediated secretion in vitro was assessed. This assay relies on the fact that when \textit{Salmonella} cultures are grown under T3SS-1-inducing conditions (a temperature shift from 25°C to 37°C), they secrete a distinct set of proteins (T3SS-1 effectors known as Sips and Sops) via T3SS-1 into the culture supernatant. These proteins are the major constituents of the culture supernatant when cultures are grown in this way. A nalidixic acid-resistant mutant of \textit{Salmonella} serovar Typhimurium, \textit{Salmonella} serovar Typhimurium strain 4/74 (11), was used throughout these studies. Bacteria were cultured overnight in 10 ml Luria Bertani (LB) broth at 25°C with agitation. Cultures were diluted 10-fold into LB broth. Test compounds were added to a final concentration of 100 $\mu$M, a concentration which effectively inhibits \textit{Yersinia} T3SS (13). Control cultures were incubated with a dimethyl sulfoxide (DMSO) vehicle at the same concentration as that present in the test samples (0.1% vol/vol). Cultures were incubated at 37°C with agitation for 4 h. Samples were taken for bacterial enumeration at intervals during the incubation period. At 4 h of incubation, bacteria were pelleted by centrifugation, and the supernatant was passed through a 0.45-$\mu$m-pore-size low protein-binding filter. Secreted proteins were precipitated by the addition of trichloroacetic acid (final concentration, 10% vol/vol) and collected by centrifugation at 10,000 $\times$ g for 30 min at 4°C. Protein pellets were washed with acetone and resus-

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pended in sodium dodecyl sulfate-polyacrylamide gel electro-phoresis (SDS-PAGE) loading buffer. Proteins were purified from cultures containing similar numbers of bacteria, and identical volumes of reagents were used during processing; thus, any differences in the amounts of protein were not due to differences in the numbers of bacteria. Identical volumes of protein samples were analyzed by SDS-PAGE and Coomassie blue staining, and the amounts of proteins from samples cultured in the presence of inhibitors were compared to amounts from the DMSO control sample by eye. Significant inhibition was arbitrarily determined by whether the compounds resulted in overall lower amounts of protein on the Coomassie gel than that of the control. Several compounds were found to impair T3SS-1-dependent protein secretion (data not shown). Based on this initial assessment, INP0007 and INP0403, previously described as compound 1 and compound 11, respectively, in the literature (13) and sharing a common structural motif present in acylated hydrazones of salicylic aldehydes (Fig. 1a), were identified as having the greatest effect on the level of secreted proteins (Fig. 1b) and were chosen for further analysis. The inhibition of T3SS-1-mediated protein secretion was not a result of inhibition of bacterial viability and growth, as viable bacterial counts were the same in the presence or absence of the compounds at all time points examined (Fig. 1c).

Both INP0007 and INP0403 acted in a dose-dependent manner and were not cytotoxic. To determine the optimal concentration of a compound required to inhibit Salmonella T3SS-1 for use in further studies, we repeated the secreted protein experiment for INP0007 and INP0403, using a range of concentrations from 10 to 100 μM. Bacteria were cultured under T3SS-1-inducing conditions in LB medium supplemented with DMSO or INP0403. Secreted proteins were isolated from culture supernatants at 4 h and were subjected to SDS-PAGE followed by Coomassie staining. (b) INP0007 and INP0403 were added to HeLa cells at concentrations ranging from 0.1 to 100 μM and incubated for 3 h. Cell viability was determined using CellTiter96 AQueous one-solution cell proliferation assay (Promega) and is expressed as the percentage of DMSO control. Values represent the means of three independent experiments performed with triplicate samples. Error bars indicate standard deviations from the mean.
vine serum were seeded in 96-well plates and incubated overnight at 37°C in 5% CO2. INP0007 and INP0403 were added to the HeLa cells in a range of concentrations from 0.1 to 100 μM and incubated at 37°C in 5% CO2 for 3 h. Cytotoxicity was measured using a CellTiter96 AQueous one-solution cell proliferation assay (Promega) according to the manufacturer’s instructions. After 3 h, neither INP0007 nor INP0403 had a toxic effect at concentrations of 0.1 to 10 μM (Fig. 2b). Any slight cytotoxic effect of INP0403 at the highest concentration of 100 μM was offset by the fact that the compounds were added to bacterial cultures at 100 μM but were then further diluted in the in vitro assays. Thus, effects seen in vitro would be attributable to the specific activity of the compounds on T3S and not any cytotoxic effect.

