

***IN VITRO* SUSCEPTIBILITY OF *CHLAMYDIA TRACHOMATIS* TO
LPS-BINDING POLYAMINES AND CELLULOSE ETHER POLYMERS:
TOWARDS THE DEVELOPMENT OF A MICROBICIDE AGAINST
CHLAMYDIA INFECTION**

BY

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ABSTRACT

Chlamydia trachomatis is the causative agent of the most prevalent sexually transmitted bacterial infection in the United States. Antibiotic therapy is currently effective in treating *Chlamydia* infection; however, the vast majority of infected individuals are asymptomatic. In women, untreated cases of *Chlamydia* infection can lead to serious reproductive health consequences. In the current absence of a safe and effective vaccine, my study focused on development of a vaginally-delivered topical microbicide as an alternative strategy for prevention of *Chlamydia*.

The paucity of a well-established method suitable for large-scale analysis of *in vitro* *Chlamydia* infection is a major limitation in the development of novel anti-*Chlamydia* compounds. In my effort to identify compounds that can be considered as microbicide candidates, I developed two automated methods for enumeration of *Chlamydia* that are amenable to a large-scale study. The automated immunofluorescence image-based assay uses computational analysis of microscopic images, and allows automated phenotypic characterization and classification of *Chlamydia*-infected host cells. The second method utilizes a host cell viability assay, and offers a facile approach with reduced liquid-handling requirements, as well as the ability to simultaneously assess anti-*Chlamydia* and cytotoxic properties of compounds. Both methods yielded enumeration of *Chlamydia* infection that is comparable to the conventional manual microscopy while drastically reducing the time requirements for analysis.

Using the automated image-based method, I performed a compound screen to test *in vitro* susceptibility of *C. trachomatis* to small subsets of compounds for the two main components of microbicides; excipient and active ingredient. Excipient candidates were

cellulose ether polymers, commonly used in vaginal gels and have pharmaceutical properties that favor their use in the preparation of controlled-release formulations for long-term administration of vaginal microbicide. Candidate active ingredients were analogues of an antibiotic polymyxin B (PMB) rationally designed to target lipid A portion of Gram-negative lipopolysaccharide (LPS). A wide range of anti-chlamydial activity was observed among cellulose ether polymers, and 14 out of 18 PMB analogues exhibited greater than 60% inhibition of *Chlamydia* growth.

Additionally, I was interested in studying the biological role of chlamydial lipooligosaccharide (LOS), using small molecule DS-96 targeting lipid A as a chemical probe. DS-96 effectively blocked chlamydial attachment and entry steps, suggesting that chlamydial LOS plays a role in these steps. These data were supported by the observation that a high level of inhibition by DS-96 was maintained through centrifugation, which is known to enhance *Chlamydia* infection but thought to override the attachment and entry mechanisms. Together, these data demonstrated that targeting chlamydial LOS is effective in blocking *Chlamydia* infection prior to the bacterial entry, and therefore, has a high potential to be a suitable approach for prevention of *Chlamydia* infection. Furthermore, utilizing DS-96 as chemical tool, my study expanded upon the current understanding of the biological significance of chlamydial LOS.

This work is dedicated to my little sister
Ayu; because of her, I found the
determination and strength to pursue and
complete this journey.

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Chapter I.

Introduction

Public Health Impact of *Chlamydia*

The phylum *Chlamydiae* represents a unique group of obligate intracellular bacteria, which are estimated to have been diverged from the planctobacterial phylum approximately two billion years ago, whereas the pathogenic species diverged from environmental *Chlamydiae* some 700 million years ago [1, 2]. The members of the family *Chlamydiaceae* infect a diverse array of hosts including birds, reptiles, amphibians, and mammals, causing acute and chronic diseases in humans and animals. [3] The earliest description of *Chlamydia* infections can be found in reference to the symptoms of trachoma in ancient Chinese and Egyptian manuscripts [4]. Two chlamydial species that commonly infect humans are *Chlamydia trachomatis* and *Chlamydia pneumoniae*. Human infections caused by *Chlamydia* are associated with blindness, sterility, and atherosclerosis, and have a tremendous impact on public health.

C. trachomatis causes severe infections of the mucosal epithelium of eyes and urogenital tract, and has traditionally been classified into serovars based on the differential serospecificity to the major outer membrane protein (MOMP) [5]. Serovars A to C typically cause the ocular infection trachoma, which is the leading cause of preventable blindness worldwide [6, 7]. The number of people with active trachoma is estimated to be over 40 million, with additional 8.2 million people with trachomatous trichiasis, the blinding stage of the disease [8]. In addition to ocular infection, urogenital

tract chlamydial infections are the most commonly reported sexually transmitted bacterial infection with an estimated annual incidence of around 92 million cases in the world [9].

Serovars L1 to L3 and their variants cause lymphogranuloma venereum (LGV), which is an invasive sexually transmitted infection (STI) with the dissemination of the infection to lymph nodes facilitated by macrophages [10, 11]. In contrast to LGV, genital infections caused by serovars D-K are restricted to the urogenital mucosa and commonly result in urethritis, cervicitis, and epididymitis [12]. The majority of *C. trachomatis* infections (approximately 70%) are asymptomatic, presenting problems in detection, treatment, and control [13]. Genital chlamydial infections, as STI in general, are of greater concern in women since the manifestation and consequences are more damaging to the reproductive health of women than men [14].

C. trachomatis infections typically last for many months, and an estimated 15 to 20% of acute genital *Chlamydia* infections are cleared spontaneously by adaptive immune responses [15, 16]. However, undetected and untreated infections can ascend to the upper genital tract and result in subsequent sequelae such as pelvic inflammatory disease (PID), which may lead to more severe complications including life-threatening ectopic pregnancy and infertility in women [17-19]. It is estimated that approximately 8-10% of untreated *C. trachomatis* infections will progress to PID [20]. In addition, genital *Chlamydia* infection has been shown to associate with cervical cancer development and contribute significantly to increased rate of HIV transmission [21, 22]

C. pneumoniae is primarily a respiratory pathogen that causes acute upper respiratory tract infections. According to a serological study, more than 60% of adults have been infected during their lifetime [23]. Infection by *C. pneumoniae* is responsible

for an estimated 10% of community-acquired pneumonia and 5% of bronchitis and sinusitis cases [23]. Chronic infections by *C. pneumoniae* have also been associated with the development of chronic inflammatory conditions such as atherosclerosis [24], arthritis [25], asthma [26], and chronic obstructive pulmonary disease (COPD) [27].

Current Challenge in Prevention and Treatment of *C. trachomatis* Infection

Although *Chlamydia* infections are highly treatable with oral antibiotics, the effectiveness of antibiotic therapy approach in controlling the transmission of *Chlamydia* infection is limited due to the fact that the vast majority of infected individuals are asymptomatic and do not seek treatment. Despite decades of research effort, an effective vaccine is currently unavailable for prevention. While condom use is an effective barrier against the pathogen, many women are unable to negotiate consistent condom use with their sexual partners [28]. And therefore, more attention is now given to female-controlled methods for the prevention of STI.

When considering antibiotic therapy, bacterial resistance to antibiotics is an increasing clinical concern and threat to public health. Antibiotic resistance, however, has not been observed frequently with *Chlamydia*. This is because *Chlamydia* constitutes a distinct evolutionary lineage and grows within relative isolation, and their isolated niche limits the opportunity for acquisition of antibiotic resistance genes from other organisms [29]. Although repeated infections are relatively common (5 to 20%) for *C. trachomatis* infections under appropriate therapy, these repeated cases generally result from patient failure to comply with the proper regimen, rather than from emergence of antibiotic resistance [30].

The current recommended treatment is a 7-day regimen of doxycycline (a tetracycline derivative) or a single dose of azithromycin [31]. Tetracycline and its derivatives are considered treatment of choice for *Chlamydia* infections in both animals and humans, because they are effective, have low toxicity, and are cost effective, as opposed to more costly azithromycin [32, 33]. To date, the only reported case of antibiotic resistance acquired through horizontal gene transfer in *Chlamydia* species is the resistance mediated by the *tet(C)* island in a swine pathogen *C. suis* [34, 35]. *In vitro* horizontal transfer of the *tet(C)* island from *C. suis* to other species of *Chlamydia* including recent clinical *C. trachomatis* isolates was demonstrated by co-infection of cell culture [36]. Therefore, acquisition of *tet(C)* facilitated by contact between tetracycline-resistant and -sensitive *Chlamydia* strains in a natural setting, and infection and selection in individuals treated with tetracycline is a possible scenario.

In addition, since asymptomatic infections escape detection and result in delayed diagnosis and treatment, the infected individuals unknowingly contribute to the spread of the infection and development of adverse reproductive complications. Screening and treatment programs have been implemented worldwide in an attempt to reduce the burden of *Chlamydia* and its sequelae. While the control program has contributed to a reduced incidence of PID [37], it has also been reported that the early detection and therapy attempts are associated with enhanced susceptibility to reinfection at the population level [38]. This phenomenon has been called the arrested immunity hypothesis and points out that early initiation of treatment leads to interruption of the acquisition of protective immunity [38].

Vaccination as a preventive strategy has made substantial progress towards eradication of certain infectious organisms [39]. The development of a protective vaccine for *Chlamydia* can significantly reduce the prevalence of infection. However, despite decades of research effort and progress, no protective vaccines are available for clinical use, and development of an effective vaccine against *C. trachomatis* remains a major challenge. It has been well demonstrated that previous exposure to *C. trachomatis* does not provide robust immunity against reinfection [13]. The initial approach of the whole-organism based vaccine against trachoma using inactivated *Chlamydia* EBs was carried out by several groups [40-45]. Although these vaccines produced short-term protection evidenced by decreased incidence in onset of the disease, as well as decreased intensity of the disease in some individuals, they also resulted in heightened severity of disease when individuals subsequently became infected [46]. These observations lead to the assumption that both immunoprotective and immunopathogenic immune responses are induced by intact *Chlamydia* [46]. Therefore, despite significant success in clinical trials, the focus of vaccine research has shifted from whole-organism based to subunit vaccine to avoid the risk of immunopathology [47].

