Inhibition of the Protein Phosphatase CppA Alters Development of *Chlamydia trachomatis*

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ABSTRACT Chlamydiae are obligate intracellular Gram-negative bacterial pathogens that undergo an essential, but poorly understood, biphasic developmental cycle transitioning between the infectious elementary body and the replicative reticulate body. Ser/Thr/Tyr phosphorylation has been increasingly recognized for its role in regulating bacterial physiology. *Chlamydia* spp. encode two Hanks'-type kinases in addition to a type 2C protein phosphatase (PP2C; CppA) and appears capable of global protein phosphorylation. While these findings substantiate the importance of protein phosphorylation in *Chlamydia*, the physiological impact of protein phosphorylation remains enigmatic. In this study, we investigated the *in vivo* role of CppA by using recombinant protein point mutants and small-molecule inhibitors. Recombinant CppA (rCppA) amino acid point mutants based upon missense mutations identified in growth-deficient *Chlamydia trachomatis* strains exhibited reduced, but not a complete loss of, phosphatase activity toward *p*-nitrophenyl phosphate (pNPP) and phosphopeptides. To more directly explore the importance of CppA in chlamydial development, we implemented a chemical "knockout" approach using derivatives of 5,5'-methylenedisalicylic acid (MDSA). Several MDSA derivatives significantly reduced CppA activity *in vitro* and the growth of *C. trachomatis* L2, *C. trachomatis* D, and *Chlamydia muridarum* in a cell culture infection model. The inhibition of *C. trachomatis* L2 growth was more pronounced when treated at earlier infection time points, and the removal of the inhibitors after 12 h postinfection did not rescue progeny production. Our findings revealed that altered CppA activity reduces chlamydial growth and that CppA function is likely crucial for early differentiation events. Collectively, our findings further support the importance of the protein phosphorylation network in chlamydial development.

IMPORTANCE *Chlamydia* is a significant cause of disease in humans, including sexually transmitted infections, the ocular infection trachoma, and pneumonia. Despite the critical roles of protein phosphatases in bacterial physiology, their function in pathogenesis is less clear. Our findings demonstrate that CppA, a broad-specificity type 2C protein phosphatase (PP2C), is critical for chlamydial development and further substantiate reversible phosphorylation as a key regulatory mechanism in *Chlamydia*. Additionally, our work highlights the potential of CppA to serve as a novel target for future therapeutic strategies and supports the feasibility of designing more potent PP2C phosphatase inhibitors for *Chlamydia* and other pathogenic bacteria.

KEYWORDS *Chlamydia trachomatis*, bacterial development, physiological effects of drugs, protein phosphatase, protein phosphorylation

Chlamydiae are highly prevalent, obligate intracellular bacterial pathogens that are responsible for a broad range of diseases in humans and animals (1, 2). *Chlamydia trachomatis* is a major public health concern as infections may lead to serious sequelae, including blindness from trachoma as well as infertility, ectopic pregnancies, and pelvic

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inflammatory disease (PID) from sexually transmitted infections (3–6). In addition, infections with the respiratory pathogen *Chlamydia pneumoniae* have been associated with the development of asthma and atherosclerosis (7, 8). Chlamydial infections in animals are ubiquitous and represent a significant threat to the agriculture industry (2). Of note, *Chlamydia psittaci* and *Chlamydia abortus* infect economically important domesticated animals, causing psittacosis and abortions, respectively, in ruminating animals (9). Additionally, the zoonotic potential of these pathogens also places wildlife and animal husbandry workers at risk for infection. A detailed understanding of chlamydial biology is critical for the development of novel therapeutics and vaccines to treat and prevent chlamydial infections.

Chlamydia undergoes a biphasic developmental cycle within a eukaryotic host cell, transitioning between the infectious elementary body (EB) and the replicative reticulate body (RB) (10, 11). The attachment of the EB to a mucosal epithelial cell triggers endocytosis, creating a host-derived membrane vacuole termed the inclusion (12). Following internalization, the EB differentiates into the replicative RB form and propagates within the expanding inclusion. RBs then asynchronously differentiate back into EBs and exit the cell by lysis or inclusion extrusion for subsequent infection of neighboring cells or transmission to a new host (13). While EB to RB and RB to EB transitions are essential steps in the developmental cycle, the signals and mechanisms that mediate these processes are not fully understood.

Phosphorylation-based cell signaling is mediated by the dynamic interplay between protein kinases and phosphatases, enabling the cell to sense and respond to a wide variety of signals (14). Protein kinases phosphorylate target substrates by the addition of phosphate groups on specific amino acid residues (typically Ser, Thr, Tyr, His, and Asp), which in turn modulate enzyme activity, subcellular localization, and protein-protein interactions (15–18). In contrast, protein phosphatases serve to reverse the actions of protein kinases by catalyzing the removal of phosphate groups (19). While common in eukaryotes, reversible Ser/Thr/Tyr phosphorylation in bacteria has gained increasing attention as a major posttranslational mechanism of regulation (20). Bacterial Ser/Thr and Tyr protein kinases and phosphatases are widely distributed and play important roles in multiple biological processes, including metabolism, development, and virulence (21–24). Moreover, the phosphoproteomes of several bacteria, including several pathogens, indicate that protein phosphorylation is an integral dynamic feature of bacterial physiology that impacts virulence (25–29).

The physiological role of Ser/Thr/Tyr phosphorylation in *Chlamydia* is unclear but appears to have important implications for development. The phosphoproteome of *Chlamydia caviae* reveals differences not only in the functional clustering of phosphoproteins but also in the phosphoprotein abundance between the two developmental forms (30). The EB phosphoproteome contains an increased number of phosphoproteins in comparison to that of the RB form, with the largest class being involved in central metabolism and energy production. In RBs, the overall phosphoprotein levels appear to be significantly reduced compared to those of EBs, and proteins associated with protein synthesis and folding are enriched. Additionally, the genes encoding the two validated protein kinases, PknD and Pkn1, are conserved across *Chlamydia* spp., and the inhibition of PknD from *C. pneumoniae* inhibits bacterial growth in cell culture (31–33).

