A small-molecule compound belonging to a class of 2,4-disubstituted 1,3,4-thiadiazine-5-ones inhibits intracellular growth and persistence of *Chlamydia trachomatis*

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*Chlamydia trachomatis* is one of the most common sexually transmitted pathogens in the world and often causes chronic inflammatory diseases that are insensitive to antibiotics. The type 3 secretion system (T3SS) of pathogenic bacteria is a promising target for therapeutic intervention aimed at bacterial virulence and can be an attractive alternative for the treatment of chronic infections. Recently, we have shown that a small-molecule compound belonging to a class of 2,4-disubstituted 1,3,4-thiadiazine-5-ones produced through the chemical modification of the thiohydrazides of oxamic acids, designated CL-55, inhibited the intracellular growth of *C. trachomatis* in a T3SS-dependent manner. To assess the feasibility of CL-55 as a therapeutic agent, our aim was to determine which point(s) in the developmental cycle CL-55 affects. We found that CL-55 had no effect on the adhesion of elementary bodies (EBs) to host cells but significantly suppressed EB internalization. We further found that CL-55 inhibited the intracellular division of reticulate bodies (RBs). An ultrastructural analysis revealed loss of contact between the RBs and the inclusion membrane in the presence of CL-55. Finally, we found that our T3SS inhibitor prevented the persistence of *Chlamydia* in cell culture and its reversion to the infectious state. Our findings indicate that our T3SS inhibitor may be effective in the treatment of both productive and persistent infections.

INTRODUCTION

*Chlamydia trachomatis* is one of the most common sexually transmitted pathogens in the world and is a very successful intracellular parasite. During the intracellular stage of its life cycle, it resides within a membrane vacuole in the cytoplasm known as an ‘inclusion body’, avoiding degradation in lysosomal compartments. *Chlamydia* has a unique biphasic developmental cycle with two distinct morphological forms: the replicative, intracellular reticulate bodies (RBs) and the extracellular infectious elementary bodies (EBs). Research from multiple teams has demonstrated that an encounter with specific stimuli can transform *Chlamydia* into a third state called ‘persistence’ (Clark et al., 1982; Harper et al., 2000), which has been defined as a reversible interruption of productive intracellular chlamydial growth mediated by environmental factors (Beatty et al., 1994). Persistence is an alternative outcome of a productive bacterial infection where a subpopulation of the bacteria becomes ‘invisible’ in response to prolonged antibiotic treatment, warding off innate and adaptive immune responses, causing little or no symptoms in the infected host, and possibly unnoticed by the diagnostician (Bavoil et al., 2000).

Since Hsia et al. (1997) first discovered direct evidence that chlamydiae exploited a type III secretion system (T3SS), it has become clear that chlamydial protein secretion is essential for both survival and virulence. This specialized secretion machinery is an essential virulence mechanism
utilized by a variety of Gram-negative pathogens to translocate anti-host effector proteins directly into the host cells. T3SS contributes directly to chlamydial pathogenesis through a secretion of effector proteins affecting host cellular processes (Valdivia, 2008) and immunostimulatory capability (Prantner & Nagarajan, 2009). The chlamydial T3SS is present and functional at each stage of the developmental cycle and is essential for the regulation of intracellular development, whereas T3SS in other organisms is essential for virulence but unimportant for survival (Wilson et al., 2006). There is still little evidence of T3SS function in persistent *Chlamydia*, but the essential role of this secretion machinery in the intracellular survival of *Chlamydia* during the normal life cycle gives reason to suggest that T3SS is important for persistence. Hoare et al. (2008) hypothesized that chlamydiae persist as enlarged RBs for long periods by maintaining a number of T3SS injectisomes above a detachment threshold. A larger atypical RB in a smaller inclusion would necessarily make more contact with the membrane and would maintain a sufficient number of T3SS needles and never fall off; thus, the RB would never differentiate to an infectious EB.

Owing to the prevalence and morbidity of diseases associated with *C. trachomatis*, the development of efficacious therapeutics is of high priority. Antibiotics can resolve an acute infection, but aberrant persistent forms are more recalcitrant to antibiotic treatment.