For a successful vaccine against genital *C. trachomatis*, an antigen capable of stimulating both cell-mediated protective CD4⁺ Th1 response and B-cell antibody immunity in the genital mucosa is desirable [48]. Effective prevention of *Chlamydia* also requires a vaccine that provides coverage against the multiple naturally occurring *C. trachomatis* serovars [13]. Candidate chlamydial antigens can be categorized into four classes based on location and timing of expression during the developmental cycle [49]. The first class is the membrane proteins such as MOMP, outer membrane complex

protein B (OmcB), and polymorphic membrane proteins (Pmps). Surface exposed proteins are desirable targets for their susceptibility to antibody recognition, and also thought to be more efficient in inducing cellular immune responses than cytoplasmic proteins [50]. Other classes include chlamydial cytoplasmic proteins such as heat shock protein 60 (hsp60), inclusion membrane proteins such as various Inc proteins, and effector proteins of the type III secretion system (T3SS) that are secreted into host cytosol.

Vaginal Microbicide

Although immunization would be an ideal strategy for controlling the transmission of STIs, protective vaccines are currently unavailable for most STIs including *C. trachomatis*. New alternative prevention strategies are needed to combat the worldwide increase in STIs. Microbicides are vaginally or rectally administered compounds aiming at reducing the transmission of STIs across the mucosal tissue by forming a chemical, physical, and biological barrier against the pathogens. They can be formulated as various forms including gels, creams, foams, or vaginal rings. One advantage of microbicides over other preventative strategies such as condom use is that vaginal microbicides have the ability to greatly empower women, especially those who lack the societal or economic power, by allowing them to control their sexual health without the consent of the partner. Therefore, a successful microbicide is expected to provide women with an affordable self-managed option for prevention of STIs.

The first vaginal microbicide to be tested was a commonly used spermicidal non-specific surfactant nonoxynol-9 (N-9) [51-53]. The antiviral activity of N-9 *in vitro* was

first reported in 1985 [54, 55], which initiated the search for microbicides for prevention of sexual transmission of infectious pathogens. Evidence from both *in vitro* [54-56] and *in vivo* [57, 58] studies demonstrated activity of N-9 against human immunodeficiency virus (HIV). In addition to HIV, N-9 also inactivates other STI pathogens *in vitro*, including *C. trachomatis* [59-61] and herpes simplex virus [62, 63]. Multiple studies also reported *in vivo* activity of N-9 against *C. trachomatis* and *N. gonorrhoeae* [64-66]. However, it quickly became apparent that surfactant-based products perturbed the protective epithelial barrier among frequent users, potentially leading to increased transmission of infection [51, 53, 67]. These disappointing results led to discontinuation of the development of N-9 as a microbicide. More recently, after two decades of clinical trials testing a wide array of products, tenofovir, a nucleoside reverse transcriptase inhibitor, was tested in CAPRISA 004 trial and demonstrated to be moderately protective against male-to-female sexual transmission of HIV in a large-scale phase IIb clinical trial [68]. In this trial, 1% tenofovir gel reduced the acquisition of HIV-1 by 39% overall, and by 54% among women who used the gel correctly more than 80% of the time [68].

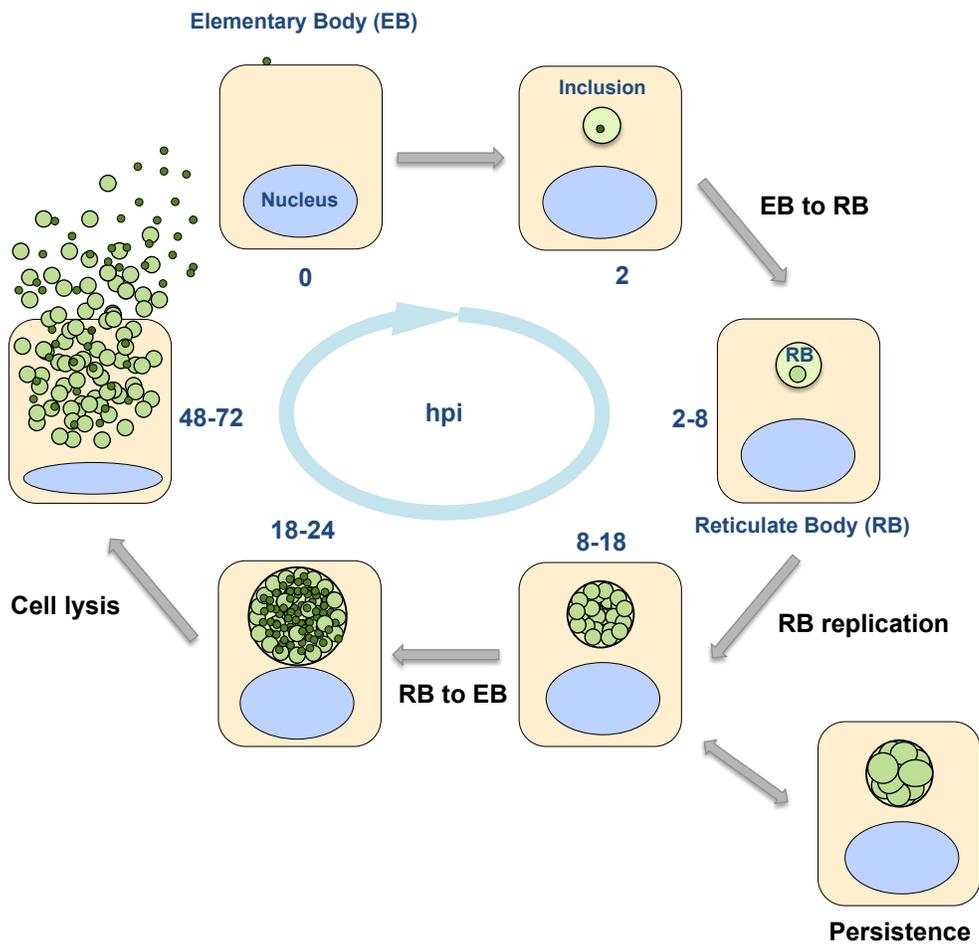
Due to the severity of the disease, the majority of microbicide research is focused on HIV prevention rather than other STIs. Although an effective microbicide is not yet approved for clinical use, over 70 microbicide candidates are currently in preclinical development [69]. There are several categories of compounds among the microbicide candidates in current trials, including vaginal defense enhancers, which facilitate the colonization of natural vaginal flora by maintaining the vaginal pH, surfactants, which disrupt the membrane of pathogens, and entry or replication inhibitors [70]. To date, four trials have assessed vaginal microbicide effects on *C. trachomatis* [71-74].

Developmental Cycle of *Chlamydia*

Despite their diversity of host range, disease pathology, and biological properties, *Chlamydia* species share the unique biphasic developmental cycle in which the organism exists in two morphologically and functionally distinct forms: metabolically inert infectious elementary bodies (EBs) and metabolically active noninfectious reticulate bodies (RBs) [75, 76] (Fig. 1.1). The infectious EBs are relatively small (approximately 0.3 μm in diameter) and their nucleoid is highly condensed by chlamydial histone-like proteins Hc1 and Hc2 [77-79]. The outer membrane of EBs consists of proteins that are highly cross-linked by disulfide bonds, and the chlamydial outer membrane complex (COMC) is made up of a number of cysteine-rich proteins, ensuring osmotic stability in the extracellular environment [80, 81].

Chlamydia infection is initiated by attachment of EB to the host cell surface followed by internalization into the host cell. The mechanisms of these processes are reviewed in the proceeding section “*Chlamydial Attachment and Entry*”. Upon entry, the EB resides within a membrane-bound parasitophorous vacuole, termed inclusion, which interacts with host vesicle trafficking pathways to acquire essential lipids [82-84]. *Chlamydia* actively remodels inclusion membrane to intersect exocytic pathway and avoid fusion with host cell lysosomes [85, 86]. Differentiation of EBs into larger (0.8 to 1 μm in diameter) RBs begins immediately following internalization [76]. This transition involves events such as decondensation of chlamydial chromosome, early-gene transcription, and reduction of the disulfide-linked outer membrane of EB, which results in loss of rigidity [87-89]. For *C. trachomatis* LGV, the transition from EB to RB is

Fig. 1.1 Developmental cycle of *Chlamydia trachomatis* LGV. The infectious but metabolically inactive form of the organism, elementary body (EB) attaches to the host cell, where the bacterium induces its own endocytosis. After internalization, the EB remains within a parasitophorous vacuole, termed an inclusion, and differentiates to the non-infectious but metabolically active reticulate body (RB). RBs undergo multiple rounds of replication by binary fission before asynchronously transforming back into progeny EBs, which are subsequently released from the inclusion vacuole to infect neighboring cells. Under altered environmental conditions, such as presence of antibiotics, immunological responses, or nutrient and iron deprivation, *Chlamydia* enters a non-replicating and non-infectious persistent form, which is reversible when the stress is removed. Hours post infection (hpi) is indicated by blue numbers.



complete by approximately 8 hour-post-infection (hpi). RBs undergo repeated cycles of binary fission to replicate within the expanding inclusion, leading to asynchronous differentiation back to EBs (~18 to 24 hpi) [90]. By 48 to 72 hpi, depending on the species, the developmental cycle is complete and *Chlamydia* are released from the host cell through two distinct mechanisms: cell lysis or a packaged release of *Chlamydia* termed extrusion [91]. Once released, progeny EBs proceed to initiate a new round of infection of neighboring cells.

Under stressful growth conditions, *Chlamydia* can deviate from the normal developmental cycle and transition into a persistence state. Chlamydial persistence can be induced by a wide range of factors including the presence of antibiotics, immunological responses, or nutrient and iron deprivation [92, 93]. During persistence, *Chlamydia* forms morphologically aberrant RBs in which the organism remains viable and chromosomes continue to divide, but replication and differentiation to EBs are inhibited [94]. It has been suggested that *Chlamydia* uses this aberrant stage to survive and persist inside the host cell for extended periods of time and augment the disease process [47]. The normal developmental cycle can be generally recovered upon removal of the persistence-inducing stimuli [93].

Chlamydial Attachment and Entry

The initial interaction of *Chlamydia* with the eukaryotic cell membrane is a critical step in establishing a productive infection. However, the mechanisms governing the attachment and entry processes have been elusive. Although the natural targets of *Chlamydia* infections are mostly mucosal and submucosal surface, *Chlamydia* is capable

of efficiently infecting a wide range of non-phagocytic mammalian cells, suggesting that the chlamydial adhesins recognize conserved cellular receptors, or that multiple redundant strategies exist to ensure attachment and entry of the bacteria [90, 95]. Identification of chlamydial adhesins and host cell receptors involved in the attachment process has been complex, and no clear consensus on the overall process currently exists. This is thought largely due to distinct biological differences between various chlamydial species or serovars, as well as the use of different mammalian host cells [95].

