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Lipopolysaccharide-Binding Alkylpolyamine DS-96 Inhibits *Chlamydia trachomatis* Infection by Blocking Attachment and Entry

Ichie Osaka, P. Scott Hefty

Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas, USA

Vaginally delivered microbicides are being developed to offer women self-initiated protection against transmission of sexually transmitted infections such as *Chlamydia trachomatis*. A small molecule, DS-96, rationally designed for high affinity to *Escherichia coli* lipid A, was previously demonstrated to bind and neutralize lipopolysaccharide (LPS) from a wide variety of Gram-negative bacteria (D. Sil et al., Antimicrob. Agents Chemother. 51:2811–2819, 2007, doi:10.1128/AAC.00200-07). Aside from the lack of the repeating O antigen, chlamydial lipooligosaccharide (LOS) shares general molecular architecture features with *E. coli* LPS. Importantly, the portion of lipid A where the interaction with DS-96 is expected to take place is well conserved between the two organisms, leading to the hypothesis that DS-96 inhibits *Chlamydia* infection by binding to LOS and compromising the function. In this study, antichlamydial activity of DS-96 was examined in cell culture. DS-96 inhibited the intercellular growth of *Chlamydia* in a dose-dependent manner and offered a high level of inhibition at a relatively low concentration (8 μM). The data also revealed that infectious elementary bodies (EBs) were predominantly blocked at the attachment step, as indicated by the reduced number of EBs associated with the host cell surface following pretreatment. Of those EBs that were capable of attachment, the vast majority was unable to gain entry into the host cell. Inhibition of EB attachment and entry by DS-96 suggests that *Chlamydia* LOS is critical to these processes during the developmental cycle. Importantly, given the low association of host toxicity previously reported by Sil et al., DS-96 is expected to perform well in animal studies as an active antichlamydial compound in a vaginal microbicide.

With over 1.3 million new cases reported each year, infection due to *Chlamydia trachomatis* is the most commonly reported infection in the United States (1). Although *C. trachomatis* infections are highly treatable with appropriate antibiotics upon detection, the vast majority (70 to 90%) of infections in women are undiagnosed due to the high asymptomatic rate and progress untreated (2). Untreated *C. trachomatis* infections can result in severe long-term consequences in women, including pelvic inflammatory disease (PID), ectopic pregnancy, and infertility (3). In addition, *C. trachomatis* infections have been linked to increased risks of acquisition and transmission of HIV (4). These concerns associated with *C. trachomatis* infection highlight the urgent need for novel preventative strategies; however, despite ongoing efforts, protective vaccines against *Chlamydia* currently are unavailable for clinical use. In the absence of effective vaccines, one alternative approach for prevention is the use of vaginally delivered topical microbicides, which offer the possibility of a female-controlled strategy for the prevention of sexually transmitted infections at the time of initial exposure.

Blocking infection in an early stage of pathogenicity (i.e., adherence and entry) before bacteria gain access to the host is a desirable preventative approach, as often evidenced in the efforts to produce sterile immunity in vaccine development. Bacterial surface molecules are potential targets for this purpose, because they often play essential roles in establishing a productive infection. One such molecule is lipopolysaccharide (LPS) of Gram-negative bacteria. LPS is a major constituent of the outer membrane and plays a crucial role for the survival of the organisms by maintaining membrane integrity and providing a permeability barrier (5–7). LPS has also been suggested to play a role in bacterial adhesion as well as being a prominent virulence-determining factor for several Gram-negative organisms, and it may be a target molecule in the development of antimicrobial agents (8–11).

A small molecule, DS-96, a synthetic alkylpolyamine, was previously developed to neutralize the endotoxicity of LPS in the pathogenesis of Gram-negative septic shock (Fig. 1A) (12). The molecule was rationally designed based on a nuclear magnetic resonance (NMR)-derived model of the interaction between *Escherichia coli* LPS and the antibiotic polymyxin B (PMB) (12–15). The cationic polypeptide antibiotic PMB is well known for its ability to bind to and neutralize the anionic lipid A moiety, which elicits a strong immune response when present in systemic circulation (16). The pharmacophore necessary for optimum binding and neutralization of LPS was determined through the model and used to synthesize various polycationic amphiphiles with a spermine backbone, and eventually it led to the discovery of a novel alkylpolyamine, DS-96 (12). In their study, Sil et al. demonstrated that DS-96 was capable of binding to LPS with high affinity and neutralized LPS endotoxicity of a variety of Gram-negative bacteria with potency indistinguishable from that of PMB (12). Importantly, daily administration of DS-96 at concentrations 10-fold higher than the fully protective dose resulted in no detectable toxicity in a mouse study (12). In *Chlamydia*, PMB treatment of the organism has been demonstrated to significantly decrease infectivity in cell culture infection (17–19) and also to impair the bacterial membrane integrity (20). Despite its effectiveness, clinical

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Address correspondence to P. Scott Hefty, pshefty@ku.edu.

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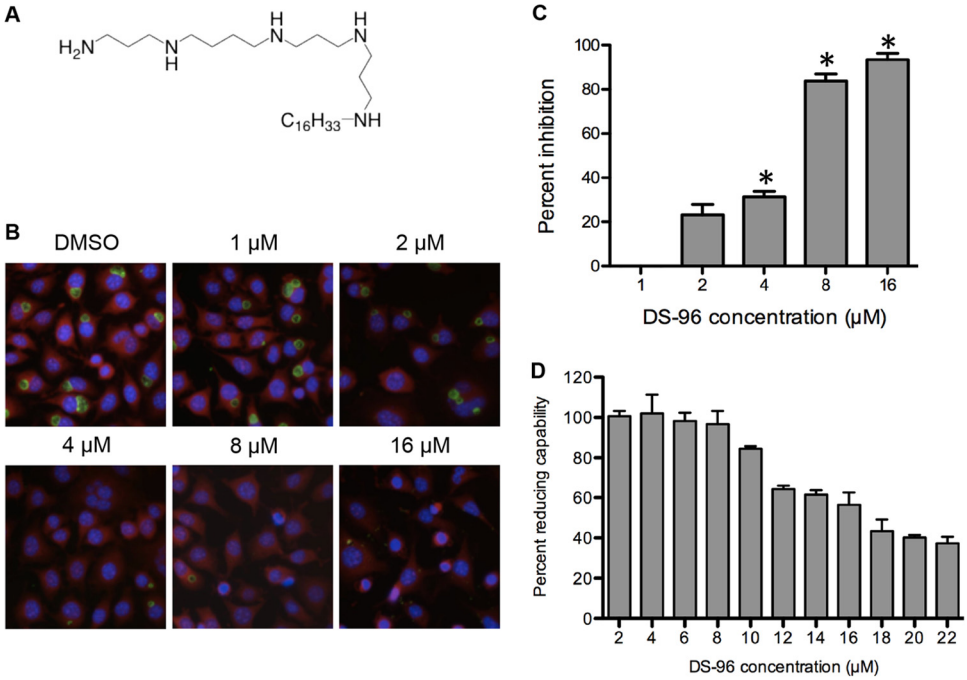