While multiple studies have explored protein phosphorylation in *Chlamydia*, much less is known about the chlamydial protein phosphatases. We previously validated the pan-chlamydial type 2C protein phosphatase (PP2C), CppA, from *C. trachomatis* and demonstrated its ability to dephosphorylate Ser/Thr/Tyr residues *in vitro* (34). However, the *in vivo* relevance of CppA is not known. We hypothesize that CppA modulates protein phosphorylation status, and consequently protein function, to control chlamydial development and growth. Consistent with our hypothesis, a forward genetic screen using chemical mutagenesis to generate a collection of growth-deficient *C. trachomatis* mutants contained five strains with missense mutations mapping to critical residues in CppA (35). Similarly, studies with other pathogens, including *Salmonella*

FIG 1 Multiple-sequence alignment of chlamydial CppA homologs. Sequences for CppA from *C. trachomatis* 434/Bu (CtL2; [YP_001654588.1](#)), *C. trachomatis* D/UW-3/CX (CtD; [NP_219764.1](#)), and *C. muridarum* Nigg (Cm; [WP_010230685.1](#)) were aligned using Clustal Omega and processed using BioEdit (73). Identical residues are highlighted in black and conserved residues are highlighted in gray using a 100% shading threshold. The eight absolutely conserved PP2C residues are represented by asterisks, and critical residues involved in catalysis are indicated with triangles (74). Arrows indicate amino acids mutated in this study: purple arrows, characterized catalytic aspartate mutants (D38N and D199N) (52); red arrows, chemically induced mutations identified by Kokes et al. (G39E, M40I, G46E, G105E, and G124E) (35).

enterica, *Bacillus anthracis*, and *Staphylococcus aureus*, have shown that PP2C phosphatases are critical for virulence (36–38). In this report, we sought to determine the importance of CppA in chlamydial growth by confirming that the mutations in CppA identified from the chemical mutagenesis screen (35) negatively affect CppA phosphatase activity and that chemical inhibition reduces growth in cell culture. Our *in vitro* studies with recombinant CppA (rCppA) mutants and *in vivo* inhibition studies with 5,5'-methylendisalicyclic acid (MDSA) and derivatives reveal that reduced or altered CppA activity reduces growth and that CppA function is likely crucial for early differentiation events. Taken together, our findings further support the importance of the phosphorylation network in the chlamydial developmental cycle and protein phosphorylation in bacterial physiology and virulence.

RESULTS

Phosphatase activity of rCppA point mutants. In a previous study, Kokes et al. generated a collection of *C. trachomatis* mutants by using a chemical mutagenesis approach and selected isolates with reduced plaque sizes (35). An analysis of this collection of growth-impaired mutants identified five strains carrying *cppA* missense mutations but no *cppA* nonsense mutations. These *cppA* mutants contained radical mutations at or near conserved PP2C residues (Fig. 1) in addition to point mutations elsewhere in their genomes. We hypothesized that the reduced growth phenotypes of the *cppA* missense mutants were due, at least in part, to defects in CppA activity. To test this hypothesis, we purified matching rCppA point mutants and performed phosphatase assays using *p*-nitrophenyl phosphate (pNPP) and phosphopeptide substrates.

Since the introduction of amino acid substitutions can lead to gross conformational changes that would otherwise prevent catalysis, limited trypsin digestion was performed on each rCppA point mutant after purification. The rCppA point mutants displayed similar degradation patterns to that of the wild-type (WT) rCppA, except the G124E point mutant, which displayed a greater amount of a 43-kDa degradation product (see Fig. S1 in the supplemental material). This band also was present in whole-cell lysate samples harvested after induction with isopropyl- β -D-thiogalactopyranoside (IPTG) (data not shown). SDS-PAGE and Western blot analyses indicated that this band corresponds to the protein affinity

FIG 2 Phosphatase activity of rCcpA point mutants. rCcpA point mutants were tested for phosphatase activity using pNPP (A) and phosphopeptide substrates (B and C). (A) Phosphatase activity was measured in the presence of 5 mM pNPP and 5 mM MnCl_2 . (B and C) rCcpA point mutants were incubated with 50 μM P-Thr [KR(pT)IRR] or P-Ser [RRA(pS)VA] peptide substrate (B) and the P-Tyr [RRLIEDAE(pY)AARG or TSTEPQ(pY)QPGENL] peptide substrates (C) in the presence of 25 mM MnCl_2 . The amount of free phosphate (P_i) generated was measured using a malachite green endpoint assay, and values from matched MBP negative-control assays for each peptide were subtracted from those of the corresponding rCcpA assay. Statistical analysis of variance (ANOVA) was performed by comparing the relative activity each rCcpA point mutant to 1 (wild-type activity); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All assays were performed at 37°C and the data represent the averages $\pm$ standard errors (SEs) from at least three independent experiments.

tag, maltose binding protein (MBP) (data not shown). For assays using the G124E rCcpA point mutant, the sample concentration was normalized to the full-length WT rCcpA preparations (3 μg).

rCcpA point mutants were assessed for their ability to dephosphorylate pNPP and synthetic phosphopeptide substrates. The phosphatase activities of rCcpA point mutants were significantly reduced but not completely abrogated, and the reduction in phosphatase activity was generally more significant for pNPP (Fig. 2A) in comparison to that for the phosphopeptide substrates (Fig. 2B and C). Additionally, rCcpA point mutants exhibited differential activities toward the substrates. For instance, the rCcpA with a G124E amino acid substitution was deficient in pNPP hydrolysis but retained the ability to dephosphorylate phosphopeptide substrates at levels $\geq 50\%$ of that of WT rCcpA. In contrast, the G105E point mutant was deficient in dephosphorylating pNPP, phosphorylated Ser (P-Ser), and P-Thr but retained moderate activity toward P-Tyr

FIG 3 Inhibitory activity of MDSA and derivatives. (A) Structure of MDSA and synthesized derivatives (C1 to C6, compounds 1 to 6). (B) rCpPA was incubated with 18.2 μM MDSA or MDSA derivatives and phosphatase activity was measured in the presence of 5 mM pNPP and 5 mM $MnCl_2$. (C and D) Phosphatase activity was assayed in increasing concentrations of MDSA (C) or ATA (D). Relative activity was calculated as the percentage of activity remaining in the presence of the compound versus the activity in the assay buffer containing DMSO. IC_{50} values were determined by nonlinear regression analysis using GraphPad Prism 5 software. Statistical analysis (ANOVA) was performed by comparing relative activity in the presence of each inhibitor to 1; **, $P < 0.01$. All assays were performed at 37°C and the data represent the averages $\pm$ SEs from at least three independent experiments.

substrates. The G39E and M40I point mutants from Kokes et al. (35) also displayed differential activities toward the phosphopeptide substrates.

Identification rCpPA phosphatase inhibitors. The results above suggest that the reduced growth phenotype of the *C. trachomatis* mutants identified in Kokes et al. could be attributed to alterations in CppA phosphatase activity (35). To further study the *in vivo* role of CppA, we attempted to inactivate *cppA* using a group II intron-based approach (39) but were unsuccessful. Additionally, the overexpression of mCherry or His-tagged rCpPA did not appear to grossly impact chlamydial growth, and the overexpression of a catalytically deficient D199N rCpPA point mutant did not result in a dominant-negative growth phenotype. The latter result was consistent with the inability of D199N rCpPA to outcompete WT rCpPA for pNPP *in vitro* (data not shown). Since we have been unable to generate a *C. trachomatis* *cppA* mutant, we sought to identify compounds that were capable of inhibiting rCpPA activity. We synthesized seven different compounds using MDSA, an inhibitor of the *S. aureus* PP2C Stp1 (40), as a preliminary scaffold and screened the derivatives against rCpPA in the presence of pNPP (Fig. 3A). Consistent with the

inhibition seen with *S. aureus* Stp1, MDSA significantly inhibited rCpPA activity. The inhibitory activity of compounds 5 and 6 were similar to that of MDSA, whereas aurintricarboxylic acid (ATA) reduced phosphatase activity levels well below that of MDSA. To determine the inhibition potency of ATA compared to that of its parent compound, MDSA, we performed phosphatase assays using various concentrations of either MDSA (Fig. 3B) or ATA (Fig. 3C). Both compounds inhibited rCpPA activity in a dose-dependent manner with ATA (50% inhibitory concentration [IC_{50}] = $3.4 \pm 0.22 \mu\text{M}$) being 5.4-fold more potent than MDSA ($18.2 \pm 0.13 \mu\text{M}$).