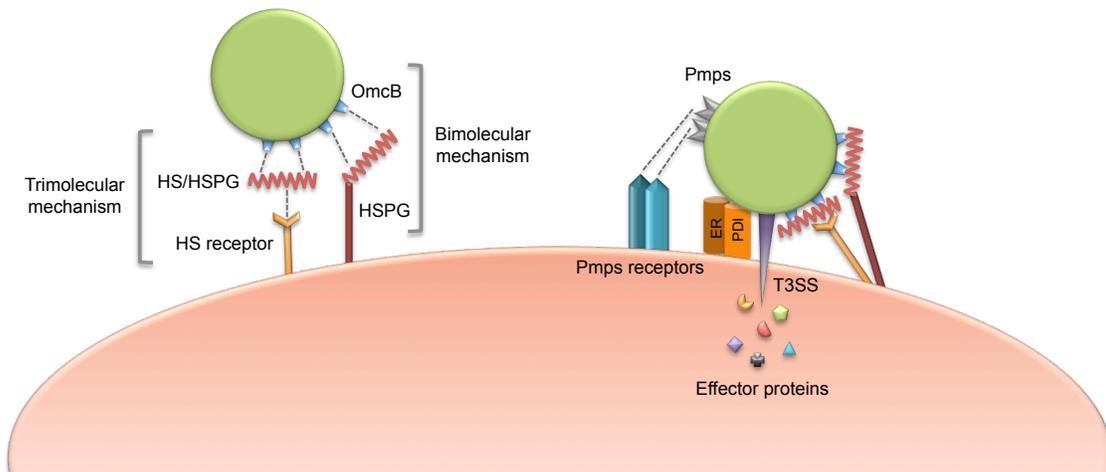
Chlamydial attachment is thought to occur in a two-step process [95]. The initial attachment of many strains and species of *Chlamydia* with the host cell appears to be through reversible electrostatic interactions [96]. This interaction can be enhanced by the addition of divalent cations to neutralize the repulsive negative charges on the bacterial and host cell surface [96, 97]. Various studies have demonstrated the involvement of heparan sulfate-like glycosaminoglycans (GAGs) in this step [98-111]. Early studies determined that pre-treatment of the host cells with heparin inhibited chlamydial attachment [112, 113]. The involvement of heparan sulfate-like GAGs in the attachment of *C. trachomatis* L2 was demonstrated when the process was inhibited following pre-treatment of EBs with heparan sulfate lyases, but this inhibition was reversible upon treatment of EBs with exogenous heparan sulfate [105]. With this observation, a trimolecular mechanism was proposed, in which soluble heparan sulfate serve as a bridge between the adhesion on the bacterial surface and the host cell receptor [105] (Fig. 1.2). Numerous studies followed and implied that heparan sulfate-like GAGs are required in the initial attachment process, although the degree of inhibition depended on the species

and serovars of *Chlamydia*, the host cell lines, and experimental settings [109, 111, 114-119].

Fig. 1.2. Generalized model for chlamydial attachment to the host cells (adapted from *Hegemann and Moelleken* [126]). (A) Reversible interaction is via binding of OmcB to heparan sulfate (HS) and/or heparan sulfate proteoglycan (HSPG) via the bimolecular or trimolecular mechanism. HS and HSPG may be soluble form or associated with host cell surface. (B) Irreversible interaction. Pmp proteins bind to unidentified host cell receptors. Surface localized PFI and/or estrogen receptor (mER) may serve as a structural component of the host cell receptors.

A. Reversible interaction

B. Irreversible interaction



One of the chlamydial proteins thought to bind GAGs is OmcB, which is the second most abundant outer membrane protein highly conserved among *Chlamydia* species [120]. Binding of OmcB and GAGs have been demonstrated by numerous studies including heparin affinity chromatography [99] and surrogate expression systems [103, 121]. The GAG-binding domain has also been mapped to its N-terminal portion of the protein[99].

Following the reversible step, an irreversible interaction of *Chlamydia* with host cell receptors takes place. This temperature-dependent irreversible process was originally demonstrated by analysis of chemically mutagenized Chinese hamster ovary (CHO) cell lines resistant to *Chlamydia* infection [122, 123]. Among numerous candidates, the family of Pmps has been identified as essential chlamydial adhesins [124, 125]. Substantial evidence has also been presented for involvement of Protein Disulfide Isomerase (PDI), which is a component of the estrogen receptor complex [126]. While many other host cell molecules have been proposed to play a role in this process, PDI is the only host cell protein that has been demonstrated to be essential for attachment of multiple species and serovars of *Chlamydia* [127]. A complex role of PDI in attachment was demonstrated by decreased binding of *C. trachomatis* to a CHO cell line containing defective PDI, where the inhibitory effect on chlamydial attachment was complemented by addition of full length PDI [127]. Subsequent study revealed that PDI is necessary for *Chlamydial* attachment. However, the bacteria do not bind directly to the host cell-associated PDI as a receptor, and rather binds to a cell surface protein that requires structural association with PDI [128]. A hypothetical model of chlamydial attachment

process based on the current understanding has been proposed by *Hegemann and Moelleken* and summarized in Fig. 1.2 [126].

Similarly to the attachment process, various and sometimes conflicting studies have been reported regarding the multistep chlamydial entry, suggesting that *Chlamydia* may utilize multiple mechanisms to ensure their internalization, and the route is dependent on the chlamydial species or the host cell type [129]. Internalization of *Chlamydia* into the host cell occurs either by clathrin-mediated endocytosis, via caveolin-rich domains, or lipid rafts [91, 130, 131]. For *C. trachomatis*, the predominant role of the clathrin-mediated entry has been reported [91]. Uptake of *Chlamydia* into non-phagocytic host cells is actively induced by chlamydial factors. Regardless of the chlamydial species or host cell type, a common feature of *Chlamydia* entry is a small GTPase-dependent actin cytoskeleton remodeling at the site of entry [132]. Like many Gram-negative intracellular pathogenic bacteria, *Chlamydia* utilizes Type III secretion system (T3SS) to inject bacterial effector proteins into the host cell cytosol to modulate cellular responses [133]. Secretion of T3S effector proteins into the host cell immediately following the irreversible attachment step has been demonstrated [134]. A T3S effector protein, Tarp (translocated actin recruiting phosphoprotein), is conserved among all pathogenic *Chlamydia* species examined, and is associated with actin polymerization and cytoskeletal arrangement [134]. Tarp is present in EBs in an unphosphorylated state and translocated upon contact with host cells, where it is tyrosine phosphorylated by host cell kinase [134, 135]. One of the well studied host cell components demonstrated to play a critical role in this process is PDI. Interestingly, whereas the enzymatic activity of host

PDI is not required for chlamydial attachment, it has shown to be absolutely essential for the entry process [128].

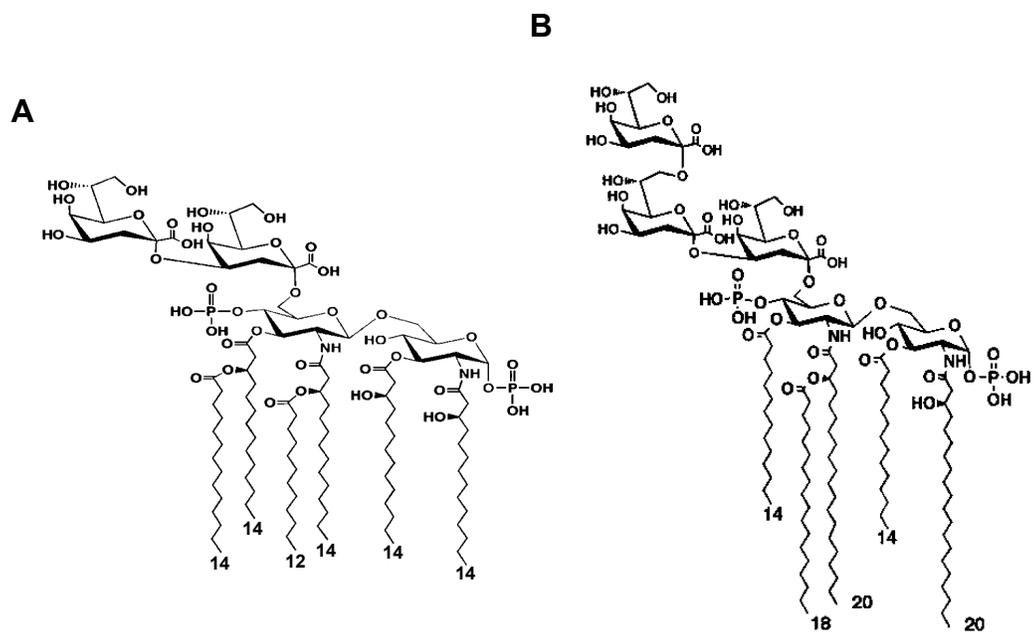
***Chlamydia* Lipooligosaccharide**

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria, typically comprising approximately 10-15% of the total molecules in the outer membrane and estimated to make up about 75% of bacterial surface area [136]. LPS typically consists of three structural parts including lipid A, a core oligosaccharide, and an O-specific polysaccharide chain of repeating oligosaccharide units (Fig. 1.3 A). Since the original discovery as a toxin within heat-inactivated *Vibrio cholerae* in 19th century, LPS has historically been known as heat-resistant “endotoxin” [137]. Once released from the outer membrane of Gram-negative bacteria, LPS is recognized by the innate immune system via toll-like receptor 4 (TLR4), triggering production and release of a cascade of cytokines including tumor necrosis factor α (TNF- α), interleukin1 (IL1), and interleukin 6 (IL6) [138]. Systemic release of these inflammatory mediators during Gram-negative infection can lead to septic shock [139].

LPS is also recognized for the essential roles it plays in the pathogenesis and survival of Gram-negative bacteria. LPS contributes greatly to the structural integrity and stability of the outer membrane, and to the protection against the external stress factors surrounding the bacteria [140]. Owing to their surface exposure, LPS also plays a pivotal role in various interaction of bacteria and the host cell or the external environment. Specifically, LPS has been suggested to play a role in bacterial adhesion as well as being

Fig. 1.3. Chemical structure of *E. coli* Kdo₂-Lipid A and *Chlamydia* Kdo₃-LipidA.

E. coli and *Chlamydia* differ in their 3-Deoxy-d-manno-octulosonic acid (Kdo) content and the number and chain length of fatty acid residues, but share a conserved glucosamine backbone. (A) *E. coli* Kdo₂-Lipid A. (B) *Chlamydia* Kdo₃-LipidA.



a prominent virulence-determining factor for several Gram-negative organisms [141-145].