FIG 1 DS-96 exhibits inhibition of *C. trachomatis* infection in a dose-dependent manner. (A) Chemical structure of DS-96. (B) *C. trachomatis* EBs were treated with various concentrations of DS-96 (0 to 16 μM) or DMSO (mock treatment) for an hour prior to infection. L929 cells were infected with pretreated EBs and cultured for 24 h before fixation and immunostaining. *Chlamydia* was stained with anti-MOMP antibody (green), and host cells were counterstained with DAPI (nuclei; blue) and Evan's blue (cytoplasm; red). (C) Inhibition of inclusion formation among the samples treated with various concentrations of DS-96 was quantified 24 hpi using the automated enumeration method iBAChIE (32). Percent inhibition was determined based on the number of inclusion-positive cells in DS-96-treated samples relative to DMSO-treated samples. Error bars indicate standard deviations from the triplicate samples. The concentration at which 50% of infection is inhibited (IC_{50}) was calculated to be 5.1 μM using a linear regression analysis in the concentration range of 1 to 8 μM. An asterisk indicates a significant difference from the untreated control ($P < 0.001$ by Student's *t* test). (D) Cytotoxic effect of DS-96 on L929 cells was examined using a redox indicator, alamarBlue. The concentration of DS-96 that decreased the reducing capability to 50% of that of DMSO-treated cells (CC_{50}) was calculated to be 17.5 μM using a linear regression analysis in the concentration range of 6 to 22 μM.

use of unmodified PMB is limited due to the associated side effects on the eukaryotic hosts (21).

C. trachomatis, like many other nonenteric mucosal pathogens, possesses lipooligosaccharide (LOS) rather than LPS in its outer membrane. Chlamydial LOS differs from a typical LPS in that it consists of a trisaccharide core of 3-deoxy-D-manno-2-octulopyranosylonic acid (Kdo) rather than disaccharide in LPS, and it lacks the repeating O-antigen polysaccharide side chain, similar to rough enterobacterial LPS (22). Nonetheless, LOS and LPS share a conserved bisphosphorylated glucosamine backbone of lipid A at which interaction with PMB takes places. In contrast to enteric bacteria, chlamydial lipid A has been shown to exhibit low endotoxicity, reportedly due to the lower number of hydrocarbons and the longer chain length (23, 24). In addition, while LPS is an important structural component for the membrane integrity of Gram-negative bacteria, the outer membrane of the infectious, extracellular form of *Chlamydia* is highly disulfide cross-linking of cysteine-rich proteins, which is expected to provide structural rigidity and stability (25, 26).

While numerous studies have reported on the antigenic and immunogenic properties of *Chlamydia* LOS, knowledge concerning the biological functions of LOS in the virulence-defining biphasic developmental cycle and pathogenesis of *Chlamydia* is limited. The developmental cycle of these obligate intracellular bacteria begins with the attachment of the infectious but metabolically inert elementary body (EB) to the surface of nonphagocytic epithelial cells. This attachment is a two-step process involving the initial reversible electrostatic interaction with glycosaminoglycans followed by a more specific irreversible binding to putative cell surface receptors (27). Following attachment, the EB induces its own uptake by initiating a signal transduction cascade of the host cell that leads to recruitment and reorganization of the actin cytoskeleton at the site of attachment (28). Once inside the host cell, the EB converts into a larger metabolically active reticulate body (RB) which replicates by binary fission within a membrane-bound vacuole, termed inclusion. After several rounds of replication, RBs asynchronously transform into EBs and are released by host cell lysis or extrusion (29). Recently, Nguyen et al. used a chemical inhibitor of lipid A biosynthesis to demonstrate the crucial role of chlamydial LOS in proper conversion of RBs into infectious EBs (30). Interestingly, inhibition of LOS synthesis had little effect on RB replication, highlighting the importance of LOS in EB function. The biological significance of LOS during early infectious stages of the developmental cycle, such as binding and entry, remains to be elucidated.

Given the demonstrated high affinity of DS-96 for LPS of other Gram-negative bacteria and conserved structural features between LPS and chlamydial LOS, it was hypothesized that DS-96 inhibits *Chlamydia* infection by binding to the lipid A moiety and compromising the function of LOS that is crucial during the developmental cycle. To study this hypothesis, the inhibitory activity

of DS-96 during various stages of *C. trachomatis* infection was examined in cell culture.

MATERIALS AND METHODS

Bacteria and cell culture. *C. trachomatis* lymphogranuloma venereum (LGV) serovar L2/434/Bu EBs were purified from infected L929 cells using a 30% Renografin density gradient and stored in sucrose-phosphate-glutamate (SPG) at −80°C until use, as previously described (31). L929 cells were routinely cultured in RPMI 1640 culture medium (Mediatech, Inc., Manassas, VA) supplemented with 5% fetal bovine serum (FBS) (Thermo Fisher Scientific, Liverpool, NY) and 10 μg/ml gentamicin (MP Biomedicals, Santa Ana, CA) at 37°C in a humidified atmosphere of 5% CO_2. *C. trachomatis* serovar D/UW-3/Cx was isolated from infected HeLa 229 cells, and *C. muridarum* strain Nigg was purified from infected L929 cells as described previously (31).

Compound preparation. DS-96 was a kind gift from Sunil David (University of Kansas) and was obtained in powder form. The compound was dissolved in 100% dimethylsulfoxide (DMSO) to a stock concentration of 1 mM and stored at 4°C. Heparan sulfate (HS; Sigma-Aldrich, St. Louis, MO) was obtained in powder form, dissolved in deionized water to a stock concentration of 500 μg/ml, and stored at −80°C.

alamarBlue cytotoxicity assay. L929 mouse fibroblast cells were plated in a 96-well plate (Bioexpress, Kaysville, UT) at a density of 8×10^4 cells/ml, 200 μl/well, and incubated overnight. DS-96 was serially diluted in Hanks' buffered salt solution (HBSS; Mediatech, Inc., Holly Hill, FL) and incubated for 2 h at room temperature. Following the incubation period, cells were washed once with HBSS and supplemented with RPMI 1640 culture medium without phenol red, and 10% (vol/vol) alamarBlue reagent (Life Technologies, Grand Island, NY) was added. The samples were incubated at 37°C for 24 h. After 24 h, the absorbance was measured in a PowerWave microplate spectrophotometer (BioTek, Winooski, VT) at 570 nm (reduced) and 600 nm (oxidized) with KC^4 data collection and analysis software (BioTek, Winooski, VT). The results were expressed as the percentage of alamarBlue reduced by cells in the presence of DS-96 relative to alamarBlue reduced by control cells in the absence of the compound, using a formula provided by the manufacturer: $[(\varepsilon_{ox})\lambda_2 A\lambda_1 - (\varepsilon_{ox})\lambda_1 A\lambda_2]/[(\varepsilon_{ox})\lambda_2 A'\lambda_1 - (\varepsilon_{ox})\lambda_1 A'\lambda_2] \times 100$, where ε_{ox} is the molar extinction coefficient of the alamarBlue oxidized form, A is the absorbance of test wells, A′ is the absorbance of the positive-control well (untreated), λ_1 is 570 nm, and λ_2 is 600 nm. A linear regression equation ($y = -4.1979x - 123.51$, where y is viability of host cells and x is DS-96 concentration) was generated in the concentration range of 6 to 22 μM using Excel. The 50% cytotoxic concentration (CC_{50}) was calculated using this equation.