Therapeutic agents that target virulence determinants of pathogenic bacteria have become an increasingly promising alternative to antibiotics (Zigangirova & Gintsburg, 2011; Zigangirova et al., 2012). T3SS proteins are attractive targets for ‘anti-virulence’ compounds because they are often essential to the virulence of widely distributed Gram-negative bacterial pathogens of plants, animals and humans. Recently, whole-cell-based high-throughput screens have been performed to identify T3SS inhibitors and have identified several classes of small-molecule compounds (salicylidene acylhydrazides, salicylanilides, sulfonylaminobenzanilides, benzimidazoles and a thiazolidinone) and three natural products as effective agents against a number of pathogenic bacteria that utilize the T3SS, including *Yersinia*, *Chlamydia*, *Salmonella*, enteropathogenic *Escherichia coli* and *Shigella* (Kauppi et al., 2003; Pan et al., 2007; Grier et al., 2010; Garrity-Ryan et al., 2010).

Recently, we developed a novel T3SS inhibitor, designated CL-55, via the chemical modification of thiohydrazides of oxamic acids and performed an experimental screening of their derivatives. The compound, 4-(3-ethoxy-4-hydrobenzyl)-5-oxo-5,6-dehydro-4H-[1,3,4]-thiadiazol-2-(2,4-difluorophenyl)-carboxamide, demonstrated high stability, solubility and low cytotoxicity. CL-55 inhibited the intracellular growth of different *Chlamydia* species in a dose-dependent manner and decreased the translocation of the type III secretion effector IncA (Zigangirova et al., 2012a). CL-55 possessed antibacterial activity *in vivo* and was able to control *C. trachomatis* (serovar D) vaginal shedding, ascending infection, and inflammation in the upper genital organs in DBA/2 mice (Koroleva et al., 2015).

To assess the feasibility of CL-55 as a therapeutic agent, our aim was to determine which point(s) in the developmental cycle CL-55 affects. We found that it did not affect EB adhesion but significantly suppressed EB internalization. At the intracellular developmental stage, CL-55 inhibits RB division when added up to 16 h post-infection (p.i.). An ultrastructural analysis revealed a loss of contact between the RB and the inclusion membrane in the presence of CL-55. Treatment with the tested compound caused a complete disruption of the enlarged aberrant bodies and inhibited a reversion of penicillin-induced persistence of *C. trachomatis*.

**METHODS**

**Bacterial strains and cell lines.** The reference strain *C. trachomatis* serovar L2 (ATCC VR 902B) and the McCoy cell line, a hybrid cell line of human synovial cells and mouse fibroblasts (Institute of Cytology RAS, Russia), were used in this study.

**Cell infection with *C. trachomatis* and assessment of inhibitor effects.** McCoy cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FBS and 2 mM glutamine at 37 °C in a 5 % CO₂ atmosphere. Cells were grown in 24-well plates for 24 h. McCoy cells were infected with *C. trachomatis* EBs at an m.o.i. of 1 in DMEM containing 0.4 % glucose, 5 % FBS and 2 μg cycloheximide ml⁻¹ and were then centrifuged for 1 h at 1500 g (Rotanta 460R; Hettich).

CL-55 was dissolved in DMSO and added to the growth medium at concentrations ranging from 12.5 to 50 μM. The diluent, 0.1 % DMSO without CL-55, was added to the controls.

For the assessment of infective progeny accumulation, McCoy cells were harvested for reinfection after 48 h of cultivation, as described elsewhere (Bashmakov et al., 2010). Serial dilutions of lysates were inoculated onto the McCoy cells. Infected McCoy cells were grown for 48 h on coverslips in 24-well plates, fixed with methanol and incubated for 30 min with a monoclonal FITC-conjugated species-specific antibody against the major outer-membrane protein of *C. trachomatis* (Bio-Rad).

To assess our results, we used a fluorescent microscope (Axioskop Plus; Zeiss). The semi-quantitative analysis was based on the counting of infected cells in 20 random visual fields and calculating the mean number of inclusion-forming units (i.f.u.) ml⁻¹ of the specimen. Every experiment was repeated three times.

