Small molecule inhibitors of type III secretion in *Yersinia* block the *Chlamydia pneumoniae* infection cycle

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Abstract Intracellular parasitism by *Chlamydiales* is a complex process involving transmission of metabolically inactive particles that differentiate, replicate, and re-differentiate within the host cell. A type three secretion system (T3SS) has been implicated in this process. We have here identified small molecules of a chemical class of acylated hydrazones of salicylaldehydes that specifically blocks the T3SS of *Chlamydia*. These compounds also affect the developmental cycle showing that the T3SS has a pivotal role in the pathogenesis of *Chlamydia*. Our results suggest a previously unexplored avenue for development of novel anti-chlamydial drugs.
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1. Introduction

Chlamydia spp. are obligate intracellular Gram-negative bacteria that have a unique biphasic developmental cycle, including two bacterial forms: a metabolic inert form termed elementary bodies (EBs) that are able to enter host cells by endocytosis and the metabolically active, replicating and non-infectious reticulate bodies (RBs) [1].

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Abbreviations: C. pneumoniae, Chlamydia pneumoniae; *C. trachomatis, Chlamydia trachomatis*; EB, elementary body; RB, reticulate body; T3SS, type three secretion system; TARP, tyrosine phosphorylated protein; Incs, inclusion membrane localized proteins; *Y. pseudotuberculosis*, *Yersinia pseudotuberculosis*; FCS, fetal calf serum; PBS, phosphate buffered saline; MOI, multiplicity of infection; p.i., post infection; *Hsp90*, Human 90 kD heat shock protein gene; ompA, outer membrane protein A gene; wt, wild-type; nd, not detectable; DAPI, 4′,6-diamidino-2-phenylindole; Ysc, *Yersinia* secretion

Although the complete genomes of several *Chlamydia* species are known [2–4], there are as yet no tools for genetic manipulation of these organisms. The involvement of a type three secretion system (T3SS) in the infectious cycle has been suggested [5,6]. T3SSs are well-recognized virulence systems in Gram-negative bacteria, e.g. human and animal pathogens including *Yersinia* spp., *Pseudomonas aeruginosa*, *Shigella flexneri*, *Salmonella typhimurium*, enteropathogenic *Escherichia coli* and *Chlamydia* spp. [7]. The T3SS allows direct transfer of effector proteins into the host cell cytosol, creating an environment supporting bacterial survival and proliferation by targeting specific host proteins [7]. Components of the T3SSs have been characterized and found to be conserved among organisms possessing them. Unlike many bacterial pathogens whose T3SS genes are grouped into large genomic islands, the chlamydial components are scattered in several clusters throughout the genome. Several studies have reported a functional T3SS in *Chlamydia* spp. [6,8–10] and these reports show similarities and differences between chlamydial T3SSs. This can be exemplified by the finding that *Chlamydia pneumoniae* seems to lack the phosphorylation site of the T3SS-associated tyrosine phosphorylated protein, TARP, proposed to be needed at the site of entry for *C. trachomatis* [11].

When *C. trachomatis* establishes its protective compartment (inclusion), the bacterium secretes a set of unique proteins such as the inclusion membrane localized proteins (Incs) via a T3SS-dependent mechanism [6,12]. Importantly, some Incs have indeed been reported to be localized to the inside of the *C. pneumoniae* inclusion [8].

We have previously identified potential T3SS inhibitors using a secretion linked reporter-gene assay to a screen of a library of 9400 chemical compounds in viable *Yersinia pseudotuberculosis* [13]. These hits were further investigated and a class of acylated hydrazones of salicylaldehydes proved to be specific T3SS inhibitors capable of preventing translocation of *Yersinia* effector proteins in a HeLa cell infection assay [14]. Importantly, no obvious negative effects could be observed on the eukaryotic cells at concentrations inhibiting T3SS, further underscoring the potential of these compounds as in vivo T3SS virulence blocking agents. The homology between T3SSs of *Yersinia* and *Chlamydia* suggested to us that inhibitors against the T3SS in *Yersinia* might also be capable

of blocking the T3SS in *Chlamydia*. We and another research group have recently shown that T3SS inhibitors belonging to the class of acylated hydrazones of salicylaldehydes cause a dose- and growth phase-dependant inhibition of *C. trachomatis* [15,16]. In the present study, we show that the T3SS inhibitor INP0010 specifically inhibits the *C. pneumoniae* developmental cycle whereas INP0400 prevents intracellular replication of both *C. pneumoniae* and *C. trachomatis* in ex vivo infection models. Addition of the inhibitors led to a 10-fold decrease in transcription of the T3SS genes CPn0824 and CPn0702, respectively. In contrast transcription of *incB* was in fact elevated twofold after addition of INP0010 while *incC* transcription was unaffected by the compound. However, addition of INP0010 did not allow the detection of the two effector proteins IncB and IncC. These results indicate that the small molecule inhibitors obtained (acylated hydrazones of salicylaldehydes) interfere with the T3SS of *Chlamydia* and indicate an important role for T3SS in the chlamydial life cycle. In addition our results indicate the possibility to develop novel therapeutic agents for treatment of *Chlamydia* infections. Moreover, these findings will also facilitate studies concerning theT3SS-associated molecular mechanisms of virulence in *Chlamydia*, since these inhibitors constitute a good substitute for the lack of classical genetic methods and will allow us to employ a chemical genetics approach.

2. Materials and methods

2.1. Compounds

Compound INP0010 and its analogues INP0007, INP0406 and INP0400 (shown in Fig. 1) were synthesized from commercially available hydrazides and salicylaldehydes, as described previously [13,14]. Stock solutions (10 mM) in DMSO were prepared and this solution or a diluted DMSO solution was added to the assay mixtures. The final DMSO concentration was kept below 1%.