***Chlamydia* infection and inhibition assays.** Purified *C. trachomatis* L2 EBs were diluted to the appropriate concentrations in HBSS. DS-96 or DMSO, as a mock treatment control, was diluted in either HBSS alone or HBSS containing diluted EBs to the appropriate concentrations according to treatment type. The mixtures of bacteria and compound were incubated for an hour at room temperature. Following this incubation, the mixtures were added to a monolayer of cells. Cells were incubated at room temperature for 2 h. For the centrifugation-assisted infection, the samples were centrifuged at 900 × *g* for 1 h. After the infection period, cells were washed once with HBSS, fresh culture medium was added, and the solution was incubated at 37°C in an atmosphere of 5% CO_2. For the treatment of host cells, diluted DS-96 or DMSO in HBSS containing no bacteria was transferred to L929 cell monolayers and incubated for an hour at room temperature. Following the incubation period, the compound was removed and cells were washed before bacteria were added. For other treatment conditions, bacteria were pretreated or DS-96 was added to culture medium at the indicated time period. Twenty-four hours postinfection (hpi), cells were washed once with HBSS and fixed with 100% methanol for 10 min at room temperature. Samples were kept in phosphate-buffered saline (PBS) at 4°C until the time of analysis. To calculate the 50% inhibitory concentration (IC_{50}) for the dose-dependent inhibition analysis (Fig. 1C), a linear regression equation ($y = 12.624x - 16.326$, where y is inhibition level and x is the DS-96 concentration) was generated in the concentration range of 1 to 8 μM using Excel.

Immunofluorescence microscopy. Following fixation, samples were stained with a MicroTrack *C. trachomatis* culture confirmation test (Syva Co., Palo Alto, CA) diluted to 1:40 in PBS for an hour in the dark, followed by a 5-min stain with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS. DAPI was removed, and 90% (vol/vol) glycerol in PBS was added to prevent rapid fading of fluorescence during analysis. For manual analysis, plates were stored in the dark at 4°C until imaging. Samples were visualized using an Olympus ix71 inverted fluorescence microscope (Center Valley, PA) with a 40× objective and 0.55 numerical aperture. Images were captured using a QImaging QICAM digital camera, 12-bit Mono Fast 1394 cooled camera, and QCapture Suite software, version 3.1.3.5 (QImaging Corp., Surrey, BC, Canada). Image-based automated quantification using CellProfier and CellProfiler Analyst (iBAChIE) was performed as described previously (32).

Antibody staining of *C. trachomatis* EBs. For staining of *C. trachomatis* EBs with anti-LOS antibody, EBs were incubated with 8 μM DS-96 or the equivalent dilution of DMSO as a control at room temperature for pretreatment. After 1 h, 300 μl of the pretreated bacteria was transferred into 8-well μ-chamber slides (ibidi, Verona, WI). Bacteria were allowed to settle and adhere to the bottom of the plate at room temperature for 1 h. The liquid was aspirated, and bacterial samples were fixed with 100% methanol for 10 min. For immunofluorescence staining, one set of samples was stained with monoclonal anti-LOS antibody (1683; ViroStat) diluted 1:500 in PBS at room temperature for 1 h, washed with PBS, incubated with secondary goat anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, CA), and counterstained with 1 μg/ml DAPI in PBS for 5 min. A parallel set of samples was stained with primary goat anti-MOMP antibody (1621; ViroStat) diluted 1:500 in PBS for an hour, washed with PBS, incubated with secondary donkey anti-goat Alexa Fluor 568 (Invitrogen, Carlsbad, CA) at 1:1,000 dilution for an hour, and stained for 5 min with 1 μg/ml DAPI in PBS. Samples were viewed with an Olympus ix71 inverted fluorescence microscope (Center Valley, PA) with a 40× objective and 0.55 numerical aperture. Images were captured using a QImaging QICAM digital camera, 12-bit Mono Fast 1394 cooled camera, and QCapture Suite software, version 3.1.3.5 (QImaging Corp., Surrey, BC, Canada).

Attachment assay. Purified *C. trachomatis* L2 EBs were diluted in HBSS, and DS-96 was added to a final concentration of 8 μM for pretreatment of the bacteria for an hour at room temperature. An equivalent dilution of DMSO mock treatment was used as a negative control, and 500 μg/ml HS was used as a positive control for inhibition of chlamydial attachment. L929 cells were infected with compound-treated EBs and incubated at 4°C for an hour. Following infection, cells were washed three times with cold HBSS to remove nonspecifically bound EBs, fixed with 100% methanol, and stained with a MicroTrack *C. trachomatis* culture confirmation kit. Images were captured using SlideBook (Intelligent Imaging Innovations, Inc., Denver, CO) software and an Olympus IX81 inverted microscope (Center Valley, PA) equipped with a 1.45-numerical-aperture, oil-immersion 100× objective and a Hamamatsu electron microscope charge-coupled device (EM-CCD) camera.

Entry assay. Purified *C. trachomatis* L2 EBs were diluted in HBSS and incubated with 8 μM DS-96 at room temperature for an hour for pretreatment. An equivalent dilution of DMSO mock treatment was used as a negative control. The inocula were added to L929 cells and incubated for 2 h at room temperature. Following the incubation period, the inocula were removed, cells were washed, and fresh culture media were added. Infected cells were incubated at 37°C for 3 h to allow sufficient time for bacterial attachment and entry. At 3 hpi, culture medium was removed and cells were washed once with PBS. Differential staining was performed to distinguish the bacteria that remained extracellular from those that gained entry into the host cells. To stain the extracellular bacteria, the cells were incubated with primary goat anti-MOMP antibody (1621; ViroStat)

diluted 1:500 in PBS for an hour at room temperature prior to permeabilization of host cells. The cells were washed three times with PBS, fixed, and permeabilized with 100% methanol for 10 min at room temperature. The cells were washed once with PBS to remove methanol and incubated with secondary donkey anti-goat Alexa Fluor 568 (Invitrogen, Carlsbad, CA) at a 1:1,000 dilution for an hour. Following three washes with PBS, the cells were incubated with primary mouse anti-*Chlamydia* antibody (ab21019; Abcam) diluted 1:500 for an hour at room temperature, washed three times with PBS, and incubated with secondary goat anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Following three washes with PBS, cells were incubated with 1 μg/ml DAPI in PBS for 5 min to label host cell nuclei. Images were captured using SlideBook (Intelligent Imaging Innovations, Inc., Denver, CO) software and an Olympus IX81 inverted microscope (Center Valley, PA) equipped with a 1.45-numerical-aperture, oil-immersion 100× objective and a Hamamatsu EM-CCD camera.