**Estimation of CL-55 effect on adhesion and internalization stage.** For estimation of the CL-55 effect on *C. trachomatis* adhesion, EBs were added to 24-well plates with coverslips coated with McCoy cell monolayers. The EBs were allowed to attach in the presence or absence of 50 μM CL-55 for 1 h at 4 °C. The medium with the inhibitor was removed, the cells were washed three times with PBS, and the plates were cultured for 48 h in DMEM without inhibitor.

To assess the effect of CL-55 on *Chlamydia* internalization, EBs of *C. trachomatis* were allowed to attach to McCoy cells in the absence of CL-55 for 1 h at 4 °C. The cells were washed with PBS and transferred to 37 °C in the presence of 50 μM CL-55 to permit internalization. Two hours later, the cells were washed three times with PBS and grown for 48 h in DMEM.
To assess chlamydial growth, the cells were fixed with methanol, stained with a monoclonal FITC-conjugated species-specific antibody against the major outer-membrane protein of *C. trachomatis* (Bio-Rad) and analysed (Cornelis, 2006).

**Automatic image processing method for the quantitative analysis of intracellular chlamydial growth.** To improve the objectivity and reproducibility of the image assessment, we developed in-house automatic immunofluorescent image processing software that generates quantitative data on intracellular chlamydial growth. The software measures the chlamydial inclusion area in each infected cell from digital images of cell cultures and performs further statistical analysis of the data (Artyukhova & Samorodov, 2013; Artyukhova et al., 2013). To perform automatic quantification of chlamydial inclusions, we collected photos of 20 random fields of each sample. All images were uploaded into the program, and the size of each chlamydial inclusion in infected cells was automatically evaluated.

**Transmission electron microscopy (TEM).** McCoy cells were cultured and infected with *C. trachomatis* in six-well plates and then harvested from the plates with trypsin–versene solution. Cell pellets obtained by centrifugation for 10 min at 1500 r.p.m. (Rotanta 460R; Hettich) were fixed with Ito–Karnovsky fixative solution, followed by post-fixation with OsO4 and treatment with aqueous uranyl acetate to provide contrast. The specimens were subsequently dehydrated in an ascending series of alcohol concentrations (50, 70, 96 and 100 % ethanol), infiltrated in a 1 : 1 (v/v) mixture of LR White resin and C. Resin, polymerized at 56°C for 24 h. Ultrathin sections were prepared, treated with a lead solution to provide contrast (Reynolds, 1963) and analysed using a JEOL 100B transmission electron microscope with an accelerating voltage of 80 kV.

**Penicillin-induced persistence in *C. trachomatis*.** Penicillin-induced persistence was established by the addition of 750 IU penicillin (OAO Biosynthesis) at 1 h p.i. Infected cell monolayers treated with penicillin were grown for an extra 24 or 48 h, stained for immunofluorescent analysis and harvested for TEM. CL-55 was added at 24 and 48 h p.i., and cultures were incubated for an additional 24 h, then stained for immunofluorescent analysis and harvested for TEM. To analyse the impact of CL-55 on reversion from penicillin persistence, the cultures were washed to remove penicillin, incubated for an additional 24 h in the presence or absence of CL-55, and then harvested for TEM. To measure *Chlamydia* viability, the cultures were harvested, and the viable infectious yield was estimated in a reinfection test.

**Statistical analysis.** Statistical data analysis was performed in STATISTICA software. All data in Figs 2 and 3 were presented as the mean ± 95 % confidence interval. Since the initial observations were not distributed normally, the Mann–Whitney U test was used for pairwise group comparisons.

**RESULTS AND DISCUSSION**

**CL-55 decreases the size of inclusions and the number of bacteria inside inclusions in a dose-dependent manner**

Previously, we demonstrated that CL-55 suppressed the development of *C. trachomatis* infection in cell culture in a dose-dependent manner and reduced the numbers of infectious progeny with complete inhibition of infectivity at 50 μM CL-55 (Zigangirova et al., 2012a).

Here, we assessed the question of CL-55 activity towards different stages of the chlamydial life cycle. Electron micrographs revealed a dose-dependent reduction of bacterial numbers per inclusion. As a result, the inclusion bodies gradually decreased in size (Fig. 1a–d, framed areas). Chlamydial inclusions in the CL-55-treated cultures primarily contained RBs (Fig. 1b–d). Chlamydial infection in cell culture was dramatically inhibited with 50 μM CL-55.