MDSA and derivatives also were screened against shrimp alkaline phosphatase (SAP), a metal-dependent non-PP2C-type phosphatase (41, 42). In contrast to rCpPA, SAP activity was not inhibited by MDSA, ATA, compound 5, or compound 6 in the presence of Mg^{2+} or Mn^{2+} (see Fig. S2A), suggesting that MDSA and derivatives are more selective for rCpPA. Since rCpPA activity toward pNPP is dependent on Mn^{2+} and our compounds contain negatively charged moieties, we addressed whether inhibition by MDSA or ATA was due to a chelation mechanism. Phosphatase assays were performed at a fixed inhibitor concentration (IC_{50}) in the presence of either 5 mM or 20 mM Mn^{2+} . Phosphatase activity remained near 50% in comparison to that of the dimethyl sulfoxide (DMSO) control regardless of the metal concentration (Fig. S2B). These results suggest that the inhibitory activities of MDSA and ATA were independent of Mn^{2+} concentration and were consistent with the inability to inhibit SAP, which also requires divalent metal ions for catalysis (Fig. S2A) (41).

We next performed kinetic assays over a range of pNPP concentrations in the presence of MDSA or ATA at their respective IC_{50} s to gain insight on the mechanism of inhibition. The presence of the inhibitors resulted in increases in K_m s for MDSA and ATA and a minor decrease in V_{max} for MDSA and no change in the V_{max} for ATA (see Fig. S3A and B), suggesting that MDSA and ATA inhibit rCpPA activity in a competitive manner.

Effect of MDSA and derivatives on chlamydial growth. For infection experiments, we initially focused on compounds that inhibited rCpPA activity *in vitro*. While compounds 5 and 6 were significantly active against rCpPA, their activities were similar to that of the parent compound, MDSA. Consequently, compounds 4 to 6 in addition to compound 3, the carboxymethyl derivative of compound 6, were not further explored in this study. Since MDSA and ATA were both capable of inhibiting rCpPA activity *in vitro*, we examined their effect on chlamydial growth in cell culture. We infected mouse L2 fibroblast cells with DFCT1 (*C. trachomatis* L2 with pGFP::SW2) in the presence of various concentrations of MDSA or ATA and then assessed infectious progeny production using the inclusion forming unit (IFU) assay. MDSA inhibited chlamydial growth in a dose-dependent manner (Fig. 4A). In contrast to the pNPP results, ATA was only slightly more effective than MDSA in reducing chlamydial growth (Fig. 4B), and higher doses of ATA led to a reduction in host cell viability (see Fig. S4). As an alternative, we tested compound 2, a carboxymethyl MDSA derivative with a reduced polar surface area compared to that of MDSA. Treatment with compound 2 reduced chlamydial growth in a dose-dependent manner, and inhibition was 75-fold more potent in comparison to that of MDSA (Fig. 4C). The IC_{50} s for MDSA ($2.12 \pm 0.23 \text{ mM}$) and compound 2 ($28.4 \pm 3.36 \mu\text{M}$) were not toxic (defined as at least 90% viability versus DMSO) to L2 cells on the basis of neutral red uptake assay data (Fig. S4).

To address whether MDSA and the methyl-protected MDSA derivatives reduce progeny production by altering host cell processes, L2 cells were pretreated with the inhibitors for 4 h, washed, and then infected with DFCT1 in the absence of the inhibitors. We found that the pretreatment of host cells with 2.12 mM MDSA, 28.4 μM compound 1, or 28.4 μM compound 2 yielded a similar progeny level as the DMSO control (see Fig. S5A). In addition, we assessed the impact of these inhibitors on the phosphatase activity of L2 cell lysates (Fig. S5B). Compounds 1 and 2 did not inhibit L2 cell phosphatase activity in comparison to that of the DMSO control, whereas MDSA marginally reduced phosphatase activity by 15%.

FIG 4 Treatment of *Chlamydia* with rCpA inhibitors. L2 cells were infected with DFCT1 at an MOI of 0.05 in the presence of increasing concentrations of MDSA (A), ATA (B), and C2 (C). *Chlamydia*-infected cells were then harvested at 44 hpi, and viable infectious yield was determined using the IFU assay. Relative IFU produced represents inclusion-forming units per milliliter in the presence of the inhibitor versus that with DMSO. IC_{80} values were determined by nonlinear regression analysis using GraphPad Prism 5 software. The data represent the averages $\pm$ SEs from at least three independent experiments.

Sensitivity of *C. trachomatis* D and *Chlamydia muridarum* to MDSA and derivatives. Since CppA is a pan-chlamydial PP2C phosphatase, we hypothesized that other *Chlamydia* species would be susceptible to MDSA and derivatives. Similar to that in the growth assays with DFCT1, we treated *C. trachomatis* D- and *C. muridarum*-infected cells with 2.12 mM MDSA, 28.4 μ M compound 1, or 28.4 μ M compound 2 at 0 hours postinfection (hpi) and examined infectious progeny production from samples harvested at 44 hpi. While the inhibitory activities of compound 2 were similar for all three *Chlamydia* species tested, the inhibition of chlamydial growth was differential for MDSA and compound 1 (Fig. 5A). The treatment with MDSA resulted in a 70% to 80% reduction in DFCT1 and *C. muridarum* progeny production and greater than 95%

FIG 5 Assessment of MDSA and methyl-protected derivatives on infectious progeny production and inclusion size for DFCT1, *C. trachomatis* D, and *C. muridarum*. DFCT1, *C. trachomatis* D, and *C. muridarum* were used to infect L2 cells in the presence of 2.12 mM MDSA, 28.4 μM C1, or 28.4 μM C2. (A) At 44 hpi, EBs were harvested and the titers determined using the IFU assay. Relative IFU produced represents IFU per milliliter in the presence of the inhibitor versus that with DMSO, and the data represent the averages $\pm$ SEs from at least three independent experiments. (B to D) DFCT1 (B), *C. trachomatis* D (C), and *C. muridarum* (D) inclusion areas at 40 hpi were determined using ImageJ. Statistical analysis (ANOVA) was performed by comparing inclusion areas in the presence of each inhibitor to that of the DMSO control; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The data represent the averages $\pm$ standard deviations (SDs) from at least three independent experiments.

reduction for *C. trachomatis* D progeny production. In contrast, the treatment with compound 1 inhibited progeny production by 98% for *C. muridarum*, 64% for *C. trachomatis* D, and 36% for DFCT1. In addition to *Chlamydia*, we also assessed the effect of these inhibitors on the growth of free-living Gram-negative and -positive bacteria. The growth of *Escherichia coli* and *Listeria monocytogenes* was not inhibited by MDSA, compound 1, or compound 2 at concentrations 1.8-fold or higher than those tested for the *Chlamydia* species (see Fig. S6A and B).