RESULTS

DS-96 inhibits *Chlamydia* infection in a dose-dependent manner. To begin examining the effect of DS-96 on *Chlamydia* infection, *C. trachomatis* L2 EBs were pretreated with various concentrations of the compound, ranging from 0 to 16 μM. The standard procedure for *Chlamydia* infection of cell culture consists of a 2-h infection of eukaryotic cell monolayers at room temperature to facilitate attachment. This is followed by washing to remove unbound bacteria and then placement at 37°C to fully enable chlamydial entry. In the inhibition assay, EBs were preincubated with DS-96 for an hour before addition to cell monolayers. This was to allow sufficient time for the interaction to take place between DS-96 and the target molecule, chlamydial LOS, prior to exposure of EBs to host cells. Exposure of infectious EBs to concentrations of DS-96 greater than 1 μM resulted in inhibition of chlamydial growth, as indicated by a decrease in the percentage of host cells containing inclusions after 24 h (Fig. 1B and C). Over a concentration range of 0 to 16 μM, a dose-dependent inhibition of infection was observed with greater than 80% inhibition at 8 μM relative to the DMSO mock-treated culture. More than a doubling in the number of infected cells was observed as the DS-96 concentration was reduced from 8 to 4 μM (83.9% and 31.3% inhibition, respectively). The 50% inhibitory concentration (IC_{50}) was calculated to be 5.3 μM using a linear regression analysis in the concentration range of 1 to 8 μM. Interestingly, the sizes of individual inclusions after 4 μM DS-96 treatment were much smaller than those of DMSO mock-treated sample (Fig. 1B), although this was not quantified. This observation may be explained by the ability of multiple EBs to infect a single cell and form much larger inclusions, suggesting that fewer bacteria, on average, infected individual cells as a result of treatment with 4 μM DS-96. In concert with this hypothesis, inhibition levels observed between treatments with 2 and 4 μM DS-96 are similar; however, the inclusions at 2 μM are larger and similar in size to those from DMSO-treated samples. An alternative hypothesis is that DS-96 is slowing *Chlamydia* entry and/or developmental progression (e.g., replication), resulting in smaller inclusions at certain concentrations. As *Chlamydia* is an obligate intracellular organism, it is crucial to ensure that the observed inhibitory effect is due to the direct action of the compound on *Chlamydia* rather than an indirect cytotoxic effect on the host cell. Although no detectable toxicity was reported in the murine model (12), DS-96 has not been reported previously in cell culture. To evaluate the possible cytotoxicity of DS-96, a resazurin-based assay (alamarBlue) was used to quantitatively assess host cell metabolic activity following exposure to DS-96 (Fig. 1D). This assay indicated that no significant cytotoxicity was observed following exposure of DS-96 concentrations up to 8 μM. At 16 μM, the reducing capability of the host cell decreased to approximately 60% of the untreated cells, which is consistent with host cell rounding evident at 16 μM treatment by microscopy (Fig. 1B). The 50% cytotoxic concentration (CC_{50}) was calculated to be 17.0 μM using a linear regression analysis in the concentration range of 8 to 22 μM. Based on the inhibitory effect and absence of cytotoxicity at 8 μM, this concentration was used for subsequent studies.

DS-96 blocks the early events of the chlamydial developmental cycle. In order to better understand the underlying inhibitory mechanism of DS-96, infected samples were treated with DS-96 at different time periods during the developmental cycle, and *Chlamydia* growth was examined. When infected cells were treated with DS-96 after bacteria were allowed to bind and begin entering host cells, no significant effect on bacterial growth was observed (data not shown). This suggests that DS-96 is membrane impermeable and, consequently, does not have access to the bacteria once they are internalized into host cells. It could also indicate that LOS is not essential once *Chlamydia* has gained access to the cell and begun replicating. Therefore, the effect of DS-96 treatment prior to the internalization of EBs was examined further (Fig. 2). The treatment conditions included pretreatment of EBs for an hour prior to addition to host cells, coincubation of DS-96 and EBs with the host cell, and addition of the compound after EBs were allowed to incubate with host cells (Fig. 2A). As *Chlamydia* relies on the host cell for survival and replication, it was also important to ensure that DS-96 exposure to the host cell does not perturb the cell and indirectly affect *Chlamydia* infectious processes. Therefore, the host cells were pretreated with DS-96 for an hour prior to incubation with the bacteria. As expected, the compound was most effective in inhibiting *Chlamydia* infection when bacteria were pretreated prior to infection (95.5%) (Fig. 2B). When DS-96 and the bacteria were simultaneously added to the cells (coincubation), the level of inhibition was decreased to 55.4%, indicating that the compound requires sufficient time to establish an interaction with its target. When compound was added after EBs had been incubated with host cells for 2 h, DS-96 exhibited no inhibitory effect on *Chlamydia* growth, supporting the earlier observation that the compound may be unable to cross the eukaryotic cell membrane. Pretreatment of host cells prior to infection resulted in only a minimum level of inhibition (8.6%). Together, these observations support that DS-96 blocks *Chlamydia* infection during the early stages of the developmental cycle, specifically prior to chlamydial entry into the host cell.