2.2. Mammalian cell lines

The human epithelial cell line HEp-2 (ATCC-CCL23, American Type Culture Collection), HeLa cells and the mouse fibroblastic cell line McCoy were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS, PromoCell), 20 mM HEPES, 8 μg/ml gentamicin (Schering-Plough), 1 μg/ml amphotericin B (Gibco), and 2 mM L-glutamine (Sigma). Cells were incubated at 37 °C in the presence of 5%

CO_2. J774 cells were used in the *Yersinia* ex vivo infection model as described below. All cell lines and bacterial strains were negative for *Mycoplasma* infection (*Mycoplasma* detection kit, Stratagene).

2.3. Yersinia ex vivo infection model

J774A cells were seeded into a 96-well plate (7×10^4 cells/well) in DMEM (Gibco) with 10% FCS and gentamicin (3 μg/ml), grown for 12 h at 37 °C in 5% CO_2. Wild-type (wt) *Y. pseudotuberculosis* YPIII (pIB102) and the translocation-deficient mutant, *yopB* (YPIII (pIB604)) strains were grown in LB-broth supplemented with 25 μg/ml kanamycin, diluted 1/10 in DMEM and incubated on a rotary shaker at 26 °C for 1 h followed by 2 h at 37 °C. The J774A cells were washed once with phosphate buffered saline (PBS) and 50 μl fresh DMEM containing the different compounds and 50 μl of T3SS induced *Y. pseudotuberculosis* ($OD_{600} = 0.002$) was added, giving a final MOI of 10. After 16 h of infection CalceinAM (Molecular Probes; Invitrogen) in 20 μl PBS was added to a final concentration of 1 μM and the plate was incubated for 40 min at 37 °C in 5% CO_2. CalceinAM is enzymatically transformed to a green fluorescent molecule in healthy cells (LIVE/DEAD® Viability/Cytotoxicity Kit *for mammalian cells*, Invitrogen;) [17], while cells that are killed during the infection stays unstained (Fig. 2A). The fluorescence was then read in the microtiter plate reader (TECAN GENios); at 485/535 nm and the results were confirmed using fluorescent microscopy. Each compound was tested three times in this assay.

2.4. Chlamydia strains and growth conditions

C. pneumoniae strain T45 [18] and *C. trachomatis* serovar L2 (ATCC VR-902B) were propagated in HEp-2 cells as previously described [18].

2.5. Chlamydia infection and immunofluorescence

HEp-2 cells were seeded onto glass cover slips in a 24-well tissue culture plate and incubated for 24 h prior to infection. Complete medium containing INP0010, INP0406 or INP0400 was added to the cells resulting in final concentrations ranging from 2 to 30 μM. *C. pneumoniae* or *C. trachomatis* was added at a multiplicity of infection (MOI) of 1–5 and the plate was centrifuged at 2500 rpm for 1 h at 37 °C. As a control, heat-inactivated *C. pneumoniae* (30 min in 60 °C) was used. After 2 h incubation at 37 °C in 5% CO_2, the cells were washed twice with ice cold PBS and the medium was changed to complete medium with 0.5 μg/ml cycloheximide (Sigma) and compounds were added as described above. Infected cells were stained with *Chlamydia* culture confirmation kit 48 h p.i. (Pathfinder; Bio-Rad Laboratories) and visualized by immunofluorescent confocal microscopy (magnification 400–1000×). Digital images were processed using the Adobe Photoshop software (Adobe Systems Inc.). The protocol used for immunofluorescence staining with polyclonal anti-IncB and anti-IncC was as described previously [8]. Minor modifications
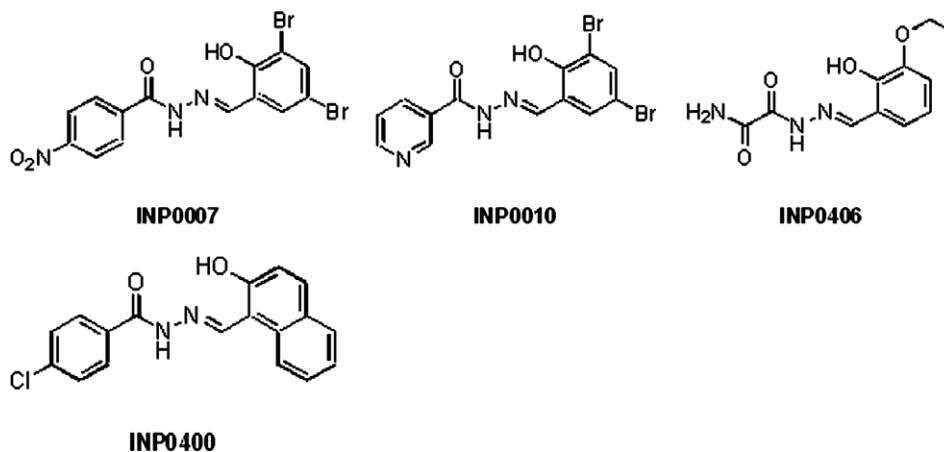