Like many other nonenteric mucosal pathogens, *Chlamydia* possesses lipooligosaccharide (LOS) rather than LPS in their outer membrane. Chlamydial LOS resembles a rough LPS of enterobacteria lacking the repeating O-antigen polysaccharide side chain present in a typical LPS (Fig. 1.3 B) [146]. All *Chlamydiaceae* share a family-specific epitope composed of a trisaccharide of 3-deoxy-D-manno-oct-2-ulosonic (Kdo₃) residues of the sequence α -Kdo(2→8)- α -Kdo(2→4)- α -Kdo, rather than a disaccharide (Kdo₂) in LPS, whereas the lipid A contains a conserved glucosamine disaccharide backbone structure (1,4'-bisphosphorylated β (1→6)-linked) [146]. In contrast to enteric bacteria, chlamydial lipid A has been shown to exhibit low endotoxicity, reportedly due to the fewer number of hydrocarbons and the longer chain length [147, 148].

Whereas LPS is an important structural component for the membrane integrity of Gram-negative bacteria, the outer membrane of *Chlamydia* EB harbors the intra- and intermolecular cysteine cross-linked proteins, which are expected to provide structural rigidity and stability [149, 150]. *Nguyen et al.* recently proposed the role of chlamydial LOS in the expression and stability of EB-specific outer membrane proteins [151]. It was demonstrated that the biosynthesis of chlamydial LOS was essential for production of infectious EBs. Specifically, the expression of an EB-specific outer membrane protein OmcB was inhibited in the absence of LOS while MOMP and Hc1, EB-specific histone-like protein, were properly expressed.

A general role of LOS in *Chlamydia* infectivity was reported by *Fadel and Eley* by demonstrating that infection of cell culture 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 [152]. A role of chlamydial LOS in infection has also been suggested by Ajonuma and colleagues [153]. It was demonstrated that CFTR (cystic fibrosis transmembrane conductance regulator) functions as a cell-surface receptor for entry and internalization of *C. trachomatis*, and LOS appears to be the ligand for CFTR [153]. Despite various studies and reports, the precise role of LOS in the developmental cycle of *Chlamydia* remains elusive.

Collaborator's Contributions

CellProfiler pipeline was designed and created by collaborative efforts of Dr. David Moore, Heather Shinogle, and Jeff Hills. Figure 2.1. “Overview of Image Analysis and CellProfiler Modules (Pipeline)” was generated by Jeff Hills. Taryn Cansler performed the preliminary studies in the development of the automated image-based method.

Chapter II.

Development and Validation of Novel Analysis Methods for *Chlamydia* Infection

Abstract

The paucity of effective tools for large-scale compound screening is a major limitation in the identification of novel antichlamydial compounds. The conventional method for quantification of *Chlamydia* infection using fluorescence microscopy typically involves time- and labor-intensive manual enumeration, which is not applicable for a large-scale analysis required for an inhibitory compound screen. In this study, two methods are developed for rapid and accurate enumeration of *Chlamydia* infection in cell culture. An immunofluorescence image-based method was customized for fully automated quantification of *Chlamydia* infection using the freely available open-source image analysis software program CellProfiler and the complementary data exploration software program CellProfiler Analyst. The method yielded enumeration of different species and strains of *Chlamydia*, as well as the inhibitory capability of established antichlamydial compounds comparable to the conventional manual methods while drastically reducing the analysis time. In the second method, a redox indicator resazurin was utilized to measure *Chlamydia* infection by quantifying the redox capability of infected host cells. This assay also provided measurements comparable to those of the conventional microscopy method while reducing time and liquid-handling requirements for analysis. Overall, our studies show that two methods are highly effective tools for automated quantification of *Chlamydia* infection and assessment of antichlamydial activities.

Introduction

A major limitation in the development of antichlamydial compounds is the lack of a well-established method for quantifying *Chlamydia* infection that is adaptable to a high-throughput screening format. The paucity of high-throughput screening methods is heavily influenced by the obligate intracellular growth characteristics and the inability for axenic cultivation of *Chlamydia*. The standard technique for quantification of *Chlamydia* infection is the immunofluorescence assay (IFA), which relies on visual detection of fluorescently stained bacteria and host cells, typically within a relatively low number of fields (e.g., 3 to 10). This process is labor-intensive and not suitable for large-scale screening required for antimicrobial compound development. The limitations of manual enumeration are evident; the process is labor intensive, field selection is prone to subjectivity, and results are based on a relatively restricted data set.

In this chapter, we describe two novel methods that were developed for rapid enumeration of *Chlamydia* infection in cell culture. The first method is an immunofluorescence image-based method, which was customized for fully automated quantification of *Chlamydia* infection using the freely available open-source image analysis software program CellProfiler and the complementary data exploration software program CellProfiler Analyst [154]. In the second method, an alamarBlue (resazurin) assay was adopted to measure *Chlamydia* infection by quantifying the redox capability of infected host cells [155].

CellProfiler is freely available open-source computational image analysis software (<http://www.cellprofiler.org>), which has been shown to be an effective tool for quantifying visual information in a variety of biological images, particularly in large-

scale imaging experiments [156]. The complement software, CellProfiler Analyst, performs analysis of image-derived quantitative information as defined within a very large collection of image-feature measurements (i.e., a cytological profile) produced by CellProfiler. CellProfiler Analyst contains a supervised machine-learning system that is intuitive for development of image analysis algorithms via visual perception using a user-friendly interface [157, 158]

CellProfiler and CellProfiler Analyst were adapted for identification and enumeration of *Chlamydia* infection. For validation of the method, cell culture infections of the commonly utilized laboratory strain (LGV-2) and the more clinically relevant strain (serovar D) of *C. trachomatis* were analyzed. The accuracy and expediency of the automated method were compared to those of the conventional visual inspection. To evaluate the efficacy of the method to identify antichlamydial compounds, infections were measured following treatment with four anti-*Chlamydia* molecules (tetracycline [159, 160], polymyxin B [161-163], hydroxyethyl cellulose [164], and anti-*C. trachomatis* polyclonal antibody) that have contrasting mechanisms of inhibitory activity toward *Chlamydia*.

While the IFA-based assay, which allows visualization of host cells and bacteria, is the preferred approach for accurate quantification of the intercellular growth of *Chlamydia*, automation of image acquisition requires specialized instrumentation such as an automated fluorescence microscope and extensive computational resources, which may not be readily available in some laboratories. Therefore, we thought to develop a facile and inexpensive method using a microplate spectrophotometer.

AlamarBlue (resazurin) is a colorimetric cell viability indicator widely used to monitor eukaryotic cell proliferative activity [165-167]. The redox dye resazurin enters the cytosol in the oxidized form (blue) and is converted to the reduced form, resorufin (red) [168]. The reduced and oxidized forms of alamarBlue can be separately measured by a spectrophotometer and used to determine the reduction capability of cells, which reflects the status of mitochondrial function and cell viability. One significant advantage of alamarBlue over other metabolic indicators is that the compound allows continuous monitoring of cells [169]. This is due to the fact that the compound does not interfere with the activity of the respiratory chain and, therefore, is nontoxic to the cells [169]. AlamarBlue has found applications in quantitative analysis of cell viability [170], proliferation [171], cytotoxicity [172], and drug susceptibility [173, 174] in both bacterial and eukaryotic systems as well as high-throughput screening of antimicrobial compounds in microorganisms such as *Mycobacterium tuberculosis* [175] and *Staphylococcus aureus* [176]. In this study, alamarBlue assay was adapted for enumeration of *C. trachomatis* infection by measuring the lysis of infected host cells as indicated by a decrease in cell viability. Similar to the validation study for the image-based assay, the ability of the alamarBlue assay to evaluate anti-*Chlamydia* activity was assessed by measuring *Chlamydia* infection levels in the presence of the well-established inhibitors.

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 as previously described [177], and stored in sucrose phosphate glutamate (SPG) at -80°C until use. *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 previously described [177]. L929 cells and HeLa 229 cells were routinely cultured in RPMI 1640 tissue 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% CO₂. Cells were plated in 96-well plates (Bioexpress, Kaysville, UT) at density of 7×10^4 cells/mL, 200 µL/well, and incubated overnight prior to infection. In order to minimize edge effect (well-to-well variations in number of cells), plated cells were incubated for 1 h at room temperature prior to incubation at 37°C in an atmosphere of 5% CO₂, as previously described [178].

Microbicide and antibiotic preparation

Polymyxin B sulfate (Enzo Life Science, New York, NY) and tetracycline hydrochloride (USB Corporation, Cleveland, OH) were obtained in powder form. Stock solution of polymyxin B sulfate was prepared in Hank's Buffered Salt Solution (HBSS, Mediatech, Inc., Holly Hill, FL) to a concentration of 10 mM. Tetracycline was dissolved in sterile water to a stock concentration of 1 mg/mL. Hydroxyethyl cellulose (HEC,

90 kDa, Sigma-Aldrich, St. Louis, MO) was prepared as a stock solution at a concentration of 12% w/v in water. The HEC gel (polymeric liquid) was adjusted to pH 4.5 using 10 M NaOH and stored at 4°C. At the time of the assay, 12% HEC gel was diluted to a final concentration of 2.8% w/v in phosphate buffered saline (PBS). The mixture was shaken for 1 h at 37°C to achieve a uniform solution, centrifuged for 1 min at 300 x g to remove any bubbles, adjusted to pH 7.0, and stored at 4°C until use.

EB dilution assay

Purified *C. trachomatis* L2 EBs were serially diluted two-fold in HBSS and transferred to ~70% confluent L929 cell monolayers (90 µL/well) in a 96-well plate. Ninety µL of HBSS alone was added to mock-infected control cells. Cells were incubated for 2 h at room temperature. Following the incubation period, the bacterial inocula were removed and cells were washed once with 200 µL of HBSS. Two-hundred µL of fresh medium (RPMI/5% fetal bovine serum/10 µg per mL gentamicin) was added to each well and cells were incubated at 37°C in an atmosphere of 5% CO₂ for 24 h.