**INP0007 and INP0403 inhibited T3SS-1-associated virulence phenotypes in vitro.** To further analyze the effect of INP0007 and INP0403 on the function of T3SS-1, we assessed whether these compounds could inhibit Sip-mediated pore-forming activity against erythrocyte membranes. Previous studies using several bacterial species have demonstrated that their respective T3SSs cause contact-dependent hemolysis of red blood cells in vitro (5, 16, 21). This is probably due to leakage of hemoglobin through the translocation pore formed in the membrane of the erythrocyte (14). Recently, a modified form of this hemolysis-based assay was used to evaluate expression of T3SS-1 in *Salmonella* in a rapid, simple, and reliable manner (10). Since DMSO was expected to affect the integrity of erythrocyte membranes, we preincubated bacteria with inhibitors prior to their use in the hemolysis assay. *Salmonella* serovar Typhimurium 4/74 was cultured overnight in LB broth at 25°C with agitation. Aliquots from the overnight culture were diluted 10-fold into prewarmed LB broth. INP0007, INP0403, or DMSO alone was added into each respective subculture and incubated at 37°C for 1 h. Bacteria were recovered by centrifugation, washed in PBS, resuspended in fresh LB medium, and assessed for their ability to lyse sheep red blood cells as described previously (10), with the modification that a red blood cell-PBS suspension was used. This assay gave an absorbance reading which was normalized to a negative control containing no bacteria to remove background lysis. The normalized readings for the DMSO or inhibitor samples were then expressed as “hemolytic activity as percentage of wild-type,” where the wild-type was untreated bacteria and was taken as 100%. Bacteria preincubated with either compound showed a reduction in contact-dependent hemolysis compared to that mediated by *Salmonella* serovar Typhimurium strain 4/74 preincubated with DMSO (Fig. 3a).

As it is well established that effector proteins delivered by T3SS-1 mediate the invasion of *Salmonella* into epithelial cells by stimulating rearrangements of the subcortical actin cytoskeleton, we assessed whether INP0007 and INP0403 would impair the invasion of HeLa cells by *Salmonella* serovar Typhimurium strain 4/74. Bacteria were cultured overnight at 25°C with agitation, diluted 10-fold into fresh LB containing INP0007, INP0403, or DMSO, and cultured for a further hour at 37°C to induce T3SS-1 effector protein expression. Bacteria were used to infect HeLa cells at a multiplicity of infection of approximately 10. The invasion assay was performed essentially as described previously (24). Briefly, following a 30-min incubation of bacteria with the HeLa cells, cells were washed with PBS, and gentamicin (150 μg/ml) was added to the culture medium for 30 min to kill extracellular bacteria, after which the HeLa cells were washed three times with PBS and lysed with 1% (vol/vol) Triton X-100 in PBS. Intracellular bacteria were enumerated by plating serial dilutions on LB agar. The presence of either INP0007 or INP0403 resulted in a reduction, 47% for INP0007 and 60% for INP0403, in bacterial invasion of HeLa cells compared to that for the DMSO control (Fig. 3b). These reductions in invasion are comparable to those seen with mutations in T3SS-1 genes required for invasion, for example, the sopE gene (17, 23).

The fact that both contact-dependent hemolysis and eukaryotic cell invasion are not completely prevented is most likely due to the reversible effect of the compounds, as observed for *Yersinia* (13), allowing the bacteria to regain some or most of their T3S activity when the compounds are washed out, in the case of the hemolysis assay, or diluted out upon addition of bacteria to cell culture medium, in the invasion assay. Nevertheless, taken together, these data suggest that both INP0007 and INP0403 inhibit T3SS-1-associated virulence phenotypes in vitro by INP0007 and INP0403 (Fig. 3a). (a) Preincubation of *Salmonella* cultures with INP0007 or INP0403 suppresses T3SS-1-mediated contact-dependent hemolysis. Bacteria were preincubated with 100 μM compounds for 1 h at 37°C, before being washed and incubated with sheep red blood cells for 4 h. Hemolytic activity is presented as the percentage of the wild-type strain, which was taken as 100%. Values represent the means of two independent experiments performed with triplicate samples. Error bars indicate standard deviations from the means. (b) Preincubation of *Salmonella* cultures with INP0007 or INP0403 inhibits bacterial invasion of eukaryotic cells in vitro. HeLa cell monolayers were infected at a multiplicity of infection of 10 with *Salmonella* serovar Typhimurium strain 4/74 cultured for 1 h in the presence of 100 μM compounds (final compound concentration of 4.5 μM in the invasion assay). Thirty minutes postinfection, extracellular bacteria were killed with the addition of gentamicin, and 1 h postinfection, cells were lysed, and viable intracellular bacteria were enumerated by plating serial dilutions on LB agar. Values are the mean log10 CFU/ml of triplicate counts, and error bars indicate standard deviations from the means.
and INP0403 are able to inhibit T3SS-1 secretion and T3SS-1-associated virulence phenotypes in vitro.