Fig. 1. Chemical structures of INP0007, INP0010, INP0400 and INP0406.
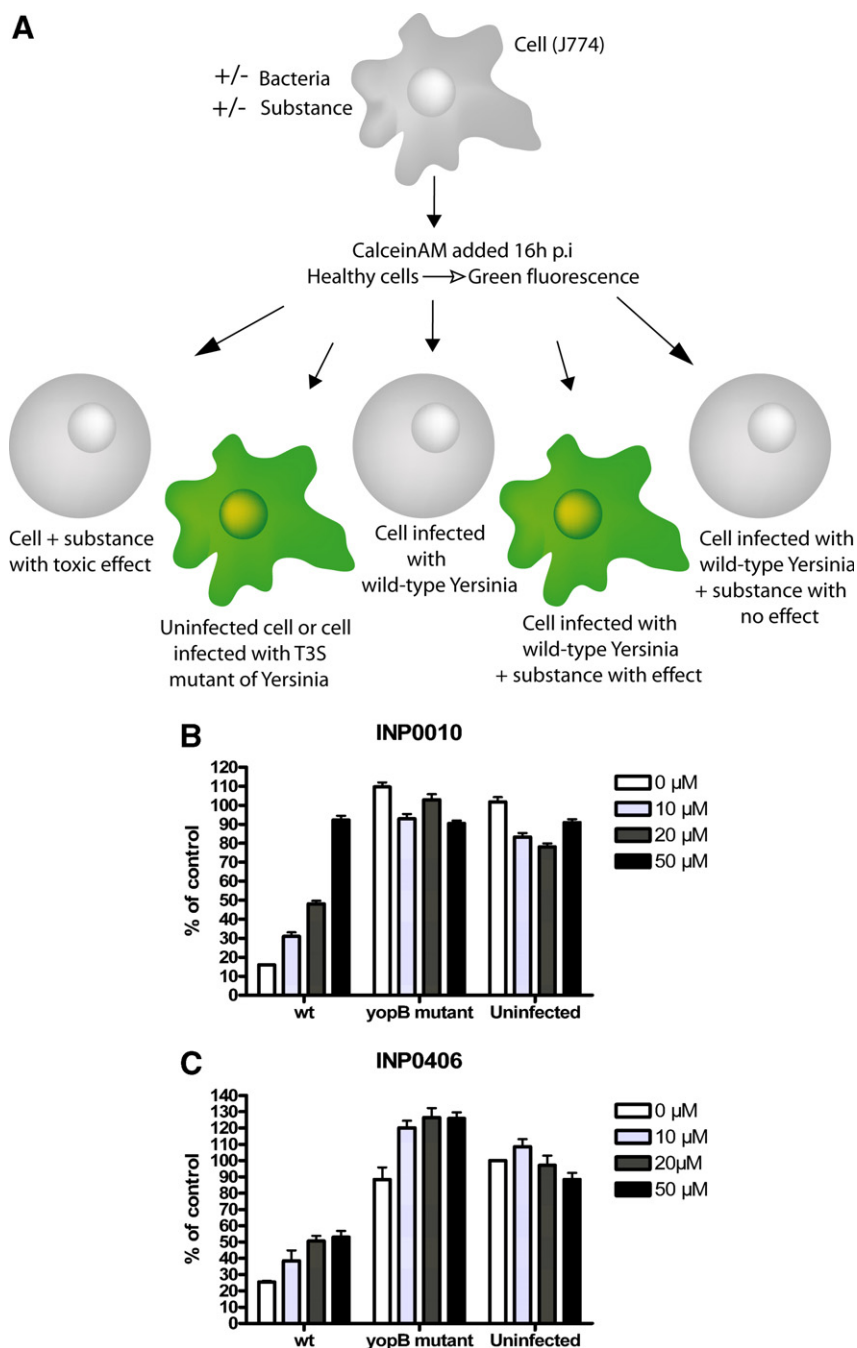
Fig. 2. INP0010 blocks yop translocation of *Y. pseudotuberculosis*. (A) Schematic illustration of the CalceinAM assay. Macrophages were infected with bacteria and different concentrations (0, 10, 20, 50 μM) of the compounds were added to the growth medium. Uninfected cells or cells infected with a T3SS mutant were stained green due to transformation of CalceinAM to a green fluorescent form by healthy cells. Cells infected and killed by the wild-type bacteria were not stained. If a compound blocked the T3SS, the amount of green staining increased relative to the increased survival of the eukaryotic cells. The different concentrations of the compounds were also added to uninfected cells, if a compound was not toxic the cells were stained green, while cells affected by a toxic compound were not stained. Sixteen hours of incubation was required since the cells are killed by YopJ that induces apoptosis between 8 and 16 h after onset of infection. (B) Survival of macrophages (J774) after treatment with INP0010 and (C) INP0406. Macrophages were infected with the wild-type *Y. pseudotuberculosis* YPIII (pIB102) and the translocation deficient mutant, *yopB* (YPIII(pIB604)). Different concentrations of INP0010 (0, 10, 20, 50 μM) were added to the growth medium. The fluorescence intensity was measured and the relative amount of living cells was calculated. As a control INP0010 was added to uninfected cells. Each bar represents the mean value of three experiments and is calculated as percent of the control (uninfected cells without compound). Error bars indicate S.D.

were done as follows: as secondary antibody Rhodamine (TRITC)-conjugated goat anti-rabbit IgG (Jackson, diluted 1:300 in BSA-PBS) was used and further, 4′,6-diamidino-2-phenylindole (DAPI) was used for staining of host cell nuclei and intracellular bacteria for 2 min.

2.6. Cytotoxicity assays

HEp-2 cells were incubated in the presence of different concentrations (10, 20 or 50 μM) of INP0010, INP0400 or INP0406 and cell viability was measured using CalceinAM, as described for the *Yersinia* ex vivo model above.

Effects of INP0010 on proliferation and cell morphology were investigated by inverted phase contrast microscopy of HEp-2 and McCoy cells cultured for 72 h at 10 μM INP0010. To exclude permanent cell changes impairing *Chlamydia* infection, HEp-2 cells were cultured for 24 h in the presence of 10 μM INP0010, thereafter washed three times with PBS and subsequently infected with *C. pneumoniae*.

2.7. Real-time quantitative PCR

Quantitative real-time PCR (iCycler iQ®Real-Time PCR Detection System; Bio-Rad) was used to monitor *C. pneumoniae* and HEp-2 cell genome copy numbers. DNA was prepared using DNeasy tissue protocol for isolation of total DNA from cultured cells (Qiagen). Oligonucleotide primers for the human 90 kD heat shock protein gene (*Hsp90*, accession number J04988) were designed using Beacon Designer software (v2.1; Premier Biosoft International, Palo Alto, CA, USA).The forward primer (5′-GGCACTTCGGGACAACTC-3′) and the reverse primer (5′-AAGATAGCAGGGCGGTTTC-3′) amplified a 170 bp fragment detected by SYBR Green. A 79 bp fragment of the *C. pneumoniae* outer membrane protein A gene (*ompA*) was amplified as previously described [18]. Amplification conditions were 3 min at 95 °C followed by 40 cycles of 30 s at 95 °C and 30 s at 60 °C according to the manufacturer's recommendations (IQ Supermix; Bio-Rad). All samples were run in triplicate and experiments were repeated three times or more. A correlation coefficient of >0.98 was set as an acceptance criteria for each qPCR run.