Antichlamydial compound inhibitory assay

C. trachomatis L2 EBs were diluted in HBSS. Polymyxin B and hydroxyethyl cellulose were serially diluted two-fold in HBSS containing EBs to the concentration range of 1 mM to 15.625 µM and 2.8% to 0.175%, respectively. Compound-EB mixtures were incubated for 60 min at room temperature. Compound-treated EB inocula were mixed using a multi-channel pipette (LabNet Excel, Edison, NJ) at the start and end of the incubation period to ensure uniformity of the inocula. The mixtures containing the

compound and EBs were then transferred to ~70% confluent L929 cell monolayers in a 96-well plate and incubated at room temperature for 2 h. Following the incubation period, the inocula were removed and cells were washed once with 200 μ L of HBSS. Two-hundred μ L of fresh medium (RPMI/5% fetal bovine serum/10 μ g per mL gentamicin) was added to each well. For tetracycline treatment, fresh medium containing appropriate concentration (0.1-0.7 μ g/mL) of tetracycline was added following two-hour inoculation period. Cells were incubated at 37°C in an atmosphere of 5% CO₂ for 24 h.

***C. trachomatis* serovar D and *C. muridarum* infection**

Purified *C. trachomatis* serovar D or *C. muridarum* were diluted four-fold in HBSS and transferred to ~70% confluent HeLa 229 or L929 cell monolayers (90 μ L/well) in a 96-well plate, respectively. Ninety μ L of HBSS alone was added to mock-infected control cells. The plate was centrifuged at 900 x g for 1 h at room temperature [177]. Following the incubation period, chlamydial inocula were removed and cells were washed once with 200 μ L of HBSS. Two-hundred μ L of fresh medium (RPMI/5% fetal bovine serum/10 μ g per mL gentamicin) was added into each well and cells were incubated at 37°C in an atmosphere of 5% CO₂ for 48 h for serovar D and 24 h for *C. muridarum* before they were analyzed.

Neutralization Assay

Guinea pig polyclonal serum against *Chlamydia* L2 EB was purchased from Abcam (Cambridge, MA). Rabbit polyclonal anti-RpoA antibody was affinity-purified using the AminoLink Plus Immobilization Kit (Thermo Scientific, Rockford, IL). Protein

concentration of purified antibody was determined to be 1.01 mg/mL by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Sera and purified antibody were serially diluted (five-fold at each step) in HBSS. Prior to infection, purified *C. trachomatis* L2 EBs were added to each sera- or purified antibody- sample and incubated for 30 min at 37°C to allow interaction between EBs and the antibody to take place [179]. HBSS alone was used as negative control. Following the incubation period, 90 µL of each sample containing antibody-EB mixture was added to a monolayer of L929 cells in a 96-well plate and incubated at room temperature for 2 h. After the incubation period, inocula were removed and cells were washed once with HBSS. Two-hundred µL of fresh medium (RPMI/5% fetal bovine serum/10 µg per mL gentamycin) was added to each well and cells were incubated at 37°C for 24 h.

Immunofluorescence assay (IFA)

At 24 or 48 hours post infection (hpi) for serovar L2 or D, respectively, infected cells were fixed with 100% methanol (200 µL/well) for 10 min at room temperature and washed once with PBS. Cells were stained with MicroTrack *C. trachomatis* culture confirmation test (Syva Co., Palo Alto, CA) diluted to 1:40 in PBS (50 µL/well) for 60 min in the dark, followed by a 5 min stain with 1 µg/mL DAPI in PBS (50 µL/well). DAPI was removed and PBS (200 µL/well) was added. Plates were stored in the dark at 4°C until imaging.

Image Acquisition

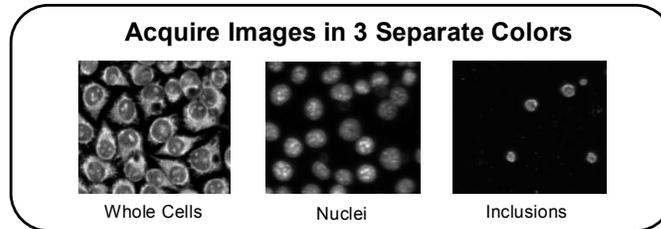
Images were automatically captured with a BD Pathway BioImager 855 microscope (Becton, Dickinson and Company, Franklin Lakes, NJ) with a 20x, 0.4 numerical aperture objective, fully equipped for multi-color capture, and an Orca ER camera (Hamamatsu Photonics, Bridgewater, NJ). The system was controlled by Attovision collection software (Becton, Dickinson and Company, Franklin Lakes, NJ), with automated infrared and image autofocus capture for 15 fields per well for a 96-well plate. Multiple plate capture was enabled by a Twister II robotic arm (Caliper Lifesciences Inc., Hopkinton, MA), which was integrated with the Attovision software.

Image Analysis, CellProfiler and CellProfiler Analyst

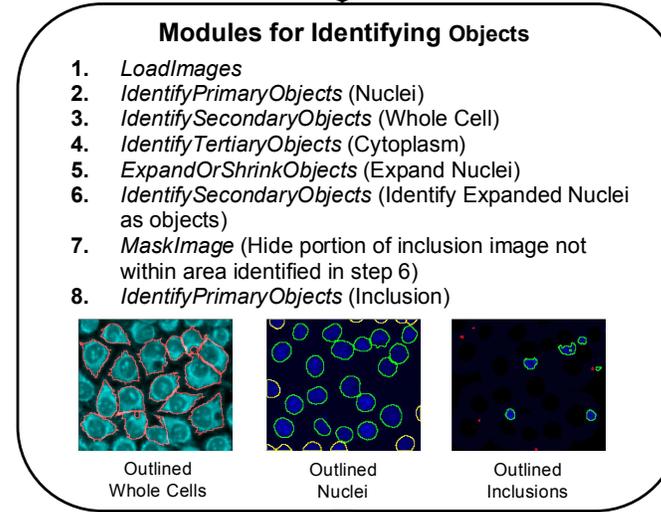
Images acquired by automated microscopy were loaded into the open-source software CellProfiler (Broad Institute, Cambridge, MA), followed by segmentation of images and identification of objects. Four main steps of the process were segmentation of nuclei, identification of whole cells, segmentation of chlamydial inclusions, and tabulation of measurements (Fig. 2.1). The first task performed by CellProfiler was identification of the objects. Using CellProfiler modules, the nuclei, referred to as primary objects within CellProfiler, were identified first from the DNA-stained images. Once the nuclei had been identified, the subsequent module used the whole cell-stained images and previously identified nuclei to identify the cell boundaries (whole cell), which were referred to as secondary objects. The area of the identified nuclei was subtracted from the whole cells to identify the cytoplasm, referred to as tertiary objects. For identification of chlamydial inclusions, CellProfiler expanded each area of nuclei by 15 pixels and used this expanded region to search for inclusions. Inclusions were then

Fig. 2.1. Overview of Image Analysis and CellProfiler Modules (Pipeline). (A) Images are acquired in three separate colors by automated microscopy. (B) A sequential image analysis commands are performed by CellProfiler based on a customized pipeline. Identification of nuclei, chlamydial inclusions, and whole-cell were performed first. (C) Following identification, measurements were collected for each objects and cytological profile of each cell was generated.

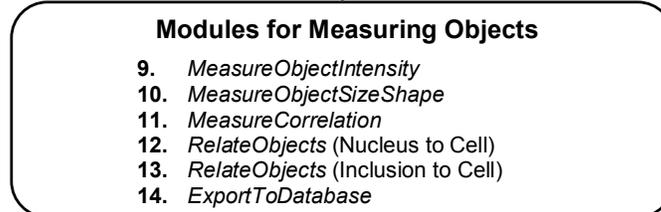
A



B



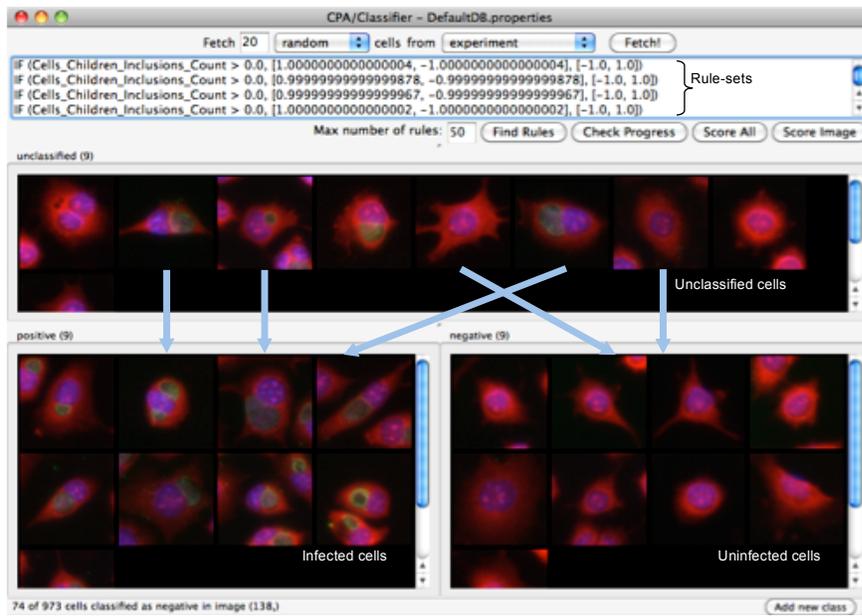
C



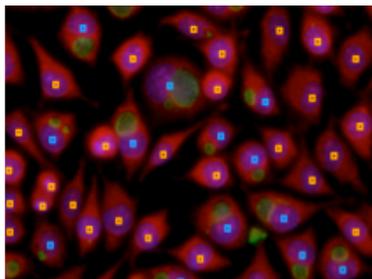
identified and segmented. The second task performed by CellProfiler was generating a cytological profile of each cell. CellProfiler measured a large number of cellular and subcellular features once all objects (nuclei, cells, cytoplasm and identified chlamydial inclusions) had been identified. These quantitative measurements consist of apparent cellular and sub-cellular features as well as an extensive amount of less evident details such as intensity of the stain, size, shape, and pixel correlation between objects. Once these measurements were taken, the cytological profile was created for each cell to be utilized by CellProfiler Analyst (Broad Institute, Cambridge, MA) and exported to a spreadsheet. Both nuclei and chlamydial inclusions were related to the identified cells so that each inclusion was assigned to only one nucleus. For enumeration of inclusion forming units (IFU), the last module within the pipeline (*ExportToDatabase*) was modified to allow for classification of individual *inclusion* rather than *cells* by CellProfiler. To enumerate infected cells, the cytological profiles were loaded into CellProfiler Analyst. Random images each containing a single cell were presented to initiate the training process. Approximately twenty images from the positive (infected) and negative (uninfected) controls were manually classified by dragging and dropping the images into the appropriate bins within the CellProfiler Analyst interface (Fig. 2.2A). Based on the cytological profiles of these classified cells, CellProfiler Analyst identified the parameters necessary to accurately distinguish between uninfected and infected cells by generating a set of 50 rules (i.e. parameters). The software then presented a new set of cells that had been classified as positive or negative based on the rules generated. Any cells classified inaccurately were manually sorted into the appropriate bin to continue the training process. To assess the accuracy of the training, images from selected wells were

Fig. 2.2. CellProfiler Analyst Drag and Drop Interface. (A) CellProfiler Analyst randomly presents cells to be manually classified using a drag and drop interface. Each cell is sorted into either infected or uninfected category. Rules are generated based on the classified cells and used to automatically distinguish between infected and uninfected cells. (B) A classified image of any well among the experiment can be opened to assess the accuracy of the classifications. A blue label represents an infected cell and an orange label represents an uninfected cell based on the current rule-set. The figure represents a portion of a classified image.