DS-96 treatment impairs *Chlamydia* attachment and entry. To determine more specifically which steps of the developmental cycle were blocked by DS-96, attachment of *Chlamydia* to the host cell was examined. *C. trachomatis* EBs were pretreated with DS-96 for an hour prior to infection and then given sufficient time (1 h) to adhere to L929 cells at 4°C. After inoculum was removed and cells were washed to remove the unbound bacteria, adherence of EBs was qualitatively examined by immunofluorescence microscopy (Fig. 3). Heparan sulfate, as a highly negatively charged polysaccharide, is known to block *Chlamydia* adhesion to the target cell *in vitro* by charge repulsion (33–37) and served as a positive control for an inhibitor of *Chlamydia* adhesion in this study. In the untreated sample (DMSO), an average of 15 to 20 EBs appeared to be associated with the host cell surface (Fig. 3). As ex-

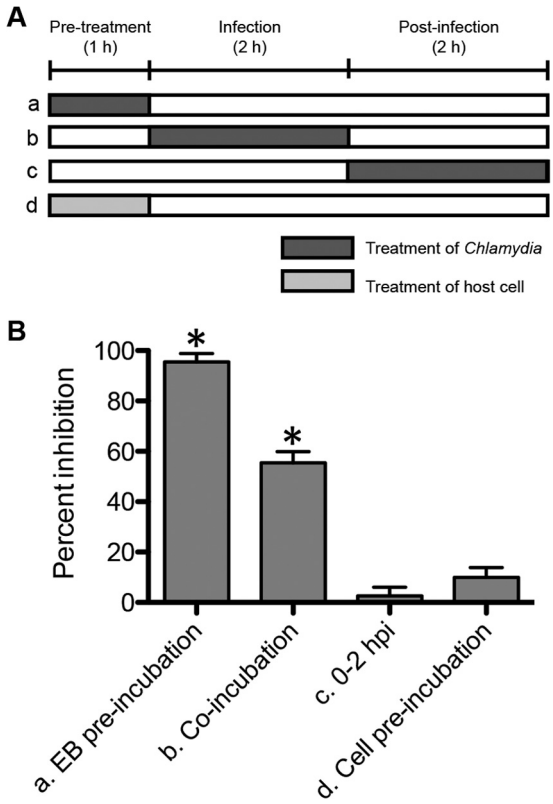

FIG 2 DS-96 inhibits initial stages of *Chlamydia* infection through interaction with EBs rather than host cells. Infection levels were compared among various treatment conditions of DS-96 (8 μM). (A) Treatment conditions. Row a, pretreatment of bacteria; row b, coincubation of bacteria and host cell; row c, addition of DS-96 to the infected culture 0 to 2 hpi; row d, pretreatment of the host cell. (B) Inhibition of inclusion formation relative to mock-treated samples was quantified for each treatment condition 24 hpi using iBAChIE (32). The percentage of infection was calculated based on the number of inclusion-positive cells in DS-96-treated samples relative to DMSO-treated samples. Error bars indicate standard deviations from triplicate samples. Asterisks indicate levels were significantly different ($P < 0.001$ by Student's t test) from similarly DMSO-treated samples.

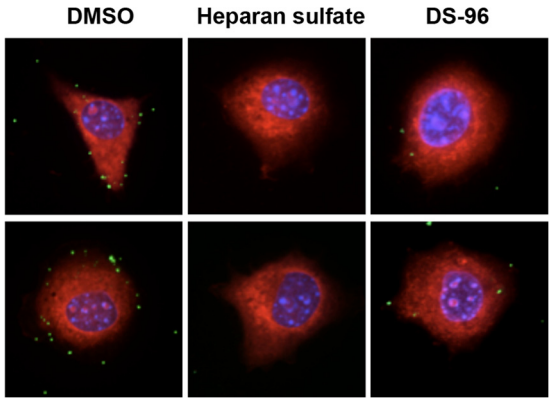

FIG 3 DS-96 blocks attachment of *C. trachomatis* EB host cell surface. Two representative L929 cells that were incubated with EBs were pretreated with DS-96 (8 μM) or mock treated (DMSO) for an hour at 4°C. Infected cultures were thoroughly washed to remove unbound bacteria, fixed with methanol, and stained with fluorescently labeled anti-MOMP (*Chlamydia*; green), Evan's blue (cytosol; red), and DAPI (nuclei; blue).

pected, bacterial adhesion was blocked when EBs were pretreated with heparan sulfate prior to infection. In samples pretreated with DS-96, inhibition was observed as a decrease in the number of EBs associated with the host cell. However, unlike that with heparan sulfate, DS-96 treatment did not result in complete prevention of bacterial adhesion; rather, the treatment decreased the number of bacteria bound to a single cell by approximately 70%, with 2 to 5 EBs per cell remaining bound to the cell surface. This observation was striking, given that 8 μM DS-96 consistently resulted in high levels of inhibition (greater than 85%) of inclusion formation throughout our study. This result indicated that the majority of EBs that remained bound to the host cell in the presence of the compound still was unable to form productive inclusions.

One possible explanation for this observation is that DS-96 blocked postattachment events in addition to bacterial adhesion. To investigate this possibility, entry of *Chlamydia* into the host cell was examined. Similar to the attachment assay, EBs pretreated with DS-96 or mock-treated (DMSO) EBs were allowed sufficient time (4 h) to attach and enter the host cell. Differential immunofluorescent staining was performed to discriminate between the internalized bacteria and those remaining on the surface of the host cell, and the samples were assessed by confocal microscopy (Fig. 4A). Two subsequent staining steps were performed before and after permeabilization of the host cell membrane to achieve labeling of the extracellular bacteria with red fluorophore and total bacteria with green fluorophore. In the representative images shown in Fig. 4A, the majority of EBs in mock-treated sample appeared green, indicating that these EBs had been internalized. When pretreated with DS-96, consistent with the observation in the attachment assay, bacterial adhesion was significantly decreased, as indicated by a decrease in the number of total EBs associated with the host cell. In contrast to the mock-treated sample, EBs associated with the host cell in DS-96-treated samples were costained and appeared yellow, indicating that these EBs remained extracellular. Quantification of the number of the extracellular and total EBs revealed that pretreatment of the bacteria with DS-96 resulted in a drastic decrease in the percentage of internalized EBs from 86.3% in mock-treated samples to 2.6% in DS-96 treated samples (Fig. 4B). These findings demonstrate that DS-96 blocks entry of *C. trachomatis* into host cells as well as blocking adherence.

The effect of DS-96 on centrifugation-assisted infection. Since the first report by Weiss and Dressler (38), it has been known that centrifugation of the chlamydial inoculum onto host cell monolayers enhances infectivity, although the degree of enhancement varies among different species and serovars. The means by which centrifugation enhances *Chlamydia* infectivity are not fully understood; however, centrifugation is thought to override binding and entry mechanisms and greatly enhance absorption of the bacteria (34). Interestingly, it has been reported that the inhibitory effect of neutralizing antibody and heparan sulfate against *Chlamydia* is overcome by centrifugation (39). To examine whether DS-96 was merely inhibiting binding and entry or also having a further effect, such as inactivation of EBs, an inhibition assay was performed with static and centrifugation-assisted infection (Fig. 5). In the presence of heparan sulfate, static infection resulted in 92.9% inhibition in *Chlamydia* growth. However, this inhibition was overcome when inoculation was facilitated by centrifugation (900 × *g* for 1 h). In comparison, when *C. trachomatis* EBs were