For absolute quantification of *C. pneumoniae* and host cell DNA, plasmid constructs were used as external standards. The plasmid construct coding for *ompA* has been described previously [18]. The 170 bp hsp90 gene was amplified by PCR and cloned into a pGEM-T Easy vector (Promega) and linearized with *Pst*I. Tenfold dilutions of the plasmid standard and genomic *C. pneumoniae* DNA dilutions between 34 copies and 3.4×10^6 copies and DNA extracted from of HEp-2 cells in dilutions between 20 copies and 2.0×10^5 copies were analyzed by RT-PCR in the same run. Plasmid and genomic standards were congruent. All obtained Ct-values from the samples were translated to gene copy number by using the standard curves obtained from the Ct-values of the genomic standards. A ratio of *C. pneumoniae*/host cell was calculated.

2.8. RNA preparation and expression of T3SS genes

Infected HEp-2 cells were harvested at 30 h p.i. using TRIzol (Invitrogen) according to the manufacturer's instructions for isolating RNA. RNA preparation were treated with RNeasy minikit (Qiagen) using the protocol for RNA cleanup with the addition of a DNase step.

In order to quantify expression levels of T3SS genes, the iScript one-step RT-PCR kit with SYBR Green (Bio Rad) was used. Primers, as described elsewhere, were specific for the 16 S RNA, *incA*, *incB*, *incC* [8] yscC, yscS and *groEL-1* [9]. The gene concentration was standardized against the expression of bacterial 16S RNA transcripts and 20 ng of mRNA template was used in all reactions. The program of the iCycler was as follows: RT reaction of 10 min at 50 °C followed by 5 min at 95 °C. The PCR was carried out during 45 cycles consisting of denaturation 10 s at 95 °C and elongation 30 s at 60 °C. A final denaturation step of 1 min at 95 °C and a final elongation step for 1 min at 55 °C were conducted.

2.9. Statistical analysis

Analysis of variance with Bonferroni's post hoc test for multiple comparisons was used to analyze differences in the ratio of chlamydial DNA copies to host cell DNA copies between INP0010 treated and untreated infections. Concentrations of mRNA transcripts for untreated or INP0010 treated cultures were compared by using Mann–Whitney *U*-test for independent samples. A *P* value less than 0.05 considered significant.

3. Results

3.1. Inhibition of the Y. pseudotuberculosis T3SS in ex vivo infection models

In a previous study, we showed that INP0007 (Fig. 1) and several analogs are inhibitors of the T3SS of *Y. pseudotuberculosis* [14]. The aim of this study was to investigate if these com-

pounds also have the ability to interfere with the T3SS of *Chlamydia*. INP0007, however, exhibits limited water solubility and we therefore focused our interest towards the more soluble analogue INP0010 (Fig. 1), that we anticipated should facilitate our studies.

However, we first had to characterize the effect of INP0010 using our well-established *Yersinia* model.

To allow rapid testing of our T3SS inhibitors in a relevant infection model, we have developed a 96-well plate high-content assay that measures both compound toxicity and blockage of the T3SS of *Yersinia* by affecting the level of phagocytosis in a macrophage model. The status of the macrophages was analyzed using CalceinAM that is enzymatically transformed into a green fluorescent molecule in healthy living cells (see Fig. 2A). This assay was used to study the potential of INP0010 to inhibit T3SS of *Y. pseudotuberculosis* after infection of J774-macrophages. Wild-type bacteria caused cell death by translocation of the effector protein YopJ into the macrophage cytosol via the T3SS [19,20] resulting in a reduced green fluorescence signal (Fig. 2A). Killed eukaryotic cells were unable to transform CalceinAM resulting in a reduced green fluorescence signal (Fig. 2A). In contrast, the *yopB* translocation-defective avirulent mutant had no effect on macrophage viability (Fig. 2B). Interestingly, for the wt strain 50 μM of INP0010 inhibited T3S and the green fluorescence signal was restored to the same level as that of uninfected cells (Fig. 2B). Microscopic visual inspection confirmed this result since it was found that the macrophages cleared wt bacteria from the medium after treatment with INP0010 in contrast to non-treated cells (data not shown). In addition, when INP0010 was added to uninfected J774 cells, no effect on cell survival was observed, i.e. the compound was not toxic to macrophages (Fig. 2B). Thus, these results show that the drug exhibits the desired functions resulting in a T3SS-minus phenotype. Based on these data, INP0010 was chosen for further studies on *Chlamydia*. We also included the compound INP0406 (Fig. 1) as negative control. This compound, which belongs to the same chemical class as INP0010, is unable to inhibit the T3SS of *Yersinia*, and as expected INP0406 showed no significant inhibition of virulence and no toxicity towards the macrophages (Fig. 2C).