A



B



opened and individual cells were automatically marked as uninfected or infected by CellProfiler Analyst based on the established rules (Fig. 2.2B). Any cell that had been classified incorrectly was manually reassigned to the appropriate bin. After the correction, a set of *refined* rules was generated and used to score every cell in every image. These steps were repeated until the system was able to accurately classify greater than 95% of cells into the correct category. Upon completion of scoring, a spreadsheet containing the number of positive and negative cells for each well was generated and exported.

AlamarBlue cytotoxicity assay

L929 cells were seeded in 96-well flat-bottom tissue culture plates (BD Biosciences, Billerica, MA) at a density of 8×10^4 cells/well and cultured until confluent monolayers were formed. Purified *C. trachomatis* was serially diluted 10-fold in Hank's Buffered Salt Solution (HBSS) (Mediatech, Inc.) and added to host cell monolayers (90 μ L/well). Ninety- μ L of HBSS alone was added to mock-infected control cells. For indicated experiments, purified *C. trachomatis* were prepared in HBSS containing 500 μ M of polymyxin B and incubated for 30 minutes. Plates were incubated for 2 h at room temperature. After infection, the chlamydial inocula were removed and cell monolayers were washed with HBSS followed by addition of phenol red-free RPMI culture medium (200 μ L/well) supplemented with 5% FBS, 300 μ g/mL glutamine (Invitrogen, Carlsbad, CA), 50 μ g/mL vancomycin, and 1 μ g/mL cycloheximide. The infection proceeded until the indicated time points. At each indicated hours after infection, 22 μ L of alamarBlue reagent (Invitrogen, Carlsbad, CA) was directly added to the medium resulting in a final

concentration of 10%. The plates were incubated at 37 °C. After 8 h of incubation with alamarBlue, the absorbance was obtained in a PowerWave microplate spectrophotometer at 570 nm (reduced) and 600 nm (oxidized) with KC⁴ data collection and analysis software (BioTek Instruments, Inc., Winooski, VT). For experiments with multiple time points, plates were kept in the incubator at 37 °C between the time points. The results were expressed as the percentage of alamarBlue reduced by infected host cells relative to alamarBlue reduced by the mock-infected control cells using a formula provided by the manufacture:

$$\frac{(\epsilon_{ox})\lambda_2 A \lambda_1 - (\epsilon_{ox})\lambda_1 A \lambda_2}{(\epsilon_{ox})\lambda_2 A' \lambda_1 - (\epsilon_{ox})\lambda_1 A' \lambda_2} \times 100, \text{ where } \epsilon_{ox} = \text{molar extinction coefficient of}$$

alamarBlue oxidized form, ϵ_{ox} = molar extinction coefficient of alamarBlue reduced form,

A = absorbance of test wells, A' = absorbance of positive control well (mock-infection),

λ_1 = 570 nm, and λ_2 = 600 nm.

Results

Method development and initial validation for the automated image-based method

Many of the challenges associated with accurate identification and enumeration of *Chlamydia* infections using an image-based method are due to diversity of inclusion maturation and size. For instance, depending on the chlamydial species or serovar, different time points need to be selected for accurate identification of chlamydial inclusions by immunofluorescent analysis. Additionally, in *C. trachomatis* infected cells, the size of inclusions may vary greatly depending on how many EBs have infected a single cell. This is because multiple EBs can infect a single cell and individual inclusions can fuse to form a much larger inclusion. Inclusion fusion of LGV strains of *C. trachomatis* has been reported to begin by 10 hpi [180, 181]. Due to these variables in chlamydial development, image-based quantification of inclusions based on limited numbers of predefined parameters are likely to result in compromised accuracy and sensitivity. The ability of CellProfiler to automatically extract quantitative cellular and subcellular features, along with the machine-learning aspect of CellProfiler Analyst were expected to be powerful tools to overcome these challenges by eliminating the need for investigators to define threshold values.

To begin testing the capability of CellProfiler and CellProfiler Analyst to accurately identify and quantify *C. trachomatis* infection, L929 cells infected by the widely utilized LGV serovar L2 strain were analyzed 24 hpi. Following image acquisition by fluorescence microscopy, CellProfiler segmented individual cells and generated a cytological profile for each cell by measuring hundreds of cellular and subcellular features. Using these cytological profiles, supervised classification features of

CellProfiler Analyst were incorporated. To begin a training session, CellProfiler Analyst presented a random selection of cells, of which 20 to 30 infected (positive) or uninfected (negative) cells were sorted into a corresponding bin via manual inspection (Fig. 2.2A). Based on the quantitative measurements within the cytological profiles of manually sorted cells, CellProfiler Analyst developed a differential rule-set (machine-learning algorithms). To evaluate this initial rule-set, the accuracy of classification was assessed for a small set of cells (~1000 cells) (Fig. 2.2B). When errors were detected in classification among this population of cells, additional training sessions were performed to refine the rule-set. These steps were repeated until the system was able to accurately classify greater than 95% of cells into infected or uninfected category. The process typically took manual classification of 30 to 50 infected and uninfected cells.

To validate the effectiveness of CellProfiler Analyst training process, the differential rule-set was applied to an image acquired from populations of cells that were not used in the training process. For an image representative of 15 fields of view, the total number of cells and the number of inclusion positive cells were counted using both the automated method and the conventional manual inspection (Table 2.1). The automated method reported a total of 1808 cells, out of which 699 cells were classified as inclusion positive. The number of total cells and inclusion positive cells reported by manual inspection were 1884 and 734, respectively. The percent infection calculated based on these numbers reported by the automated method and manual inspection were 38.7% and 39.0%, respectively. It was noted that the vast majority of cells that were classified as uninfected by CellProfiler Analyst but identified as infected by manual inspection were within highly confluent regions of the sample, where cellular boundaries were not evident

Table 2.1. Comparison of automated and manual methods for *C. trachomatis* enumeration.

	Automated	Manual	Percent difference ^d
Number of L929 Cells	1808	1884	4.12%
Number of Inclusion Positive Cells	699	734	4.88%
Percent Infection ^a	38.7%	39.0%	0.77%
Number of IFU ^b	898	931	3.61%
IFU per milliliter ^c	9.98 x 10 ³	1.03 x 10 ⁴	3.61%

^a The percent cell infection was calculated by dividing the number of inclusion positive cells by the total number of cells.

^b The number of inclusions visualized by FITC-labeling.

^c IFU/mL was calculated by dividing the number of IFU by the volume of EBs used for infection (90 μ L).

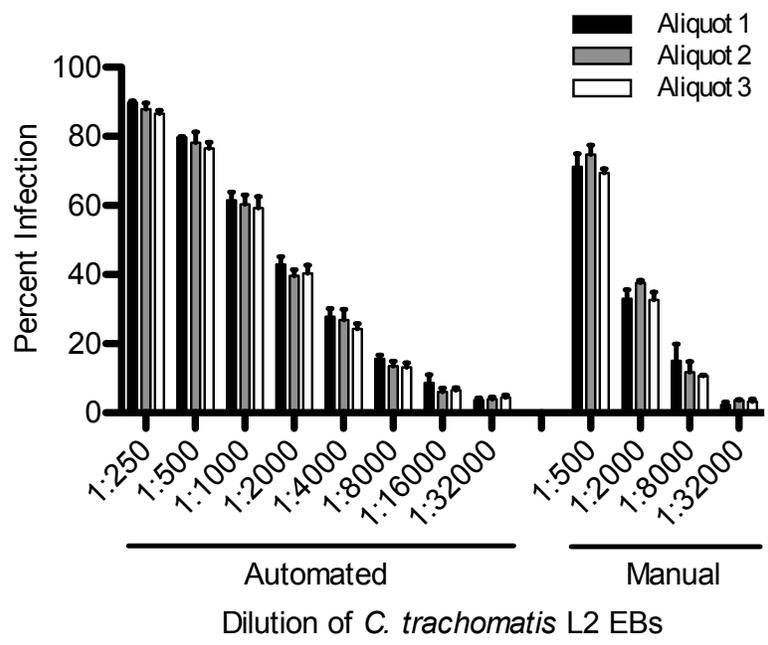
^d Percent difference between the values reported by automated method and manual counting.

and cells were often multinucleated. Together, these results strongly support that enumeration using the automated method was highly comparable to the conventional manual inspection.

Application to large-scale format

To evaluate the suitability of this automated method for large-scale application, image acquisition and quantification of *Chlamydia* infection were performed in a 96-well plate format. Sensitivity of the method was examined by measuring the infection level of cells infected with two-fold serial dilutions of *C. trachomatis* EBs. The reproducibility and precision of the method were assessed through infections by different aliquots from the same EB preparation. The use of CellProfiler and CellProfiler Analyst allowed simultaneous analysis of a total of 1080 images (72 samples, 15 image-fields per sample) within a 96-well plate. These samples included sets of eight two-fold serial dilutions of EBs in triplicate from three separate aliquots. Although a slight variance in values among the aliquots was observed, all three aliquots followed a highly similar trend of decrease across a dynamic range of infections, ranging from 89.7% to 3.7% (Fig. 2.3). Correlation coefficient values were greater than 0.99 between any of the aliquots suggesting that the three sets of data are highly correlated and similar, as anticipated for the three aliquots containing nearly identical number of infectious EBs. Standard deviations among the triplicates were less than 3.5% in all samples. Together, these data indicate that the automated method is capable of performing highly reproducible analysis among a large set of samples with high sensitivity (a dynamic range of infections) and precision (low standard deviations).