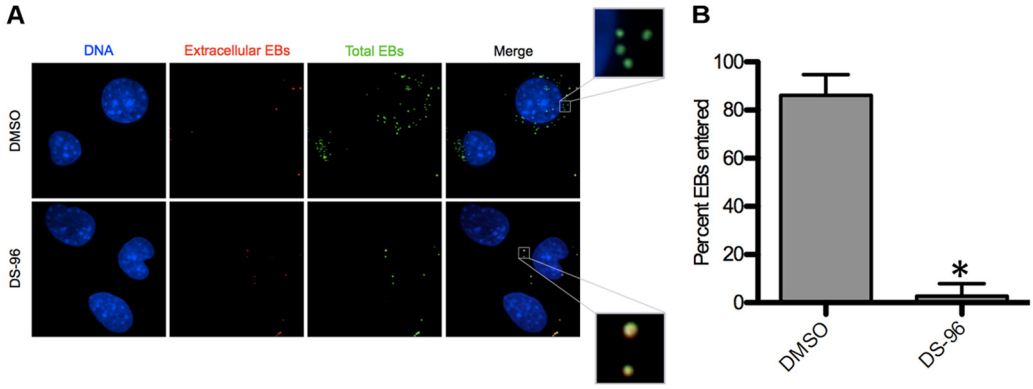

FIG 4 Entry of *C. trachomatis* EBs into the host cell is inhibited by DS-96. *C. trachomatis* EBs were allowed to attach and enter host cells. Differential immunofluorescent staining was performed 4 hpi to discriminate between the extracellular and internalized EBs. (A) Representative images of infected cells. Host cell surface-associated EBs were labeled with anti-MOMP antibody (red) prior to cell permeabilization, and total EBs (intracellular plus extracellular) were labeled with anti-*Chlamydia* antibody (green) after permeabilization. The inset shows the internalized bacteria (green) in a mock-treated sample and the extracellular bacteria (yellow) in a DS-96-treated sample. (B) Percentage of EBs internalized relative to total EBs was quantified. The asterisk indicates that levels measured were significantly different ($P < 0.001$ by Student's *t* test) from those of DMSO-treated samples.

treated with DS-96, a high level of inhibition (95.3%) was maintained through centrifugation-assisted infection. Further studies are needed to determine what postentry event(s) is perturbed by DS-96, or if infectious EBs are inactivated after exposure to the compound. Nevertheless, it is of interest that the bacteria were unable to progress through the developmental cycle even when the absorption was facilitated by centrifugation.

While *C. trachomatis* LGV strains can efficiently infect cultured mammalian cells, non-LGV strains generally require centrifugation for efficient infection in cell culture. LGV serovar L2 is a commonly used *C. trachomatis* laboratory strain due to the relative ease of cultivation and shorter developmental cycle. However, *Chlamydia trachomatis* serovars D, E, and F are more clinically relevant strains, since they are reported to be the most prevalent urogenital serovars worldwide (40). With the data revealing that the effect of DS-96 is maintained through centrifugation-assisted inoculation, an inhibition experiment was performed with *C. trachomatis* serovar D and the mouse pathogen *C. muridarum*, both of which are typically infected using a centrifugation-assisted procedure. Greater than 80% inhibition of the bacterial growth was observed for both *C. trachomatis* serovar D and *C. muridarum* at 4 μM, a concentration lower than that needed for equivalent inhibition of the *C. trachomatis* LGV strain (Fig. 6).

Treatment of EBs by DS-96 alters the binding of antibody against *Chlamydia* LOS. Considering the rational design of the compound, along with the structural similarities between *E. coli* and *Chlamydia* lipid A, it is highly anticipated that the observed inhibitory effect of DS-96 in *Chlamydia* infection is through the interaction with LOS. In an attempt to demonstrate this interaction, the immunofluorescent staining pattern of the bacteria by monoclonal antibody specific to chlamydial LOS was examined by confocal microscopy (Fig. 7). It was anticipated that binding of DS-96 to LOS could alter the antibody-binding epitope on LOS and consequently result in the absence of LOS staining. To perform this study, purified *C. trachomatis* EBs were pretreated with DS-96 or mock treated with DMSO as a control and allowed to adhere to the plastic bottom of a cell culture plate for an hour. Following methanol fixation, samples were stained with monoclonal antibody against LOS or MOMP as a control and counterstained with DAPI. When visualized by immunofluorescence mi-

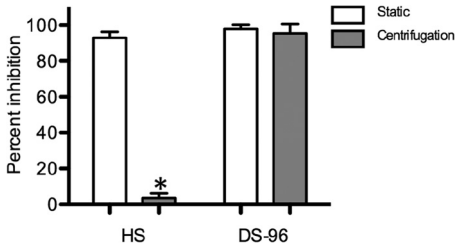

FIG 5 Inhibition by DS-96 is maintained after centrifugation. Following pretreatment of EBs with DS-96 (8 μM) or heparan sulfate (HS) for 1 h, infection was performed with or without centrifugation (900 × *g* for 1 h). Infected cultures were fixed with methanol, immunofluorescently stained, and analyzed for inhibition of inclusion formation 24 hpi using iBAChIE (32). Error bars indicate standard deviations from triplicate samples. The asterisk indicates that levels measured were significantly different ($P < 0.001$ by Student's *t* test) from those for DMSO-treated samples.

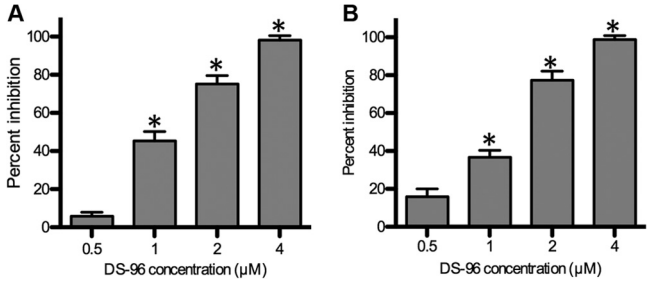

FIG 6 Inhibition of *C. trachomatis* serovar D and *C. muridarum*. Centrifugation-assisted infection was performed with *C. trachomatis* serovar D (A) and *C. muridarum* (B) in the presence of various concentrations of DS-96 and quantified 24 hpi using the automated enumeration method iBAChIE (32). Percent inhibition was counted based on the number of inclusion-positive cells in DS-96-treated samples relative to DMSO-treated samples. Error bars indicate standard deviations from the triplicate samples. The asterisk indicates that levels measured were significantly different ($P < 0.001$ by Student's *t* test) from those for DMSO-treated samples.