3.2. INP0010 inhibits C. pneumoniae but not C. trachomatis infections in vitro

C. pneumoniae and *C. trachomatis* inclusions could be observed in infected cells in the absence of INP0010 (Fig. 3a–c; j–l). In contrast, no *C. pneumoniae* inclusions were observed after treatment with INP0010 (10 μM) (Fig. 3d–f). Single stained bacteria could occasionally be observed, but no inclusions could be visualized. Identical results were observed when McCoy and HeLa cells were used. When INP0010 (10 μM) was added to *Chlamydia* infected cultures early in the developmental cycle, before EBs undergo differentiation and develops to RBs (8–12 h), no inclusions could be visualized 48 h post-infection (Fig. 3d–f). We also found that pre-treatment of the host-cells had no effect on *Chlamydia* replication, i.e. *C. pneumoniae* propagated normally when host-cells were first treated with a concentration of 10 μM of INP0010 for 4 h and then the drug was washed away prior to infection. These results collectively suggest that INP0010 targets *Chlamydia* and blocks the developmental cycle without interfering with the eukaryotic host cell.
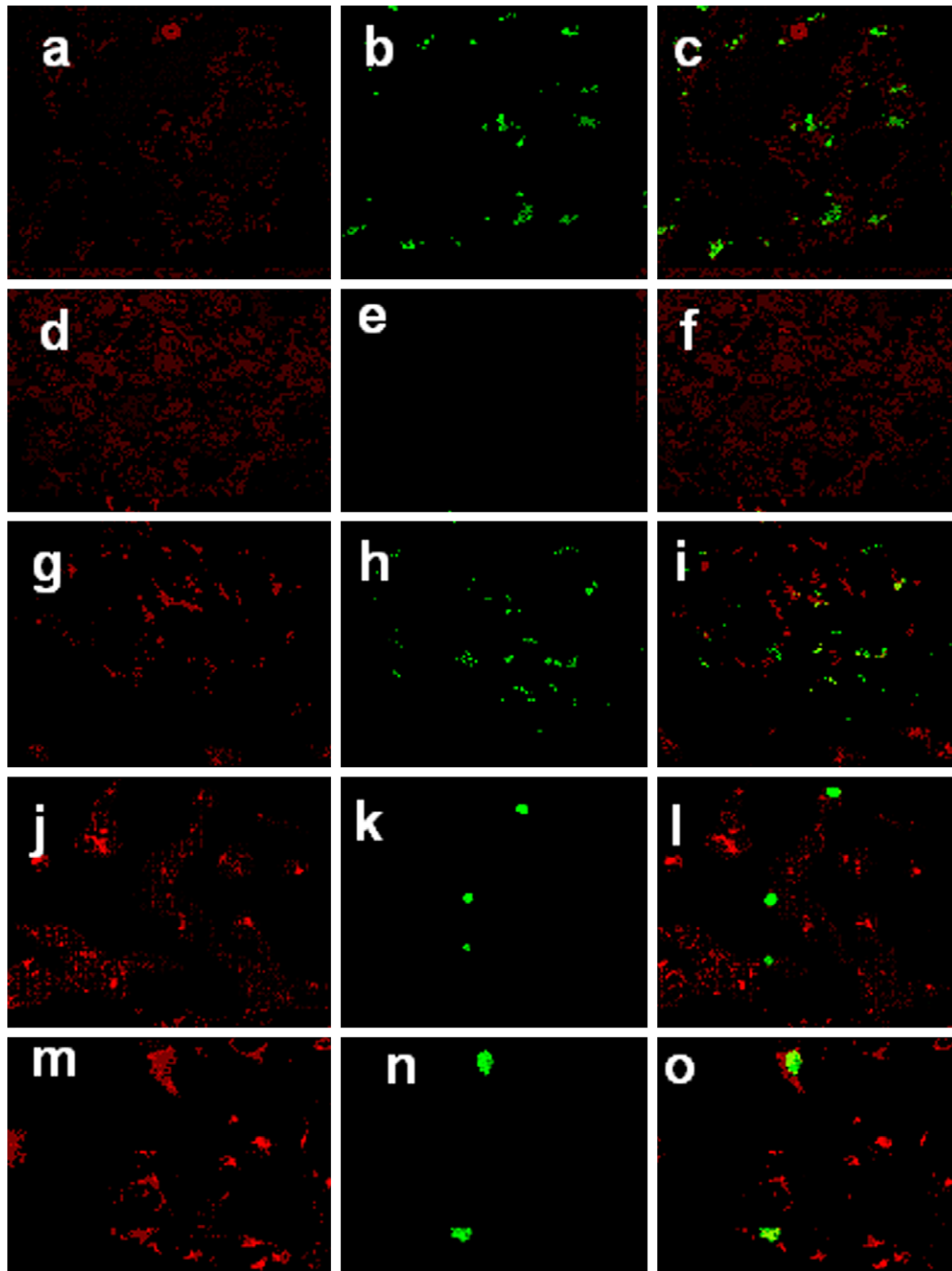
Fig. 3. Effects of INP0010 on *Chlamydia* growth in HEp-2 cells. Left column represents the Evans blue stained HEp-2 cells (red), middle column represents fluorescein isothiocyanate stained chlamydial inclusions (green) and the right column is the merged overlay of the two staining methods. All pictures were taken 48 h p.i. (a–c) *C. pneumoniae* infection (magnification, 630×). (d–f) *C. pneumoniae* infection treated with 10 μM of INP0010, no bacterial inclusions were observed (magnification, 630×). (g–i) *C. pneumoniae* infection treated with 50 μM INP0406 (magnification, 630×). (j–l) *C. trachomatis* infection (magnification, 400×). (m–o) *C. trachomatis* infection treated with 30 μM of INP0010 (magnification, 630×).

Surprisingly, when HEp-2 cells were infected with *C. tracho-matis*, INP0010 at a final concentration ranging from 10 to 30 μM no inhibitory effect could be seen (Fig. 3m–o). In this context, *C. trachomatis* can be regarded as a negative control not responding to the compound, strongly arguing for the hypothesis that INP0010 directly targets *C. pneumoniae* and that the result is not an effect of any adverse effects of the drug

on the eukaryotic host cell. Importantly, no inhibitory effect could be observed on either *C. pneumoniae* or *C. trachomatis* infections treated with INP0406 at 10–50 μM, corroborating the results obtained with *Y. pseudotuberculosis* (Fig. 3g–i), and further supporting the idea that INP0010 targets the T3SS of *C. pneumoniae*. Recently, we also showed that the compound INP0400 inhibited intracellular replication of

C. trachomatis at 10 μM, analogous as seen here with *C. pneumoniae* infected cells treated with INP0010 at 10 μM (Fig. 3d–f and [15]). Interestingly, intracellular replication of *C. pneumoniae* was similarly affected by INP400 (data not shown). Moreover, when INP0400 was evaluated in the *Y. pseudotuberculosis* ex vivo infection model it displayed the same profile as INP0010, i.e. it inhibited T3SS mediated virulence without any detrimental effect on the macrophages (data not shown). In addition, none of the tested compounds were cytotoxic for the eukaryotic host cells given at final concentrations ranging from 10, 20, or 50 μM as measured using CalceinAM (Fig. 4). These results were also supported by microscopic investigations showing that cell proliferation and morphology, independent of cycloheximide, were unaltered in HEp-2 cells grown in presence of 10 μM INP0010 for up to 72 h. However, INP0010 blocked proliferation of *C. pneumoniae* both in presence and absence of cycloheximide (data not shown).