In addition to progeny production, inclusion areas were measured to assess chlamydial growth. L2 cells were infected with DFCT1, *C. trachomatis* D, or *C. muridarum* in the presence of MDSA, compound 1, or compound 2, and inclusions were viewed using fluorescence microscopy at 40 hpi. Consistent with the reduced bacterial growth, the treatments with the inhibitors significantly decreased the inclusion area in comparison to that of the DMSO control. The treatment of DFCT1 with MDSA, compound 1, or compound 2 led to a 70%, 55%, or 66% reduction in inclusion area, respectively (Fig. 5B and S7A). Furthermore, MDSA significantly decreased *C. trachomatis* D (Fig. 5C and S7B) and *C. muridarum* (Fig. 5D and S7C) inclusion areas by 88% and 81%, respectively, and the reduction was more severe than for the other two compounds. Treatments with compounds 1 and 2 reduced *C. trachomatis* D inclusions by 40% and 51%, respectively, and *C. muridarum* inclusions by 47% and 54%, respectively.

The differential reductions in progeny production and inclusion areas of DFCT1, *C. trachomatis* D, and *C. muridarum* prompted us to investigate the inhibitory activity of MDSA on *C. trachomatis* D and *C. muridarum* CppA. We purified rCppA from *C. trachomatis* D and *C. muridarum* and performed pNPP assays over a range of MDSA concentrations to generate dose-response curves. The IC_{50} s to inhibit *C. trachomatis* D (see Fig. S8A) and *C. muridarum* (Fig. S8B) rCppA were determined to be $23.8 \pm 1.15 \mu\text{M}$ and $19.4 \pm 1.84 \mu\text{M}$, respectively, and were similar to the IC_{50} required to inhibit *C. trachomatis* L2 rCppA ($18.2 \pm 0.13 \mu\text{M}$).

FIG 6 Treatment of *Chlamydia*-infected cells with inhibitors during the developmental cycle. (A) DFCT1-infected cells were treated with either 2.12 mM MDSA or 28.4 μ M C2 at the various times indicated. (B) 2.12 mM MDSA or 28.4 μ M C2 was added to DFCT1-infected cells at 0 hpi and subsequently removed at 2, 12, and 24 hpi. EBs were then harvested at 48 hpi and the titers were determined from lysates using the IFU assay. (C and D) DFCT1 was treated with MDSA (C) or C2 (D) and the inhibitors were removed at 12 or 24 hpi. EBs were harvested at either 48 hpi or 60 hpi and progeny levels were determined using the IFU assay. Relative IFU produced represents samples harvested at either 48 or 60 hpi versus matched DMSO samples. Statistical analysis (ANOVA) was performed by comparing the relative IFU produced from each sample to 1 (A and B) or by comparing relative IFU produced between the 48 hpi and 60 hpi harvest samples (C and D); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Treatment of *Chlamydia* with inhibitors during the developmental cycle. While several proteomics studies with *Chlamydia* have been published, CppA has not been detected (43–45). In addition, we have been unable to detect CppA in EB and RB lysates by using custom rabbit polyclonal anti-CppA antibodies. However, a genome-wide transcriptome analysis of *C. trachomatis* indicates that *cppA* is expressed by 8 hpi and continuously transcribed throughout the developmental cycle, with maximal transcript levels at 24 hpi (46). To determine the importance of CppA throughout development, we treated DFCT1-infected cells with either MDSA or compound 2 at various times postinfection and assessed bacterial growth using the IFU assay. The treatment with MDSA (Fig. 6A) significantly inhibited bacterial growth in comparison to that of the DMSO control. Additionally, the reduction in progeny production was more pronounced at earlier time points (0 to 12 hpi) than at later time points (20 to 40 hpi). Consistent with the inhibition seen with MDSA, the treatment with compound 2 also inhibited bacterial growth, with progeny numbers significantly lower at earlier time points (0 to 6 hpi). This time-dependent effect of MDSA and compound 2 also could be due to the increasing numbers of bacteria present at later stages, reducing the effective concentration of the inhibitors later in the developmental cycle. To test for “dosage” effects, we performed similar experiments using 100-fold fewer bacteria than in the experiments shown in Fig. 6A and found a similar trend in which progeny levels were significantly reduced when compounds were added at earlier rather than later time points (see Fig. S9A). We further explored the impact of the inhibitors on early development by pretreating *Chlamydia* with MDSA or compound 2. While the pretreatment of *Chlamydia* and growth in the presence of the inhibitors led to a significant reduction in progeny levels consistent with treatment at 0 hpi (~75% to 80% for both

scenarios), the pretreatment and removal of inhibitors at 1 hpi only modestly reduced progeny levels (17% for MDSA and 32% for compound 2) (Fig. S9B).

We next addressed whether the removal of the inhibitors would rescue progeny production. DFCT1 was used to infect L2 cells in the presence of MDSA or compound 2 at 0 hpi. The inhibitors were subsequently removed at 2, 12, or 24 hpi by washing, and progeny production was assessed from samples harvested at 48 hpi. While the removal of the inhibitors at 2, 12, and 24 hpi did not rescue progeny production to wild-type levels, removal at 2 hpi led to a significant increase in progeny levels compared to that of the no removal control from 30% to 76% for MDSA and 28% to 74% for compound 2 (Fig. 6B). In contrast, only modest increases in progeny levels were observed when MDSA and C2 were removed at 12 (44% and 41%, respectively) or 24 (39% and 37%, respectively) hpi. Since progeny levels remained similar to that of the no removal control following inhibitor removal at 12 or 24 hpi, we performed identical experiments to that shown in Fig. 6B with the exception that *Chlamydia* was allowed to recover for an additional 12 h. While the progeny levels from the no removal control remained similar (30% for MDSA and 33% for compound 2), the extended recovery period partially rescued the progeny production. In comparison to the 48-hpi harvest samples, progeny levels from the 60-hpi harvest increased from 44% to 74% and 41% to 64% when MDSA was removed at 12 and 24 hpi, respectively (Fig. 6C). For compound 2, progeny levels increased from 39% to 63% and 37% to 55% when removed at 12 and 24 hpi, respectively (Fig. 6D).