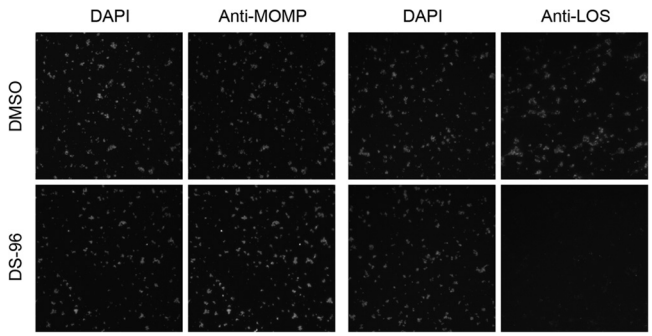

FIG 7 Pretreatment of EBs with DS-96 results in reduction of anti-LOS staining. Purified EBs were treated with DS-96 (8 μM) or DMSO and stained for LOS (A) or MOMP (B) following methanol fixation. Samples were counterstained with DAPI to visualize the total number of bacteria.

croscopy, anti-MOMP antibody stained the bacteria similarly to DAPI staining in both control and DS-96-treated samples. In contrast, while anti-LOS staining was consistent with DAPI staining in the control sample, staining by anti-LOS was completely absent from the sample treated with DS-96. Although this does not provide direct evidence for an interaction of DS-96 with LOS, it suggests that DS-96 treatment of *Chlamydia* results in altering or possibly masking the epitope recognized by the anti-LOS antibody used in this study.

The effect of phosphate on the activity of DS-96. HBSS is a common buffer in *Chlamydia* infection in cell culture, and it was used throughout this study. However, SPG generally has been accepted as a suitable transport and storage medium for *Chlamydia*. More importantly, it is commonly used in animal challenge studies. Before considering challenge studies in an animal model, the inhibition study was repeated using SPG in place of HBSS. Surprisingly, a complete lack of inhibition by DS-96 (8 μM) was observed when the cell culture inhibition assay was repeated with SPG (data not shown). To determine which component of SPG (220 mM sucrose, 8.5 mM $NaHPO_4$, 3.7 mM KH_2PO_4, 4.9 mM L-glutamic acid) was accountable for the impairment of DS-96 activity against *Chlamydia*, each constituent was added separately to HBSS, and the effect on DS-96 activity was observed in the cell culture infection (Fig. 8). In the presence of sucrose or glutamic acid, the inhibitory activity of DS-96 remained comparable to that of the control (HBSS alone), resulting in a high level of inhibition. In contrast, when $NaHPO_4$ and KH_2PO_4 were added together to HBSS, the level of infection was similar to that of the untreated sample, indicating that the activity of DS-96 against *Chlamydia* infection was impaired in the presence of these components. Considering that the molecular interaction between DS-96 and *E. coli* LPS is expected to involve formation of salt bridges between the two phosphates on lipid A and the amine groups on DS-96 (12, 13), it is reasonable to speculate that free phosphate from $NaHPO_4$ and KH_2PO_4 competitively interact with the amine for the salt bridge formation essential for the activity of DS-96. Although phosphate is commonly used in buffer solutions and is present in HBSS, the concentration of phosphate is approximately 15 times higher in SPG than in HBSS, which suggests that the phosphate component of the buffer, at high concentration, outcompetes lipid A for the interaction with DS-96. To assist in supporting this possibility, the experiment was repeated using PBS, which contains a phosphate concentration comparable to that of SPG. The data revealed that PBS blocked the activity of DS-96 similarly to SPG, supporting that phosphate is the component responsible for impairment of the activity of DS-96. Importantly, this observation gives further evidence that DS-96 is indeed interacting with *Chlamydia* lipid A in a manner similar to its interaction with *E. coli* lipid A.

DISCUSSION

In search for an active component effective against *Chlamydia* infection with potential to be considered for a vaginally delivered microbicide, the LPS binding molecule DS-96 was examined for inhibitory properties. Numerous observations within this study support that DS-96 inhibits *Chlamydia* infection of cell culture. A dose-dependent inhibition was observed with greater than 80% protection at 8 μM (Fig. 1B and C). Attachment to the host cell appears to be the stage of infection where EBs were predominantly inhibited by DS-96 treatment (Fig. 3), although entry and possibly postentry events were also affected by the treatment (Fig. 4 and 5). The possible effect on postentry events is highlighted by the inhibition observed following centrifugation-assisted infection, which is thought to override binding and entry mechanisms. This study also provided indirect evidence for the specificity of DS-96 interaction with *Chlamydia* LOS, which was demonstrated by the absence of immunofluorescence detection of LOS following DS-96 treatment (Fig. 7), as well as the effect of high phosphate concentration on DS-96 activity (Fig. 8), which is discussed further be-

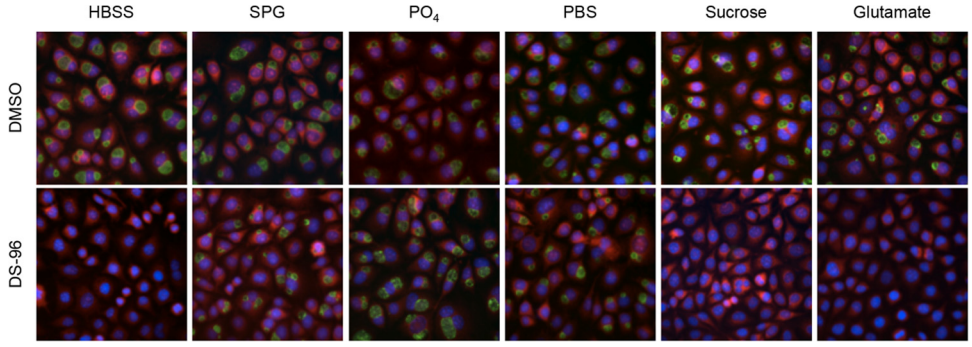

FIG 8 Phosphate impairs activity of DS-96. The inhibition assay was performed with HBSS supplemented with each constituent of SPG. Samples were fixed with methanol 24 hpi. *Chlamydia* was stained with anti-MOMP antibody (*Chlamydia*; green), and host cells were counterstained with DAPI (nuclei; blue) and Evan's blue (cytoplasm; red).

low. Together, these data support that DS-96 is an effective inhibitor of *Chlamydia* infection, albeit within tissue culture cells. A potential limitation for the clinical use of DS-96 was the observed decrease in inhibitory capability (~40%) when compound was added at the same time as infection instead of as a pretreatment (Fig. 2D). While this is of concern, cell toxicity prevented the addition and evaluation of higher concentrations of DS-96 during coincubation. This concern is tempered by observations in the original animal studies evaluating the utility and toxicity of DS-96 for sepsis (12). During these studies, high micromolar (>500 μM) concentrations of DS-96 were employed and toxicity was not detectable. This observation supports that higher concentrations of DS-96 should be tolerated during combined treatment and infection in mice, possibly achieving much higher protection. However, it is important to point out that previous toxicity studies (12) were not performed on mucosal surfaces, including vaginal exposure, that will need to be performed before clinical application can be considered.