3.3. INP0010 inhibits C. pneumoniae *multiplication in a dose-dependent manner*

INP0010 was added at different concentrations to test the efficacy of the compound. At 10 μM of INP0010, no inclusions could be observed (Fig. 5A, B). To quantify the dose-dependent effect, the inclusions in a total of 1000 infected cells from two independent experiments were counted by eye (Fig. 5B). Quantification of infecting chlamydiae was also carried out with quantitative real-time PCR at 48 h p.i. in the presence or absence of 2, 4, 6, 8, or 10 μM of INP0010 (Fig. 6). *C. pneumoniae* and HEp-2 cell genome equivalents were measured and the ratio of chlamydiae per host cell was calculated. In the absence of INP0010, there were in mean 103 (±2.3) chlamydiae per host cell. Paralleling the results obtained with immunofluorescence, the number of *Chlamydia* per host cell decreased with increasing concentration of INP0010, and addition of 10 μM resulted in 11.7 (±3.9) chlamydiae per host cell. Heat-inactivated *C. pneumoniae* was used as a control and resulted in 5.8 (±0.8) chlamydiae per host cell, not significantly different from treatment with 10 μM INP0010 ($P < 0.001$). These results indicate that INP0010 blocks proliferation of *C. pneumoniae*.
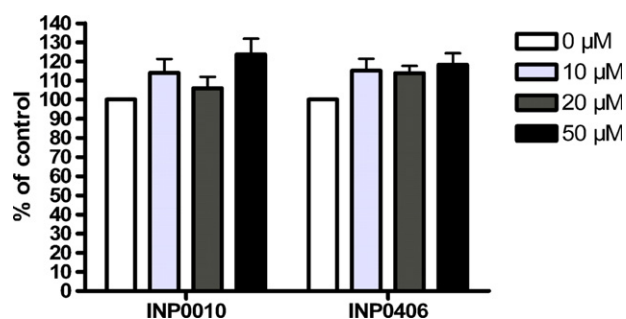

Fig. 4. INP0010 and INP0406 exhibit non-toxic effects on HEp-2 cells. Different concentrations of INP0010 and INP0406 (0, 10, 20, 50 μM) were added to the growth medium of HEp-2 cells. The CalceinAM fluorescence intensity was measured and the relative amount of living cells was calculated. Each bar represents the mean value of three experiments and is calculated as percent of the control (uninfected cells without compound). Error bars indicate S.D.

3.4. Treatment with INP0010 inhibits chlamydial effector protein secretion and leads to decreased transcription of T3SS specific genes

The above results suggested that INP0010 interferes with the T3S machinery of *Y. pseudotuberculosis*, thereby inhibiting the translocation of Yop effectors into the host cell [14]. Therefore, we asked the question if T3S mediated secretion of two effector proteins IncB and IncC of *C. pneumoniae* could be blocked by the addition of INP0010 to infected host-cells. In absence of INP0010 immunofluorescence microscopy showed that IncB was localized to the chlamydial inclusion (Fig. 7A). However, in the presence of INP0010 (10 μM), no specific staining of IncB could be observed (Fig. 7A), suggesting that IncB is not secreted in the presence of INP0010. Similarly, we were unable to detect IncC in INP0010-treated cultures (data not shown). Due to the fact that the number of bacteria was severely reduced after drug treatment these results could be questioned. To further examine the role of INP0010 as a specific inhibitor of the chlamydial T3S machinery, we analyzed also the transcription of T3SS associated genes using quantitative reverse-transcriptase PCR. Two genes were chosen as specific markers for the T3SS of *Chlamydia*, CPn0824 and CPn0702, respectively. These two genes show homology with the T3SS genes *yscS* and *yscC* of *Yersinia* [9]. We also analyzed the level of 16 S rRNA expression that was used as reference point since this transcript is known to be stably expressed in *Chlamydia* throughout the developmental cycle [8]. The *groEL-1* gene, is also known to be expressed throughout the complete developmental cycle and since it has no known connection to the T3SS this gene was selected as a marker for a gene not affected by a T3SS inhibitor. It was found that the relative amount of mRNA transcribed from *groEL-1* visa vie 16S rRNA did not differ significantly between untreated and INP0010-treated cultures (Fig. 7B). Interestingly, transcription of the *Chlamydia* T3SS associated genes, CPn0824 and CPn0702, decreased significantly after treatment of INP0010 (10 μM) ($P < 0.002$). However, when *incB* and *incC* transcription was determined it was found that while *incB* transcription was elevated twofold in the INP0010 treated cultures *incC* transcription was at the same level as in untreated cultures. Taken together these results argue strongly for the idea that the compound INP0010 specifically targets the T3SS of *C. pneumoniae* resulting in chemical attenuation of the pathogen. In addition, it also indicate that that there is a regulatory coupling between the expression of the T3SS and secretion in line with what has earlier been described for *Yersinia* [21].