DISCUSSION

Reversible phosphorylation is an integral regulatory mechanism that enables adaptive responses to changing environmental conditions (47, 48). The interplay between protein kinases and phosphatases modulates the activity of target proteins that regulate important cellular processes, including metabolism, development, and virulence (23, 24). Numerous phosphoproteome studies have shown that Ser/Thr/Tyr protein phosphorylation is widely distributed across species and that phosphorylation significantly impacts bacterial physiology and pathogenesis (20, 49, 50). Consequently, protein kinases and phosphatases have garnered interest as novel targets for therapeutic strategies (51).

In this study, we investigated the physiological role of CppA, a chlamydial PP2C phosphatase with P-Ser, P-Thr, and P-Tyr substrate specificity using a combination of rCppA point mutants and small-molecule inhibitors. Prior attempts to inactivate *cppA* were unsuccessful and suggest that *cppA* is essential for growth in cell culture. Consistent with this observation, the mutagenesis screen by Kokes et al. did not yield a *cppA* null mutant, and strains harboring missense mutations at or near conserved PP2C residues exhibited small-plaque phenotypes (35). Phosphatase assays using rCppA point mutants matching those identified by Kokes et al. demonstrated that these mutations reduce, but do not completely abrogate, phosphatase activity, suggesting that defects in CppA lead to reduced chlamydial growth. In addition, rCppA point mutants with asparagine substitutions at conserved PP2C aspartate residues (D38N and D199N) were incapable of pNPP hydrolysis, consistent with the findings of Jackson et al. (52), but were capable of dephosphorylating the phosphopeptide substrates at low to moderate levels. While the mechanistic details regarding how rCppA point mutants retain some level of activity despite mutations in the aspartate residues responsible for metal coordination are unclear, it is possible that other residues are partially compensating for the mutations and/or that rCppA is better able to bind the phosphopeptide substrates, a more physiologically relevant substrate than pNPP. Similar activity-substrate differences have been seen for other PP2C aspartate mutants/variants (53, 54). It is also possible, as suggested by recent findings from Rajagopalan et al., that our current definition of PP2C-critical residues is too rigid (59). Additionally, the differential activities of the rCppA point mutants may shed light on specific CppA amino acid residues responsible for substrate recognition or catalysis. Of note, D38N, G39E, M40I, G105E, and G124E rCppA displayed differential activities toward the phosphopeptide

substrates and can be leveraged in future studies to investigate the significance of P-Ser, P-Thr, and P-Tyr dephosphorylation *in vivo* using conditional expression mutants. Mutations at these positions may serve to slightly destabilize the active site (based on a three-dimensional [3D] threading model of CppA) through the neutralization of a negative charge (D38N) or the introduction of a larger negatively charged amino acid (G39E, G105E, and G124E), leading to an altered substrate specificity or enzyme activity (56).

To identify inhibitors of CppA, we synthesized derivatives on the basis of MDSA, a previously reported inhibitor of the *S. aureus* Stp1, and screened them against purified rCppA using *in vitro* pNPP assays (40). Both MDSA and ATA inhibited rCppA in a dose-dependent manner, and the inhibition was more significant for ATA. Additionally, compounds 5 and 6 reduced rCppA activity to levels similar to that of MDSA, whereas compounds 1 to 4 did not inhibit rCppA activity, suggesting that the spacing of the phenolic rings and/or the charge of carboxyl groups is important for inhibition. While the molecular interaction between CppA and the inhibitors is not known, our data demonstrated that the inhibition by MDSA and ATA was independent of the metal concentration and likely occurs in a competitive manner, as rCppA activity was rescued in the presence of increased concentrations of pNPP. Other studies have shown that MDSA and ATA are noncompetitive and mixed-type inhibitors of *S. aureus* Stp1, respectively (40, 57). Despite the differences in inhibition mechanisms, which may be attributed to slight structural differences between rCppA and Stp1, MDSA and ATA appear to be more selective for bacterial PP2C phosphatases, as these inhibitors do not inhibit shrimp alkaline phosphatase, a non-PP2C-type phosphatase, and exhibited mild inhibitory activity against the eukaryotic PP2C α (40, 57). In addition, we observed minimal inhibition of whole-cell phosphatase activity of L2 cell lysates in the presence of MDSA and methyl-protected MDSA derivatives. These findings support the feasibility of developing bacterial PP2C-specific inhibitors.

To investigate the *in vivo* function of CppA, we treated *Chlamydia*-infected cells with the rCppA inhibitors and assessed bacterial growth in cell culture. While MDSA and ATA inhibited chlamydial growth, the inhibition efficacy of ATA, in contrast to the pNPP results, was similar to that of MDSA. Alternatively, the treatment with compound 2, a carboxymethyl MDSA derivative, significantly reduced progeny levels, and the inhibition was 75-fold greater than that with MDSA. We suspect that the methylation of the carboxyl groups improves the diffusion through cell membranes and into the host cell cytoplasm where it is converted to MDSA by endogenous esterases (see Table S2 in the supplemental material). The esterification of small molecules is a classical prodrug approach frequently used to enhance the permeability of a parent drug by masking charged moieties (58). Ester-based prodrugs have been shown to become activated by host enzymes, including carboxylesterase, paraoxonase, acetylcholinesterase, and cholinesterase.

In addition to inhibiting the growth of *C. trachomatis* L2, MDSA and compounds 1 and 2 significantly reduced progeny production for *C. trachomatis* D and *C. muridarum*. Furthermore, the treatment of *Chlamydia* with these inhibitors also resulted in a significant reduction in inclusion areas. The reduced progeny levels and inclusion sizes are in agreement with the small-plaque *cppA* *C. trachomatis* mutants and the rCppA point mutant data. It is unclear why MDSA and compound 1 exhibited differential inhibitory activities for *C. trachomatis* L2, *C. trachomatis* D, and *C. muridarum* progeny levels and inclusion areas. The IC₅₀s of MDSA required to inhibit *C. trachomatis* L2, *C. trachomatis* D, and *C. muridarum* rCppA were similar and suggest that the differential inhibition of chlamydial growth could be due, in part, to the differences in bacterial growth rates, host-cell-type specificities, or off-target effects. While we cannot rule out the possibility that MDSA and compounds 1 and 2 inhibit other chlamydial and host proteins, the inhibitor concentrations used in the *Chlamydia* growth experiments were not toxic to L2 mouse cells (defined as at least 90% host cell viability) based on the neutral red uptake assay data. The compounds also did not inhibit the phosphatase activity of L2 mouse cell lysates with the exception of MDSA, which only slightly

decreased the phosphatase activity in comparison to that of the DMSO control, consistent with the mild inhibitory activity of MDSA toward the eukaryotic PP2C α (40, 57). In addition, pretreating L2 mouse cells with the compounds did not reduce *C. trachomatis* L2 progeny levels, indicating that MDSA and compounds 1 and 2 do not impair the host cell processes required for initial infection.