To perform a reliable estimation of CL-55 effects on *C. trachomatis* inclusion sizes, we used in-house morphometric software, based on the segregation of fibroblast cells and chlamydial inclusions according to their different colours.

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**Fig. 1.** CL-55 added at the time of infection decreases the size of inclusions and the number of bacteria inside each inclusion in a dose-dependent manner. TEM analysis of *C. trachomatis*-infected McCoy cell cultures at 48 h p.i. (a) No CL-55 added; (b) 12.5 μM CL-55; (c) 25 μM CL-55; (d) 50 μM CL-55. Frames indicate inclusions. White arrows, EBs; black arrows, RBs. Bars, 1 μm.
The results are presented in Fig. 2. The increasing concentrations of CL-55 added to the infected McCoy cells correlated with a gradual statistically significant \((P<0.01)\) decrease in the mean inclusion area (Fig. 2). At a concentration of 50 \(\mu\text{M}\), the mean area of the sporadic inclusions was 48.0 \(\mu\text{m}^2\), which is fourfold smaller than the control (193.7 \(\mu\text{m}^2\)).

Summarizing our findings from morphometric and electron microscopic methods, we concluded that the addition of CL-55 to McCoy cells infected with *C. trachomatis* decreased the number of bacteria per inclusion. Inclusions had smaller sizes and predominantly contained the RB form of the bacteria. The results presented above agree with data for other T3SS inhibitors, which also caused the formation of smaller inclusions containing only one or a few reticulate bodies. In the presence of a small molecule belonging to a class of acylated hydrazones of salicylic aldehydes known as INP0400, internalized EBs were converted to RBs, but RB multiplication was inhibited in a dose-dependent fashion, resulting in ultimately smaller inclusion bodies containing just one or a few RBs (Muschiol et al., 2006).

**CL-55 partially inhibits EB entry and effectively suppresses intracellular growth of *C. trachomatis***

Next, we investigated the effect of CL-55 on EB entry into host cells. The adhesion and internalization rates of chlamydial EBs into McCoy cells were estimated in the presence of 50 \(\mu\text{M}\) CL-55, as previously described. Addition of CL-55 to infected McCoy cells at the adhesion stage of infection led to a slight decrease in the number of cells containing detectable inclusions after 48 h of growth. The level of adhesion reduction was 10\% (1.29 \(\times\) 10\(^7\) compared to 1.87 \(\times\) 10\(^7\) i.f.u.). The effect of CL-55 on chlamydial uptake was more pronounced and caused an approximate 49\% (6.03 \(\times\) 10\(^7\) via 1.87 \(\times\) 10\(^7\) i.f.u.) decrease in the number of inclusions. Thus, CL-55 had a minor effect on the level of adhesion of *C. trachomatis* and significantly arrested its internalization.

To evaluate the progression of *C. trachomatis* through its life cycle in the presence of the inhibitor, CL-55 was added to infected cells during chlamydial intracellular growth from the time of infection up to 32 h p.i. and was then analysed by immunofluorescence at 48 h p.i. In-house-developed morphometric software was employed to identify the stages of the *Chlamydia* life cycle that were more susceptible to the inhibitor.

As shown in Fig. 3, CL-55 treatment induced a significant decrease in the mean inclusion area when added from 0 to 16 h p.i. Addition of CL-55 at later times (up to 24 h) did not have such a pronounced effect, but the inclusions were still significantly \((P<0.01)\) smaller than those in the untreated control.