4. Discussion

In a previous study, we identified INP0010 as a more soluble and equally potent analog of the well-characterized *Y. pseudotuberculosis* T3SS inhibitor INP0007 [14]. We show here that INP0010 is also a potent inhibitor of *C. pneumoniae* infection in cell culture and prevents formation of inclusions at 10 μM, supporting our original hypothesis that T3SS-specific inhibitors should be capable of blocking this evolutionarily conserved virulence system in multiple species [13]. Importantly, INP0406, a compound within the same chemical class as INP0010, did not inhibit T3SS-mediated virulence in *Y. pseudotuberculosis* and also failed to block replication of *C. pneumoniae* and *C. trachomatis*, arguing that the anti-chlamydial
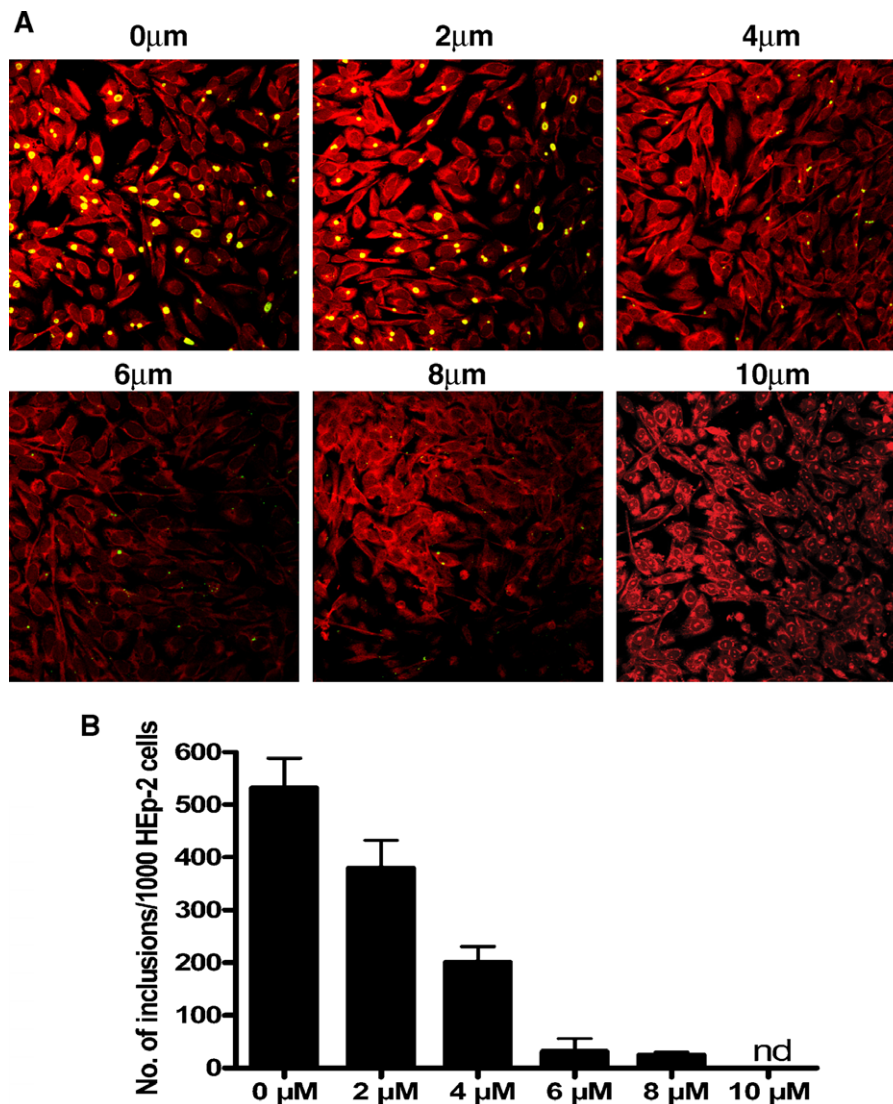
Fig. 5. Dose-dependent inhibitory effects of INP0010 on *C. pneumoniae* infection in HEp-2 cells. (A) Fluorescence staining of chlamydial inclusions in HEp-2 cells after treatment of INP0010. *C. pneumoniae* growth inhibition assay using INP0010 at concentrations ranging from 2 μM to 10 μM (magnification, 400×). To set a threshold, an inclusion was only counted when it was comparable in size and appearance to the inclusions observed in untreated cells. (B) Inhibitory dose-dependent effects quantified by immunofluorescence. The dose-dependent effect was demonstrated by counting the total number of chlamydial inclusion in 1000 cells. No inclusions could be detected at 10 μM, marked as nd (not detectable). Each bar is presented as an average from two independent experiments. Error bars indicate S.E.M.

effect of INP0010 is specific and that inhibition is not a result of a general toxic effect on the eukaryotic cell. This was also supported by the finding that INP0010 did not block replication of *C. trachomatis* although the compound had a profound effect on *C. pneumoniae* proliferation. We suggest that if INP0010 exhibited a general toxic effect on the eukaryotic cell, the drug would not discriminate between the two different chlamydial species. Our finding that INP0010 blocked secretion of the two effector proteins IncB and IncC supports the hypothesis that INP0010 targets the T3SS of *Chlamydia* per se. However, it could be argued that we failed to detect the presence of these two effectors due to the fact that the drug inhibited replication resulting in too low antigen levels. We therefore employed the more sensitive RT-PCR method allowing us to measure the relative amount of mRNA of T3SS associated genes. We observed that transcription of the two T3SS

linked genes, CPn0824 and CPn0702, was decreased 10-fold in the presence of INP0010 while the transcription of the *groEL-1* gene used as an internal control was unaffected. This result corroborates the idea that the drug specifically targets the T3SS but it also indicates that there is a regulatory link between the expression of the T3SS and secretion similarly to what has been found to be the case for *Yersinia* [19]. We observed also that transcription of *incB* showed a twofold increase while *incC* transcription was unaffected. These results indicate that expression of the IncB and IncC proteins is still at wt levels or even higher after drug treatment. Thus, the fact that we did not detect IncB and IncC after INP0010 addition is more likely an effect of the blockage of the T3SS than failure to detect the proteins. These observations could not have been made without the novel tools presented herein showing that chemical genetics can substitute for the lack of suitable genetic
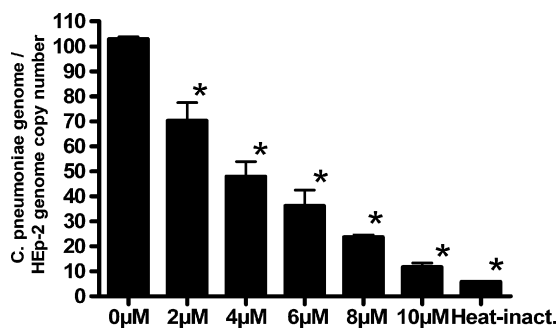
Fig. 6. Inhibition of *C. pneumoniae* infection with INP0010. Real-time PCR was used to estimate the number of bacterial genomes. The results confirmed the finding described above (see Fig. 5). Non-treated infected cells were used as positive control and cells with heat-inactivated bacteria was used as negative control, denoted as Heat-inact Cpn in the figure. Significant differences were shown between INP0010 (10 μM) untreated and treated *Chlamydia* infected cell cultures ($P < 0.05$ is marked with an asterix).