Furthermore, the inhibitors did not impair the bacterial growth of *E. coli* or *L. monocytogenes*. *E. coli* MG1655 harbors an atypical PP2C phosphatase reported to be resistant to MDSA (59), while *L. monocytogenes* harbors both Stp, a PP2C phosphatase involved in virulence, and RsbU, a PP2C-like phosphatase involved in environmental signaling (60, 61). It was previously reported that an *stp* null mutant exhibits similar growth to that of the wild type in brain heart infusion (BHI) medium, suggesting that Stp is dispensable for *in vitro* growth, consistent with our data (61). The inability to reduce the growth of bacteria that harbor an MDSA-resistant phosphatase and/or do not require a PP2C phosphatase for growth in broth culture indicates that MDSA and compounds 1 and 2 likely have limited off-target effects. As PP2C phosphatases have been shown to play important roles in signaling, stress response, and protein synthesis in nonpathogenic bacteria, these compounds may be suitable to probe the *in vivo* function of PP2Cs in other bacteria (62–65). The results from such experiments might shed light on how PP2C inhibition as a therapy might impact patient microbiomes.

We found that the treatment of *Chlamydia* with MDSA or compound 2 led to a time-dependent effect in which the treatment at earlier time points postinfection significantly reduced the progeny levels in comparison to those at later time points. The time-dependent effect of the inhibitors was most likely not due to the increasing numbers of bacteria produced during the developmental cycle, as the treatment of 100-fold fewer *Chlamydia* cells with the inhibitors also generated similar inhibition profiles and suggests that MDSA and compound 2 most likely act on early infection events. Despite the pronounced reductions in progeny production at earlier time points, the pretreatment of *Chlamydia* cultured in the absence of the inhibitors led to a significant increase in progeny production. While there are a variety of factors that could explain the increase in progeny levels following pretreatment with MDSA and compound 2, we suspect that the rigid and highly cross-linked outer membranes of EBs hindered the entry of the inhibitors into the cell (66). In support of this hypothesis, the treatment at 0 hpi and the removal of MDSA and compound 2 following EB to RB differentiation (12 and 24 hpi) did not rescue progeny production to wild-type levels, even with a longer recovery period.

We also assessed the effect of the inhibitors at different time points during the developmental cycle. The pronounced reductions in progeny levels when *Chlamydia* cells were treated at earlier infection time points and the inability to rescue progeny production following inhibitor removal at 12 or 24 hpi are supportive of CppA playing an important role during early infection. The treatment time points in which progeny levels were lowest corresponded to EB invasion and EB to RB transition (10). During these stages of the developmental cycle, the phosphoproteome landscape appears to undergo a dramatic shift as EB-specific phosphoproteins, the majority of which are involved in central metabolism and energy production, become dephosphorylated in the RB form (30). Consistent with CppA playing an important role during early infection, the removal of inhibitors at 2 hpi resulted in only a slight reduction in progeny levels. Progeny levels remained reduced when the MDSA and compound 2 were removed following the EB-to-RB transition phase (12 hpi). We hypothesize that the inhibition of CppA activity prevents the dephosphorylation of phosphoproteins in EBs, which in turn hinders the normal growth and propagation within the host. Transcriptomic data indicate that *cppA* transcripts are present by 8 hpi, and our data show that the treatment prior to this time results in reduced progeny production (46). Alternatively, since *cppA* transcripts are present throughout the developmental cycle following 8 hpi, CppA could be prepackaged into EBs where it is inhibited by MDSA or compound 2 upon the infection of

a new host cell. The failure of pretreatment followed by inhibitor removal to significantly reduce infection might be due to multiple reasons such as poor drug penetration into EBs, which possess a rigid and highly cross-linked outer membrane, and/or the dilution of the inhibitor below effective concentrations during infection (66). Alterations in PP2C phosphatase activity, leading to impaired cellular processes, have been reported in other bacterial pathogens. The hyperactivity of *Streptococcus pneumoniae* PhpP as a result of Mn^{2+} toxicity hinders cell division, and inhibition of *S. aureus* Stp1 by ATA reduces virulence in a mouse model (57, 67).

In summary, our study provides insight on the importance of CppA in the chlamydial developmental cycle. Mutations in *cppA* that were identified in the study by Kokes et al. reduce rCppA activity *in vitro*, and this suggests that altered phosphatase activity of CppA reduces chlamydial growth in the plaque assay (35). Consistent with CppA playing an important role in chlamydial development, the treatment of *Chlamydia* with derivatives of a CppA inhibitor significantly reduced bacterial growth in cell culture. Additionally, the reduction in progeny levels was more severe when *Chlamydia* cells were treated at earlier time points rather than at later time points postinfection, suggesting that CppA likely plays an essential role during early development. The inhibition of CppA leading to reduced growth is consistent with PknD inhibitor studies performed with *C. pneumoniae*, a close relative of *C. trachomatis* (33). Altogether, our findings extend the understanding of reversible phosphorylation as a critical regulatory mechanism in chlamydial development and highlight the potential of PP2C inhibitors as an approach to investigate protein phosphorylation in other bacterial pathogens and as possible novel antimicrobials.

MATERIALS AND METHODS

Cell and bacterial culture conditions. Mouse L2 fibroblast cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. To visualize inclusions, a *C. trachomatis* L2/434/Bu strain (DFCT1) harboring a green fluorescent protein (GFP)-expressing vector (pGFP::SW2; gift of Ian Clarke, University of Southampton) was generated using methods previously described (39, 68). All recombinant DNA experiments were performed in accordance with NIH section III-D-1-a and were approved by the Southern Illinois University recombinant DNA committee. DFCT1, *C. trachomatis* D/UW-3/CX, and *C. muridarum* MoPn were routinely cultured in L2 mouse cells. Confluent cell monolayers were infected with a multiplicity of infection (MOI) of 1 by centrifugation for 1 h at 545 × *g* or by rocking for 2 h at 37°C with 5% CO₂. *Chlamydia* was then grown at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS, 1 × nonessential amino acids, and 0.2 μg/ml cycloheximide. Bacteria were harvested at 40 hpi and stored at −80°C in sucrose-phosphate-glutamic acid (SPG) medium; the titer was determined using the inclusion forming unit (IFU) assay (69), and the bacteria were then used in inhibitor experiments. *Escherichia coli* DH5α was used for routine cloning procedures, and *E. coli* NEB Express (New England BioLabs) was used for protein production. *E. coli* DH5α and NEB Express were grown in LB broth or on agar supplemented with ampicillin (100 μg/ml). *E. coli* MG1655 and *Listeria monocytogenes* PTA-5236 (from ATCC) (70) used in bacterial growth experiments were maintained in LB and brain heart infusion media (broth and agar), respectively.