Preincubation of *S. enterica* serovar Typhimurium with INP0007 or INP0403 suppressed T3SS-1-induced secretory and inflammatory responses in vivo. The effect of INP0007 and INP0403 on the induction of T3SS-1-mediated intestinal secretory and inflammatory responses by *S. enterica* serovar Typhimurium was assessed in a bovine intestinal ligated loop model. *Salmonella* serovar Typhimurium 4/74 was grown overnight at 25°C with agitation, diluted 10-fold into fresh LB medium, and grown for 1.5 h at 37°C with agitation. Two approaches were taken to assess the effect of the compounds: either 100 μM compound or 0.1% (vol/vol) DMSO was added to the bacteria for the 1.5-h incubation period (preincubated), or both were added to the bacteria immediately prior to injection into the bovine intestinal ligated loops (coinjected). The experimental procedures were performed essentially as described previously (20), in accordance with the Animals (Scientific Procedures) Act of 1986 and with the approval of the local ethical review committee. Three ca. 4-week-old Friesian bull calves were confirmed to be culture negative for *Salmonella*, placed under terminal anesthesia, and underwent a laparotomy. Ligated loops (6 cm) were constructed in the mid ileum, and each treatment was tested in triplicate, with 5 ml of bacterial culture injected into discrete loops in a semirandomized order. Neutrophils were isolated from jugular blood after induction of anesthesia, radiolabeled with 111In-oxinate, and reinjected within an hour of loop inoculation as described previously (20). Twelve hours after loop inoculation, calves were killed with an overdose of sodium pentobarbitone, the loops were excised and the volume of fluid accumulated was measured, and radioactivity associated with loop contents and mucosa was quantified. Statistical analysis (*t* test) of the results, comparing the effect of the various chemical treatments to those of *Salmonella* cultured in the presence of DMSO alone, was performed using Minitab statistical software (release 14.13). *P* values of <0.05 were considered significant.

An internal control strain of *Salmonella* serovar Typhimurium 4/74 harboring a transposon insertion in prgH (prgH:: miniTn5Km2 [11]), a key structural component of T3SS-1, confirmed that the model is capable of detecting T3SS-1-mediated enteritis in calves (*P* < 0.02 for both secretory and inflammatory responses; Fig. 4a and b). The magnitude of the secretory and inflammatory responses elicited by *Salmonella*...
serovar Typhimurium 4/74 preincubated with INP0007 or INP0403 for 1.5 h prior to injection into the loops was lower than that elicited by Salmonella serovar Typhimurium 4/74 preincubated with DMSO control (Fig. 4a and b), indicating that both chemicals suppressed enteric virulence of Salmonella in vivo. The reduced secretory response elicited when Salmonella serovar Typhimurium 4/74 was pretreated with INP0403 (Fig. 4a) was statistically significant \((P = 0.0001)\) compared to that of Salmonella serovar Typhimurium 4/74 cultured with DMSO alone. However, when INP0007 and INP0403 were co-injected into loops alongside the Salmonella inoculate there was no significant suppression of secretory and inflammatory responses \((P = 0.7380 \text{ and } P = 0.9998 \text{ for INP0007; } P = 0.9690 \text{ and } P = 1.0 \text{ for INP0403; Fig. 4a and b})\). This could be explained by a rapid dilution of the chemicals by intestinal fluids and/or by their absorption by intestinal epithelia or their inactivation by intestinal enzymes. Elevated secretory or inflammatory responses were not observed for the loops injected with the chemicals or with DMSO alone.

Concluding comments. Our work, together with previously published studies of Yersinia, clearly demonstrates the potential of INP0007 and INP0403 to inhibit the activity of distantly related T3SSs, making such compounds attractive as potential antiinfective agents. By disarming a key virulence factor without affecting bacterial replication, it is anticipated that resistance to such compounds may be less likely to develop than with conventional antimicrobials. While additional studies will be required to determine the active concentration, stability, and toxicity of such inhibitors at relevant sites in vivo, we assert that further work is needed to develop the therapeutic potential of INP0007 and INP0403 and their derivatives.

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