Therefore, CL-55 did not interfere with bacterial attachment but significantly affected EB internalization. However, the CL-55 inhibitory effect on the internalization process was not complete, which suggested either that the compound did not inhibit the translocation of the ‘pre-loaded’ EB effector proteins or that the uptake of *Chlamydia* may also proceed in a T3SS-independent fashion. EBs internalized in the presence of CL-55 were converted to RBs (Fig. 1), but RB multiplication was inhibited in the presence of CL-55, and the inclusions contained fewer,
smaller RBs. Like other T3SS inhibitors, CL-55 suppressed the intracellular chlamydial growth up to the phase of RB differentiation to EB (Muschiol et al., 2006). A large number of chlamydial proteins are delivered into either the inclusion membrane or the host cytosol by the T3SS. T3SS effectors are expressed and produced by RBs at specific stages during the intracellular chlamydial infectious cycle; moreover, their function is essential for the progression of the C. trachomatis developmental cycle (Valdivia, 2008). There are at least two possibilities for the observed effect of type III secretion on C. trachomatis development. First, T3SS effectors are required for the rearrangement of the intracellular compartment to provide chlamydial growth. In the absence of these secreted proteins, the chlamydiae fail to grow. A second possibility is that the signals governing bacterial development may originate or be transduced through the type III secretion apparatus (Wolf et al., 2006).

CL-55 impairs contact between RBs and the inclusion membrane

Next, we analysed the effect of CL-55 on the ultrastructure of the chlamydial cell contact with the inclusion membrane. Electron microscopy of the untreated McCoy cells infected with C. trachomatis at 30 h p.i. showed electron-dense structures at the sites of contact of the chlamydial cells with the inner inclusion membrane, which might correspond to T3SS needle-like structures. Those structures were detected between the outer membrane of the RB cell wall and the inclusion membrane (Fig. 4).

As shown in Fig. 5, RBs failed to associate with the inclusion membrane when treated with 25 μM CL-55 added at the time of infection and analysed at 30 h p.i. The electron micrographs revealed RBs with zones of cell wall destruction and significant damage to the inclusion membrane (Fig. 5).

In 1973, Matsumoto and colleagues published electron micrographs showing rosette-like structures and projections at the surface of EBs of several Chlamydiaceae species (Matsumoto, 1973, 1982), which have become known as ‘Matsumoto’s projections’. In some of the published micrographs, Matsumoto was able to demonstrate that the projections were anchored in the cytoplasmic membrane and extended through the outer membrane of the chlamydiae and that a cluster of hexagonally arrayed projections delineated a zone of contact between the bacterium and the plasma-membrane-derived inclusion membrane (Peters et al., 2007). Matsumoto’s projections were proposed to be T3SS injectisomes (Bavoil & Hsia 1998). Later, many authors reported similar T3SS structures (Dumoux et al., 2015). Our TEM images revealed the same projections in the area of contact between the chlamydiae and the inclusion membrane (Fig. 4). These structures were sensitive to CL-55 treatment, and treatment with the compound induced lesions at the site of contact between the RBs and the luminal face of the inclusion membrane, suggesting that CL-55 directly or indirectly affected the chlamydial T3SS (Fig. 5).

CL-55 lyses aberrant chlamydial forms induced by penicillin

It is known that persistent chlamydial forms are refractory to treatment with antibiotics, so we studied the effect of our T3SS inhibitor on in vitro persistent Chlamydia. To induce persistence, we added penicillin as described elsewhere (Clark et al., 1982). The persistent state of C. trachomatis upon penicillin exposure at 24 h p.i. was confirmed by a significant decrease in viability and an atypical inclusion morphology, with large cellular forms present inside each inclusion. Electron microscopic analysis showed that penicillin induced the formation of enlarged aberrant bodies typical for chlamydial persistence. At 24 and 48 h p.i., the penicillin-treated infected cells contained smaller inclusions than the untreated controls.
The inclusions were less populated with cell-shaped bodies that were consistent with a persistent phenotype (Fig. 6). The state of Chlamydia persistence in the penicillin-treated cultures was confirmed by a huge decrease in infectious progeny. The number of i.f.u. recovered from the cultures exposed to penicillin was negligible, which was consistent with a viable but non-culturable phenotype of Chlamydia.

To monitor the effect of CL-55, we added CL-55 to the growth medium of Chlamydia cultured with penicillin at 24 and 48 h p.i., incubated it for an additional 24 h and then harvested the cells for TEM. Addition of CL-55 to C. trachomatis-infected McCoy cells treated with penicillin resulted in either total or partial membrane lysis of bacterial cells (Fig. 6).