Cloning, expression, and purification of rCppA proteins. The construction and purification of WT and mutant rCppA proteins were carried out as described previously by Claywell and Fisher (34). Unless specified, PCR was performed with Phusion polymerase (Thermo Scientific) and the primers listed in Table S1 in the supplemental material. The *cppA* gene (CTL0511) was amplified from *C. trachomatis* L2/434/Bu genomic DNA using MAL511 primers and inserted into pMAL-c4X to create a plasmid construct encoding a maltose binding protein (MBP)-CppA fusion protein. Following transformation into *E. coli* DH5α, transformants were screened by colony PCR using *Taq* DNA polymerase. A *cppA*-positive transformant was grown for plasmid isolation (designated pMAL511) and sequencing of the gene insert (Sanger sequencing performed by Macrogen USA). The cloning of *cppA* from *C. trachomatis* D/UW-3/CX (CT259) and *C. muridarum* MoPn (TC0530) into pMAL-c4X was performed using MAL511 and MALCM511 primers, respectively. For site-directed mutagenesis of conserved PP2C aspartates (52) and residues identified by Kokes et al. (35), PCR was performed using pMAL511 and 5'-phosphorylated primers to create seven different rCppA point mutants, each with one of the following amino acid substitutions: G39E (pMAL511G39E), D38N (pMAL511D38N), M40I (pMAL511M40I), G46E (pMAL511G46E), G105E (pMAL511G105E), G124E (pMAL511G124E), or D199N (pMAL511D199N). For protein expression and purification, all plasmid constructs were transformed into *E. coli* NEB Express, and rCppA proteins were affinity purified using amylose resin (34).

Synthesis of small molecules. All commercially obtained compounds and synthesized derivatives are listed in Table S2. Detailed synthetic methods and compound validation are provided in the supplemental methods. Physicochemical properties of compounds, including LogD, cLogP, and polar surface areas were predicted using SPARC from ARChem (Danielsville, GA), ChemSketch from ACD Labs

(Advanced Chemistry Development, Inc., Toronto, Canada), and MarvinSketch from ChemAxon (Budapest, Hungary), respectively.

Limited trypsin digestion analysis of rCcpA point mutants. Limited trypsin digestion assays were performed as previously described (71). Briefly, 27 μg of purified rCcpA was incubated with 2.1 μg of trypsin in 450 μl phosphate-buffered saline (PBS) at room temperature. At 5, 15, 30, and 60 min, 100- μl aliquots were then transferred to separate tubes containing 6 $\times$ Laemmli buffer and heated for 5 min at 90 to 100°C. Samples were run on 12% SDS-PAGE gels and stained with Coomassie brilliant blue.

Phosphatase assays. (i) Phosphatase activity of rCcpA point mutants. Unless specified, all *p*-nitrophenyl phosphate (pNPP) assays were performed in 100 μl assay buffer containing 50 mM Tris-HCl (pH 8), 50 mM KCl, 1 mM dithiothreitol (DTT), 5 mM pNPP, and 5 mM MnCl_2 . Three micrograms of purified rCcpA was added to the wells in duplicates, and the reactions were initiated by the addition of the assay buffer containing pNPP. The release of *p*-nitrophenol (pNP) was monitored by measuring the absorbance at 405 nm (A_{405}) every 5 min for 90 min in a 96-well plate reader (Synergy HT; BioTek) at 37°C. The amount of pNP formed was calculated using a molar extinction coefficient of $18,500 \text{ M}^{-1} \cdot \text{cm}^{-1}$, and the specific activity was defined as micromolar concentration of pNP per minute per microgram of protein. The relative activity of rCcpA point mutants represents the specific activity versus that of the WT rCcpA.

Phosphatase assays with synthetic phosphopeptide substrates were performed using Ser [RR(pS)VA], Thr [KR(pT)IRR], or Tyr [RRLI(pY)AARG or TSTEPQ(pY)QPGENL] phosphopeptides (Upstate). The reactions were carried out in 50 μl assay buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM DTT, and 25 mM MnCl_2) containing 50 μM phosphopeptide substrate and 3 μg purified rCcpA for 1 h at 37°C. The reactions were terminated by the addition of 100 μl Biomol Green reagent (Enzo Life Sciences) and then left at room temperature for 20 min to allow for color development. The amount of inorganic phosphate (P_i) released was determined by measuring the A_{620} and using a standard curve with P_i . A_{620} values from matched MBP negative-control assays for each phosphopeptide substrate were subtracted from those of rCcpA assays. The relative activity of rCcpA point mutants represents the amount of P_i released versus that of the WT rCcpA.

(ii) Inhibitor studies. MDSA and various MDSA derivatives were screened against rCcpA and shrimp alkaline phosphatase (SAP; Thermo Fisher). Each compound was dissolved in dimethyl sulfoxide (DMSO) to yield a 10 mM stock solution and tested at a final concentration of 18.2 μM . Phosphatase assays with purified rCcpA were initiated by the addition of 3 μg purified protein to the assay buffer containing pNPP and MnCl_2 . To test the inhibitory activity of each compound against SAP, pNPP assays were performed in the presence of either 5 mM MnCl_2 or 5 mM MgCl_2 . The concentration of SAP for each metal condition was adjusted to match the activity of rCcpA. SAP was serially diluted and assayed in the presence of 5 mM pNPP and either 5 mM MnCl_2 or 5 mM MgCl_2 . The concentration of SAP required to yield a similar activity to that of rCcpA was determined using a standard curve generated by plotting activity values from each metal condition against the SAP concentration. Metal rescue assays were then performed using a fixed inhibitor concentration (18.2 μM MDSA or 3.4 μM aurintricarboxylic acid [ATA]) and assay buffer containing either 5 mM or 20 mM MnCl_2 . For all inhibitor assays, the release of pNP was monitored by measuring A_{405} every 5 min for 90 min at 37°C. The relative activity was calculated as the percentage of activity remaining in the presence of the inhibitor versus the activity in the assay buffer containing DMSO.

Phosphatase assays with L2 cell lysates in the presence of 2.12 mM MDSA, 28.4 μM compound 1, or 28.4 μM compound 2 were performed using a cell density number at 100% confluence for a 96-well plate ($\sim 1.6 \times 10^5$ cells). The cells were resuspended in buffer (20 mM Tris-HCl [pH 8], 500 mM NaCl, 0.1% Triton X-100, and 10% glycerol) to a final concentration of 3.2×10^6 cells/ml before disruption by gentle sonication, and 50 μl whole lysate was transferred to a 96-well plate in duplicates. The reactions were initiated by the addition of assay buffer containing inhibitor, pNPP, and MnCl_2 . Three micrograms of purified rCcpA was used as an assay control. The relative activity was calculated as the percentage of activity remaining in the presence of the inhibitor versus the activity in the assay buffer containing DMSO.

For dose inhibition assays, phosphatase activity was measured in the presence of various concentrations of MDSA (1.95 to 125 μM) or ATA (0.14 to 35 μM). Each compound was serially diluted in assay buffer containing pNPP and then transferred to the wells in duplicates. The reactions were initiated by adding 3 μg purified rCcpA from *C. trachomatis* L2, *C. trachomatis* D (MDSA only), or *C. muridarum* (MDSA only), and IC_{50} s were determined by nonlinear regression analysis using GraphPad Prism 5 software (version 6.01).