Fig. 2.3. Comparison of automated and manual enumeration of *C. trachomatis* serovar L2 infection in 96-well format. Percent infectivity was determined for a two-fold dilution series of *C. trachomatis* EBs 24 hpi by automated and manual enumeration methods from the same samples for comparison of sensitivity, reproducibility, and precision. For the automated method, percent infectivity was calculated based on the number of infected and uninfected cells identified by CellProfiler Analyst. Error bars indicate standard deviation of the triplicate wells. For manual enumeration, cells and inclusions were counted in the three randomly selected fields from one of the triplicate wells. Error bars represent standard deviation of the mean of the three fields.



In order to compare the accuracy and efficiency of the automated method to the conventional manual inspection, manual counting of infected and uninfected cells were performed in three randomly selected fields of view from the immunofluorescent-images acquired for the automated method. Similar to the automated method, a dose-dependent decrease in infectivity over two-fold dilution of EBs was observed (Fig. 2.3). When the datasets obtained using the automated and manual methods were compared, the correlation coefficient between the two datasets was greater than 0.99 for all three aliquots, demonstrating that these datasets acquired by the two methods were highly comparable to each other.

Validation of the automated method with known chemical inhibitors

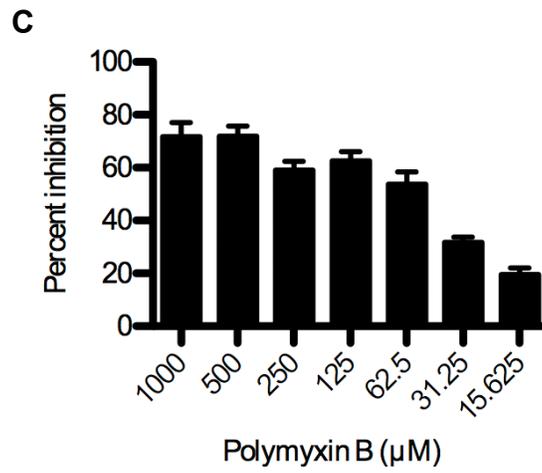
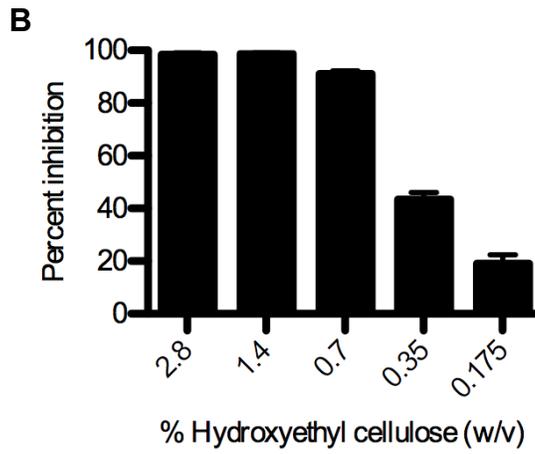
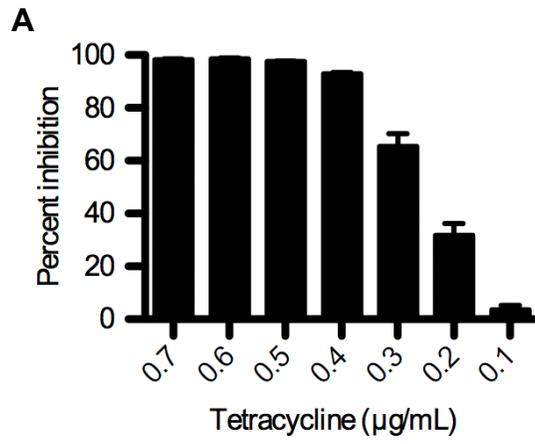
The ability of the automated method to accurately assess the inhibitory activities of compounds against *Chlamydia* infection was demonstrated using three previously known antichlamydial compounds that inhibit different stages of the developmental cycle: tetracycline, hydroxyethyl cellulose (HEC), and polymyxin B (PMB). Tetracycline is a broad-spectrum antibiotic, for which *Chlamydia* is known to be exquisitely sensitive. Tetracycline interferes with the metabolic process of *Chlamydia* by targeting translational machinery and inhibiting protein biosynthesis [159]. Complete inhibition of inclusion formation by tetracycline at a concentration of 0.51 µg/ml has been previously reported [160]. L929 cells were infected with *C. trachomatis* and treated with various compounds in a 96-well plate, and analyzed 24 hpi following automated image acquisition. The analysis by the automated method reported antichlamydial effect of tetracycline in a

dose-dependent manner with complete inhibition of inclusion formation at 0.5 µg/ml, an inhibitory concentration consistent with the previous report [160] (Fig. 2.4).

HEC is a common polysaccharide excipient used in vaginally delivered microbicides for STIs. Although excipients are typically used as delivery vehicles and are expected to be pharmacologically inactive, a 90-kDa HEC has been shown to have an inhibitory effect on *C. trachomatis* infection, purportedly as a competitive inhibitor for adhesion [164]. When EBs were treated with HEC, the inhibitory effect of HEC was directly proportional to the concentration in the range of 0.175% to 0.7% and reached complete inhibition at 1.4% (Fig. 2.4B). This proportionality between the concentration and the inhibitory effect supports a previously suggested mechanism for HEC inhibition, that HEC may act as a competitive inhibitor by competing with the host cell for the recognition site on the chlamydial surface [164].

PMB is an antibiotic that typically acts by disrupting the outer membrane of gram-negative bacteria through interaction with the lipid A portion of lipopolysaccharide [182, 183]. PMB is active against nearly all species of gram-negative bacteria, including *C. trachomatis*, and has been used as a positive control for antichlamydial activity in numerous studies[161-163]. In contrast to HEC treatment, when EBs were treated with PMB, the concentration of the inhibitor did not show a proportional effect on inclusion formation, suggesting a mode of inhibition different from that of HEC. In addition, complete inhibition of inclusion formation was not achieved even at the highest concentration of PMB, reaching maximum inhibition of approximately 70% at 500 µM. Interestingly, previous studies show dose-dependent antichlamydial activity by PMB that plateaued without reaching complete inhibition [161, 184].

Fig. 2.4. Inhibitory properties of known inhibitors on *C. trachomatis* serovar L2 infection. Inhibitory activities of three known anti-chlamydial compounds tetracycline, 90 kDa HEC, or PMB were determined using the automated image-based assay. Percent inhibition was calculated relative to the wells without treatment 24 hpi. (A) Tetracycline treatment. L929 cells were infected with *C. trachomatis* and 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, or 0.7 $\mu\text{g}/\text{mL}$ of tetracycline was added along with growth medium. Error bars indicate standard deviation of the mean of triplicate wells. (B) 90 kDa HEC treatment. L929 cells were infected with *C. trachomatis* pre-incubated with decreasing concentration of 5.6 to 0.175% of HEC for an hour prior to infection. Error bars represent the standard deviations of the mean of triplicate wells. (C) PMB treatment. L929 cells were infected with *C. trachomatis* pre-incubated with decreasing concentration of 1000 to 15.625 μM of PMB for an hour prior to infection. Error bars represent the standard deviations of triplicate wells.



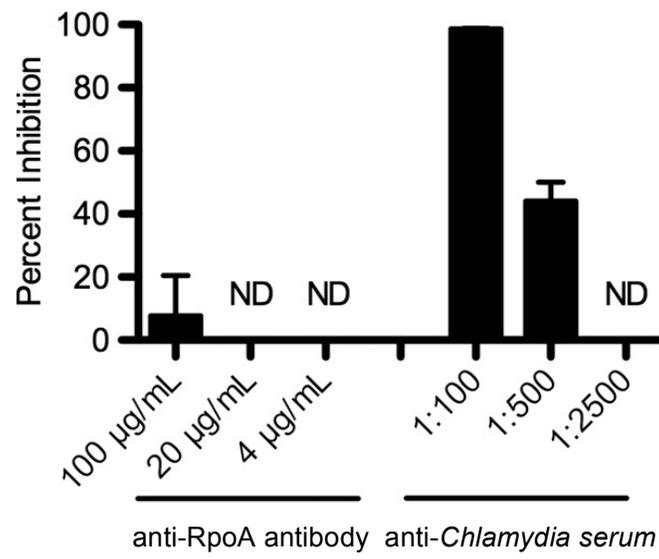
Efficacy of the automated method for measuring neutralizing antibodies

Given the current lack of an effective vaccine against *Chlamydia*, identifying and evaluating neutralizing antibodies would be extremely advantageous. The automated method was used to examine inhibitory properties of polyclonal sera for chlamydial EBs. Affinity-purified polyclonal antibody against *C. trachomatis* RpoA (bacterial cytosol RNA polymerase α subunit) was used as a negative control. *C. trachomatis* EBs were pre-treated with anti-*Chlamydia* EB serum or affinity-purified polyclonal anti-RpoA antibody prior to inoculation of L929 cells, and infection levels were analyzed 24 hpi. At the highest dose (1:100 dilution), anti-*Chlamydia* serum inhibited infection by 98.7% relative to the infection level of the untreated control samples (Fig. 2.5). Anti-RpoA antibody treatment showed insignificant neutralization activity, demonstrating that the neutralization activity of anti-*Chlamydia* serum was not due to a nonspecific reaction. When the amount of anti-*Chlamydia* serum reduced by 5-fold (1:500 dilution), the inhibitory effect was reduced to 44.1%. No inhibitory effect was observed at the lowest concentration (1:2,500). Together, these results indicate that this automated method may be used to accurately identify neutralizing antibody and protective concentrations for *Chlamydia* infection in cell culture.

Assessment of the automated assay with *C. trachomatis* serovar D and the mouse *Chlamydia* species *C. muridarum*

Due to the relative ease of cultivation and shorter developmental cycle, LGV serovar L2 is a commonly used *C. trachomatis* prototype strain in chlamydial research.

Fig. 2.5. Inhibitory properties of anti-*Chlamydia* sera on infectivity of *C. trachomatis* L2 EBs. The ability of anti-*Chlamydia* sera to neutralize the infectivity of *C. trachomatis* L2 EBs was evaluated using the automated image-based method. L929 cells were infected with *C. trachomatis* EBs pre-incubated with various concentrations of anti-RpoA antibody or anti-*Chlamydia* sera for 30 min prior to infection. Percent inhibition was calculated relative to the wells without treatment following 24 hr incubation. ND=No inhibition was detected. Error bars indicate standard deviation of the triplicate samples.