Penicillin persistence has been described as a state in which Chlamydia cells rapidly cease dividing while chromosomal and plasmid replication processes continue at the same rates, regardless of the presence or absence of penicillin (Byrne 2001). To our knowledge, only a few studies have demonstrated T3SS function during penicillin persistence (Wang et al., 2011). Wang et al. (2011) found that ampicillin-induced persistent forms showed decreased expression levels of CopN and Tarp, whereas the levels and distribution of IncA in the inclusion membrane were not significantly changed. These findings differ from those of Belland et al. (2003) in an IFN-γ model of C. trachomatis persistence in HeLa cells, in which tarp gene transcription was downregulated while copN transcription remained unchanged. These results suggest that the secretion of certain proteins varies in a stressor-dependent manner, as noted previously (Klos et al., 2009), perhaps due to differences in the mechanisms by which persistence is induced by different stressors.

We used TEM analysis to visualize the inhibitory effect of CL-55 on persistent forms of Chlamydia. We showed that treatment with the compound resulted in the total destruction of persistent chlamydial forms. Our findings indicate that a T3SS inhibitor might be effective against persistent Chlamydia.

Fig. 5. CL-55 induces lesions in the site of contact between the RBs (RBM) and the luminal face of the inclusion membrane (IM). TEM analysis of C. trachomatis-infected McCoy cell cultures treated with 25 μM CL-55 at the time of infection and analysed at 30 h p.i. Bars, 0.25 μm.

Fig. 6. CL-55 induces a total or partial membrane lysis of C. trachomatis persistent forms. TEM analysis of penicillin-induced persistent Chlamydia in McCoy cells. (a) Penicillin exposure for 24 h; (b) penicillin exposure for 24 h and an additional 24 h of incubation in the presence of 50 μM CL-55; (c) penicillin exposure for 48 h; (d) penicillin exposure for 48 h and an additional 24 h of incubation in the presence of 50 μM CL-55. Bars, 1 μm.
CL-55 inhibits reversion from penicillin persistence

Penicillin removal allows the reversion of persistence, which occurs via RB budding from the aberrant body and can be seen only in some inclusions. Furthermore, the reversion from penicillin-induced persistence seems to be very asynchronous, with the gradual transformation of the aberrant persistent forms to RBs in the inclusion over 10–20 h after penicillin was removed (Skilton et al., 2009).

The penicillin-induced persistent cultures were washed to remove penicillin to induce reversion and were then incubated for additional 24 h in the presence or absence of CL-55. TEM micrographs 24 h after penicillin removal revealed increased inclusions in untreated cultures, along with normal bacterial cells (Fig. 7b). For CL-55 treatment, no recovery of normal morphology of chlamydial cells was evident (Fig. 7c).

The viable infectious progeny were measured 24 h after penicillin removal. Reactivation from persistence was effective in recovering infectious forms, but CL-55 addition was practically lethal to the bacteria, indicating that CL-55 treatment during reversion from penicillin persistence is critical for Chlamydia.

CONCLUSIONS

Taken together, our results demonstrate that a small-molecule compound from a group of thiadiazinones inhibited productive and persistent C. trachomatis (serovar L2) infection in vitro. CL-55 treatment in the early and middle phases of the developmental cycle inhibited chlamydial RB multiplication and the differentiation of RBs into infectious EBs. Electron microscopic studies revealed that C. trachomatis RBs are typically found juxtaposed to the inner surface of the inclusion membrane during mid-cycle development, and electron-dense structures were observed at the sites of contact between chlamydial cells and the inner inclusion membrane, which might correspond to T3SS needle structures. We were able to show that CL-55 impaired the contact between RBs and the inclusion membrane, indicating T3SS-inhibiting activity. Finally, we demonstrated for the first time that a T3SS inhibitor disrupted the progression of the persistent state and the reversion process. Our findings suggest that our novel T3SS inhibitor might be effective against both productive and persistent Chlamydia infections.

ACKNOWLEDGEMENTS

We dedicate our paper to the bright memory of our dear friend and colleague Lena Kost, who passed away suddenly at the final stage of this publication. Lena made a major contribution to this study, and it was a privilege for all of us to work with her. She was a young bright scientist and a pure inspiration for all of us. She will be thoroughly missed by all of us.

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