The kinetic parameters of rCcpA in the presence of MDSA or ATA were determined by measuring the initial reaction velocities at various concentrations of pNPP (50 to 0.024 mM) with a fixed inhibitor concentration (18.2 μM MDSA or 3.4 μM ATA). pNPP was serially diluted in assay buffer with inhibitor, and the reactions were initiated by the addition of 3 μg purified rCcpA. Kinetic parameters were determined by fitting the data to the Michaelis-Menten equations by nonlinear regression analysis by using GraphPad Prism 5 software.

Host cell viability. The cytotoxicity of MDSA, ATA, compound 1, or compound 2 toward HeLa and L2 cells was assessed using the neutral red uptake assay as described by Repetto et al. (72) with slight modifications. Briefly, confluent cell monolayers were incubated with various concentrations of MDSA (0 to 3.13 mM), ATA (0 to 2.99 mM), compound 1 (0 to 100 μM), or compound 2 (0 to 100 μM) in DMEM, 10% FBS, and 0.2 $\mu\text{g}/\text{ml}$ cycloheximide (HeLa cells only) for 48 h at 37°C with 5% CO_2 . The cells were then washed 3 times with 200 μl PBS and incubated with 100 μl PBS with 0.01% neutral red (Sigma) for 2 h at 37°C with 5% CO_2 . After removing the neutral red and washing with PBS, 100 μl neutral red extraction

solution (1% acetic acid and 50% ethanol) was added followed by gently shaking. After incubation at room temperature for 20 min, the neutral red absorbance was measured at A_{570} . The relative viability was expressed as a percentage of the DMSO control values.

***E. coli* and *L. monocytogenes* growth assays.** Overnight cultures of *E. coli* MG1655 or *L. monocytogenes* PTA-5236 were diluted to a final concentration of 5.33×10^4 cells/ml in fresh LB or BHI broth, respectively, and transferred to microcentrifuge tubes containing MDSA (3 mM), compound 1 (50 μ M), compound 2 (100 μ M), ampicillin (100 μ g/ml), or DMSO. One hundred fifty microliters of each mixture was then transferred to the wells in duplicates, and the cells were grown at 37°C with shaking. Bacterial growth curves were generated by measuring the optical density at 600 nm (OD_{600}) every 30 min for 14.5 h in a 96-well plate reader, and the background was eliminated by subtracting the blank values for each time point.

***Chlamydia* growth assays.** Unless specified, the inhibitor experiments were performed in 96-well plates seeded with L2 cells, and infections were carried out at an MOI of 0.05 by centrifugation at $545 \times g$ for 1 h. *Chlamydia* was cultured at 37°C with 5% CO_2 in DMEM containing 10% FBS and $1 \times$ nonessential amino acids and supplemented with inhibitors at an IC_{50} (unless otherwise noted) or DMSO ($<1\%$) as a vehicle control for various time periods. EBs were harvested by scraping and centrifuged at $13,000 \times g$ for 10 min at 4°C. The pellets were washed 2 times with 500 μ l PBS to remove the inhibitor, suspended in SPG medium, and stored at $-80^\circ C$. The inclusion forming unit (IFU) assay was performed to assess progeny production for DFCT1 using vector-encoded GFP fluorescence as an inclusion marker or Pathfinder reagent (a fluorescein-conjugated mouse monoclonal anti-LOS antibody; Bio-Rad) to detect *C. trachomatis* D and *C. muridarum* inclusions. Fluorescent inclusions were viewed at $\times 400$ magnification using a Leica DMIL microscope fitted with a Leica EC4 camera and LAS EZ software (Leica).

For dose inhibition experiments, confluent L2 cell monolayers were infected with DFCT1 in the presence of various concentrations of MDSA (0 to 3 mM), ATA (0 to 200 μ M), and compound 2 (0 to 50 μ M). At 44 hpi, EBs were harvested and the titer was determined using the IFU assay. The relative IFU produced represents inclusion forming units per ml in the presence of the inhibitor versus that in DMSO. IC_{80} values were determined by nonlinear regression analysis using GraphPad Prism 5 software.

To assess the growth of *C. trachomatis* D and *C. muridarum*, the IC_{50} s for MDSA and compound 2 generated from dose inhibition assays with DFCT1 were used. L2 cells were infected with *C. trachomatis* D or *C. muridarum* in the presence of 2.12 mM MDSA, 28.4 μ M compound 2, or 28.4 μ M compound 1 (IC_{80} of compound 2). At 44 hpi, EBs were harvested and processed, and the titers were determined as described above.

For time course assays, L2 cells were infected with 8,000 (MOI, 0.05) or 80 (MOI, 5×10^{-4}) EBs (DFCT1) and then treated with either MDSA or compound 2 at various time points (indicated in the figures) postinfection and then harvested at 48 hpi. For inhibitor removal assays, the infections were carried out in the presence of MDSA or compound 2 and subsequently removed at 2, 12, and 24 hpi by washing with 200 μ l PBS 3 times followed by addition of medium. At 48 or 60 hpi, EBs were harvested and then assessed for progeny levels by using the IFU assay. The relative IFU produced represents samples at 48 or 60 hpi over which treatment or removal was conducted versus that for DMSO. For pretreatment experiments, DFCT1 was incubated in infection medium supplemented with MDSA or compound 2 for 1 h on ice and then directly added to the well containing L2 cells. Following centrifugation (1 hpi), the medium was either left in the well or replaced with prewarmed medium without inhibitors. EBs were harvested at 44 hpi and the titer was determined using the IFU assay. The relative IFU produced represents samples 44 hpi versus that for the matched DMSO control.

To assess the effect of inhibitors on host cell processes during early infection, confluent L2 monolayers were pretreated with 2.12 mM MDSA, 28.4 μ M compound 1, or 28.4 μ M compound 2 for 4 h at 37°C with 5% CO_2 . The cells were then washed 3 times with 200 μ l PBS and infected with DFCT1. EBs were harvested at 40 hpi and assessed for progeny production using the IFU assay.

For inclusion area analysis, L2 cells were grown on glass coverslips in 24-well plates and infected with DFCT1, *C. trachomatis* D, or *C. muridarum* in the presence of 2.12 mM MDSA, 28.4 μ M compound 1, or 28.4 μ M compound 2. At 40 hpi, cell monolayers were washed 3 times with PBS and fixed with a 3% paraformaldehyde (PFA) and 3% sucrose solution. DFCT1-infected cells were washed with PBS and mounted on microscope slides. *C. trachomatis* D- and *C. muridarum*-infected cells were permeabilized with 0.2% Triton X-100, incubated with Pathfinder reagent for 30 min at 37°C with 5% CO_2 , and rinsed 3 times with distilled water (dH_2O). All coverslips were mounted using the ProLong antifade reagent. Images were taken under $\times 630$ magnification with oil using a Leica DM4000B fluorescence microscope outfitted with a QClick camera and QCapture software (QImaging), and the areas of inclusions were measured using ImageJ (<https://imagej.nih.gov/ij/>). Representative images were processed using the Velocity software (PerkinElmer).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00419-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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