In addition to the commonly used laboratory strain *C. trachomatis* LGV, we tested the activity of DS-96 against *C. trachomatis* serovar D and *C. muridarum* (Fig. 6) as one of the most clinically prevalent serovars (41–43) and a mouse pathogen, respectively. Interestingly, lower doses of DS-96 were as effective against these strains as they were against LGV, indicating higher susceptibility. Although further studies, including animal challenge, will be required to provide more evidence, these observations suggest that serovar D and *C. muridarum* have greater requirements for LOS in their infectivity than serovar LGV. Considering that the structure of the lipid A glucosamine backbone is well conserved throughout Gram-negative bacteria (44), it is highly unlikely that the affinity of DS-96 toward lipid A varies among *Chlamydia* spp. However, the requirements for LOS in the establishment of productive infection may be different between various serovars and species of *Chlamydia* due to the differences in their tissue tropisms. The idea of differential requirements for infectivity between serovars and various species of *Chlamydia* is well accepted, especially for the specific ligand-receptor interactions during bacterial attachment and entry (27). In support of our findings, it has been reported that *C. trachomatis* serovar E was more susceptible to various LOS inhibitors than serovar LGV *in vitro* (19). Clearly, it will be critical to evaluate the efficacy of DS-96, and further developed chemical analogs, using clinically relevant serovars of *Chlamydia* in animal challenge studies.

To be a vaginal microbicide, the candidate inhibitors should have the ability to specifically target vaginal pathogens while having little deleterious effect on the normal vaginal flora. While total genomic analyses are providing a new appreciation of the vaginal microbial diversity, vaginal microbiota of healthy women typically is dominated by the hydrogen peroxide-producing Gram-positive *Lactobacillus* species, which is essential in maintaining vaginal homeostasis and providing the natural defense against pathogenic organisms (45–47). In this aspect, DS-96 designed specifically to target a virulence factor unique to Gram-negative bacteria has the potential to be effective against *Neisseria gonorrhea*, in addition to *C. trachomatis*, while having limited adverse effects on the normal vaginal flora dominated by Gram-positive *Lactobacillus* spp. While the application and utility of DS-96 for these infections is an encouraging possibility, a relatively high percentage (~30%) of women have bacterial vaginosis that can be dominated by LOS/LPS-containing Gram-negative bacteria. This high concentration of lipid A target could substantially decrease the availability of DS-96 introduced to prevent *Chlamydia* and *Neisseria* infections in this portion of the female population. Further complicating the challenge is the higher susceptibility of women with bacterial vaginosis to sexually transmitted diseases. An alternative view is that DS-96, or future chemical analogs, could be an effective addition in vaginal microbicides to treat bacterial vaginosis locally and assist in enhancing the balance of *Lactobacillus*. Future studies are certainly required to ascertain the clinical utility of DS-96 with these approaches.

Moreover, DS-96 not only is an attractive microbicide candidate for Gram-negative vaginal pathogens but also served in this study as a chemical biology tool to examine a role for LOS in *Chlamydia* biology. Despite the recent major advances in genetic systems (48–53), routine genetic manipulation of *Chlamydia* with classic techniques remains challenging. The use of inhibitors targeting specific virulence factors can substitute to facilitate the studies of this nature. Our data demonstrated that both attachment and entry processes of *C. trachomatis* were impaired in the presence of DS-96, which may reflect a role of LOS in early events during the developmental cycle, specifically binding and entry. Other studies have used *Chlamydia* LOS as a target for disruption of the developmental cycle, and various functions have been proposed (19, 30). Fadel and Eley postulated a general role of LOS in *Chlamydia* infectivity (19). They demonstrated that infection of human epithelial cells by *C. trachomatis* serovar LGV and serovar E was inhibited in the presence of purified LOS, PMB, antibody against *C. trachomatis* LOS, and synthetic Kdo (19). Similar to our study, a role of *Chlamydia* LOS in adherence was also suggested (19). More recently, Nguyen et al. examined the biological significance of *Chlamydia* LOS using LOS biosynthesis inhibitors (LpxC inhibitors) and proposed a crucial role of LOS in proper morphological transition of RBs to EBs (30). Their study revealed that a decreased number of progeny EBs was generated in the presence of LpxC inhibitors (30). They also reported that OmcB, a membrane protein specific to the EB surface, was poorly expressed in the absence of LOS (30). Our study extended the possible role of LOS to chlamydial attachment and entry steps, although the inhibitory effect may be indirect. Similar to the effect of LpxC inhibitors on the expression of OmcB, the interaction between DS-96 and LOS may disrupt other key surface molecules required for binding and entry. For instance, *Chlamydia* is known to promote entry into host cells by inducing host cell actin polymerization via injection of the type 3 secretion effector Tarp (54, 55). Considering the findings of Nguyen et al., it is possible that DS-96 treatment of LOS results in functional disruptions of other outer membrane surface molecules, such as type 3 secretion systems.

One of the unexpected but potentially revealing observations of this study was the blocking of DS-96 inhibitory effect by high phosphate concentrations. With an expectation to examine the efficacy of DS-96 *in vivo* in the near future, the inhibition experiments were repeated with SPG, a buffer commonly used in animal challenge models. To our surprise, the inhibitory effect by DS-96 was reversed in the presence of SPG (data not shown), and it was determined that phosphate was solely responsible for blocking the inhibitory activity of DS-96 (Fig. 8). The observation was further confirmed when PBS, a buffer containing phosphate at a concentration similar to that of SPG, also blocked DS-96 activity. This observation was particularly intriguing considering the molecular design of DS-96. Sil et al. reported the central pharmacophores,

which have been experimentally tested and defined for optimal recognition and neutralization of lipid A (12, 56). These pharmacophore properties include (i) two protonatable cationic functions ~14 Å apart that are necessary for simultaneous ionic interactions with the negatively charged phosphates on lipid A and (ii) appropriately positioned hydrophobic groups. Given that the ionic interaction with the phosphates on lipid A is a requisite for the compound activity, it is plausible to reason that deactivation of DS-96 is a result of the free phosphate in the buffer solution competitively binding to the amine groups on DS-96. This observation supports, albeit indirectly, the specificity of the interaction between DS-96 and *Chlamydia* lipid A. Furthermore, with the ultimate goal of developing vaginally delivered microbicide, formulation is a critical factor to be considered for the stability of the compound in the vaginal environment, where various factors may influence the activity of the compound. Deactivation of DS-96 by phosphate observed in this assay emphasizes the importance of compound formulation, especially given that the concentrations of inorganic ions, including phosphate, in the vaginal lumen are unclear (57). Nonetheless, considering its effectiveness against *C. trachomatis* infection *in vitro* along with its demonstrated low toxicity in a mouse model, it is expected that DS-96 will perform well as a microbicide candidate compound in future animal model studies.

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