methods to achieve a deeper understanding about the chlamydial T3SS at the molecular level. The finding that INP0010 did not block replication of *C. trachomatis* at the tested concentrations imply differences between the two chlamydial species and spurred us to attempt to identify an T3SS inhibitor that also inhibits *C. trachomatis* at low concentrations. Further screening resulted in INP0400 that blocked T3SS mediated virulence in *Y. pseudotuberculosis* as well as formation of inclusions both for *C. pneumoniae* and *C. trachomatis* [15]. These results indicated, in agreement with our original hypothesis, that the effect of INP0010 and INP0400 on the chlamydial replication is mediated by inhibition of the T3SS of *Chlamydia*. Further work will focus on the identification of the molecular target(s) of this class of inhibitors. Moreover, a systematic search for additional compounds using our high content CalceinAM assay, or chemical optimization of existing inhibitors should result in T3SS inhibitors with improved characteristics useful to study the different T3SSs in more detail.

In summary, the present study shows that the T3SS inhibitors, of the chemical class of acylated hydrazones of salicylaldehydes, can inhibit chlamydial growth by interfering with the T3SS without influencing the host cells. Hence, the discovery of compounds through a chemical genetics approach demonstrates the feasibility of a previously unexplored route to develop novel anti-chlamydial drugs to be used in basic and applied research. Design, synthesis and evaluation of new libraries of TTSS inhibitors against multiple species will allow us to establish quantitative structure–activity relationships that will guide us towards inhibitors either specific against a certain organism or compounds capable of inhibiting T3SSs in a broad manner.
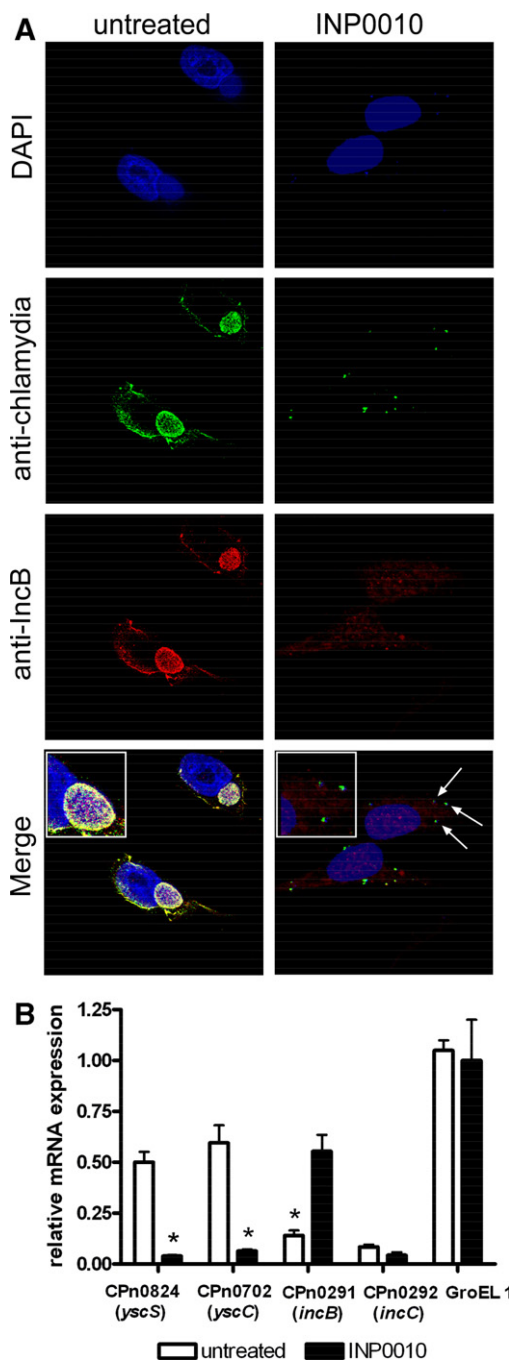
Fig. 7. *INP0010 treatment inhibits IncB secretion and down-regulates the T3SS genes CPn0824 and CPn0702.* (A) Immunofluorescence double staining of the untreated infection revealed co-localization of the bacteria (green) and IncB (red) within the peri-nuclear inclusion (yellow, left column, and insert) 48 h p.i. (magnification, 630×). No IncB-specific signal could be detected in INP0010-treated cultures at (10 μM) (right column, arrows and insert). (B) Transcription of four essential genes of the T3SS were analyzed by quantitative RT-PCR. mRNA was prepared from six independent cultures treated and not treated with 10 μM INP0010. Transcription of the T3SS associated genes, CPn0824 (*yscC*), CPn0702 (*yscS*), *incB* and *incC* was analyzed and compared to that of groEL-1 which was used as a negative control. After INP0010 treatment at 10 μM 30 h p.i., transcription of CPn0824 and CPn0702 decreased significantly ($P < 0.002$), while transcription of *incB* showed a two-fold increase ($P = 0.02$) and *incC* transcription was unaffected. The relative level of transcription of *groEL-1* did not differ between untreated and INP0010-treated cultures. 16S rRNA was used as a reference point.

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