However, trachoma serovars D, E, and F are the most prevalent urogenital strains of *C. trachomatis* worldwide and therefore of more clinical relevance [185]. *C. muridarum* is a natural pathogen of mice and is commonly used as a model for infection of the human genital tract caused by *C. trachomatis*.

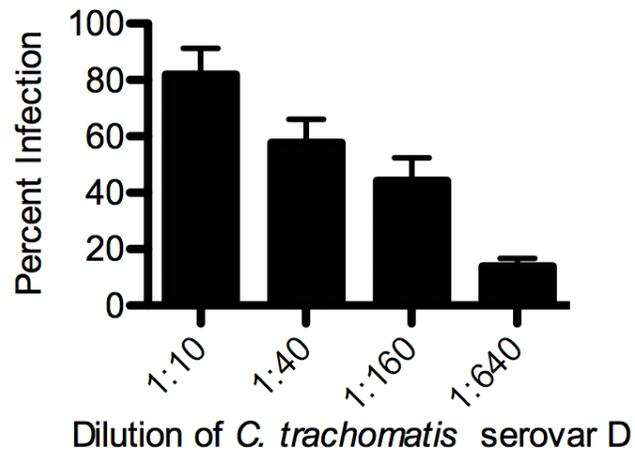
HeLa 229 cells or L929 cells were infected with 4-fold serial dilutions of serovar D or *C. muridarum*, respectively. HeLa 229 cells were used to demonstrate that various eukaryotic cells may be used as host cell lines for the automated enumeration. Due to the longer developmental cycle of serovar D than of L2, serovar D infections were quantified 48 hpi and *C. muridarum* was quantified 24 hpi. The automated method reported the infection level, ranging from 81.9% to 14.0% for serovar D and 95.7% to 4.8% for *C. muridarum* in a dose-dependent manner (Fig. 2.6). These results support that accurate enumeration of *Chlamydia* infection by the automated method is highly adaptable to different serovars and species of *Chlamydia* despite the differences in the duration of developmental cycle, and eukaryotic host cell lines.

Percentage of cells infected and total inclusion forming units

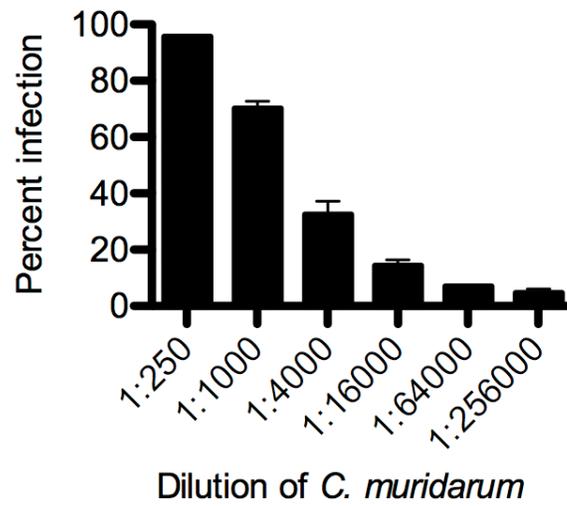
Quantification of *Chlamydia* infection typically utilizes two approaches; inclusion forming unit per milliliter (IFU/mL) or percentage of cells infected. IFU per milliliter is determined by enumerating individual inclusions formed in a given population of cells and dividing the number of total IFU by the volume of EBs used for infection. Percentage of infected cells is defined by the relative number of infected cells compared to the total number of cells (infected and uninfected). The percent infection approach considers well-to-well variation of eukaryotic cell number often observed within microplates, and

Fig. 2.6. Automated enumeration of *C. trachomatis* serovar D and *C. muridarum* infection. Percent infectivity for a four-fold dilution series of (A) *C. trachomatis* serovar D and (B) *C. muridarum* were determined by the automated method. Infections were performed in triplicate wells for each dilution. Percent infectivity was calculated based on the number of infected and uninfected cells identified by CellProfiler Analyst. Error bars indicate standard deviation of the triplicate wells.

A



B



therefore, is advantageous in applications such as inhibitory compound screening. The number of IFU within a given well is largely influenced by the number of cells available for EBs to infect. Therefore, well-to-well variation of host cell number must be considered in order to make relative comparisons of the infection levels among different wells. On the other hand, when solely considering the number of inclusions formed, the automated method may lead to underestimation of the number of inclusions. This is because the automated method utilizes a *binary classification* and does not reflect on how many inclusions are present in a single cell when multiplicity of infection (MOI) is greater than one. To demonstrate that this automated method could be easily adapted for chlamydial quantification using both of these approaches, a minor modification was applied to the CellProfiler pipeline. Specifically, the last module within the pipeline (*ExportToDatabase*) was modified to facilitate classification of individual *inclusions* rather than *cells* by CellProfiler Analyst (Fig. 2.1C). As a result, CellProfiler Analyst was successfully trained to distinguish between productive inclusions and aggregation of EBs or background staining. The precision of the automated method relative to the conventional manual inspection in determining IFU was examined (Table 2.1). IFU per milliliter calculated from the number of IFU reported by the automated methods and manual inspection were 9.98×10^{-3} and 1.03×10^{-4} , respectively. These data demonstrate that the automated method can be used for accurate quantification of *Chlamydia* infection by both of the two commonly used approaches.

Adaptation of alamarBlue cytotoxicity assay for quantification of *Chlamydia* infection

A productive developmental cycle of *C. trachomatis* strain L2 typically takes 48 to 72 hours, and is completed with the host cell lysis or extrusion [186]. In order to correlate the infection level with the host cell viability, we first determined a time-point, where the lysis of infected host cells results in a significant decrease of the overall host cell viability among the sample. In addition, to validate this method as an effective enumeration tool for *Chlamydia* infection, sensitivity of the assay was examined by analyzing samples infected with 2-fold dilution of *Chlamydia* EB. The parallel samples were also prepared and analyzed by the conventional IFA for comparison.

Table 2.2 summarizes the reducing capability of infected samples as well as the infection level determined by IFA. To statistically compare and evaluate the datasets, the Z' factor was determined. The Z' factor is a statistical parameter used to evaluate the quality of high-throughput assays, where Z' factor values greater than 0.5 are indicative of excellent low variation and signal dynamic range [187]. At 24 hpi, the reduction capability of infected sample was mostly comparable to that of mock-infected cells with exception of the three highest inoculation doses (90.64, 92.94, and 95.77% infectivity determined by IFA). Given that cell lysis upon completion of developmental cycle takes place 48-72 hpi, this decrease at the high inoculation doses is likely to be a result of cytotoxicity due to over-infection, rather than completion of the developmental cycle. At 48 and 72 hpi, the expected dose-dependent decrease in reduction capability was observed ranging from 108.17 to 6.90% and 105.87 to 4.21%, respectively, over increasing inoculation doses. The Z' factor for 48 hpi analysis was determined to be above 0.5 for the three highest inoculation doses, which corresponds to 95.77 to 90.64% infection reported by IFA. When infection was allowed to proceed for additional 24 h,

Table 2.2. Comparison of IFA and alamarBlue assay for enumeration of *C. trachomatis* infection.

Infection level (%) 24 hpi, IFA	Reducing capability of infected cells relative to the mock-infected cells								
	24 hpi			48 hpi			72 hpi		
	Average	STDEV	Z'factor	Average	STDEV	Z'factor	Average	STDEV	Z'factor
95.77	26.40	1.36	0.56*	6.90	0.68	0.83*	4.21	0.08	0.98*
92.94	61.80	3.66	-0.02	18.83	0.89	0.79*	6.13	0.28	0.97*
90.64	87.84	4.71	-2.47	46.35	0.27	0.72*	13.42	1.59	0.92*
86.70	103.00	6.16	-14.50	69.27	0.78	0.47	27.96	2.17	0.88*
63.95	107.29	2.15	-3.74	85.86	2.26	-0.47	49.17	2.91	0.79*
43.61	109.90	5.82	-3.60	96.11	1.15	-3.50	74.03	2.82	0.60*
20.80	114.35	4.43	-1.88	106.17	0.42	-1.48	85.67	1.35	0.57*
14.79	119.60	9.48	-1.88	99.23	5.43	-38.28	88.67	2.00	0.29
7.20	114.30	0.77	-1.13	102.47	0.49	-5.27	100.06	0.72	-75.83
3.06	108.15	8.51	-5.58	105.74	0.61	-1.76	100.58	0.47	-4.98
2.16	102.65	4.24	-14.41	103.46	1.90	-4.71	102.67	2.49	-2.57
1.08	120.76	8.34	-1.56	108.26	0.66	-0.94	103.06	0.50	-0.17
0.98	109.81	0.48	-2.01	108.17	2.61	-1.67	105.87	0.66	0.31
	R ² = 0.52			R ² = 0.79			R ² = 0.98		

* 1>Z-factor value ≥ 0.5 is indicative of an assay with suitable screening characteristics.

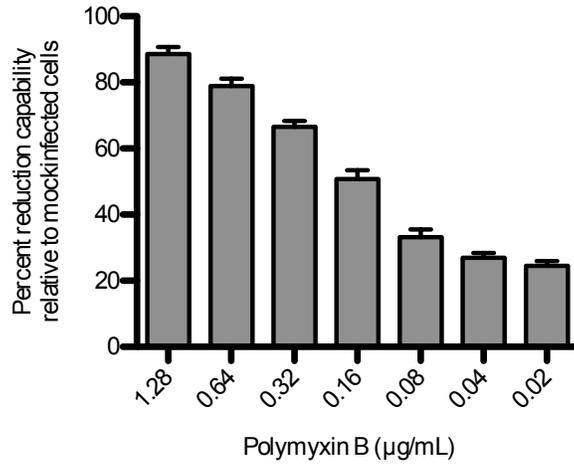
the 7 highest inoculation doses corresponding to 95.77 to 20.80% infection level resulted in Z' factor greater than 0.5, indicating that the analysis in this infection range can be performed with a high degree of confidence at 72 hpi. In order to utilize this assay in measuring infection level or assessing anti-*Chlamydia* activity of compounds, a sufficient dynamic range must be provided, and therefore, 72 hpi was chosen as the time point for the subsequent analysis. In addition, when a correlation coefficient (R^2) was used to determine the degree of similarity between the two methods, linear regression analysis between the dataset obtained from alamarBlue assay (72 hpi) and IFA resulted in a correlation coefficient (R^2) of 0.98, indicating a strong correlation between the two analyses and further supporting that enumeration of *Chlamydia* infection by alamarBlue assay is highly comparable to the conventional IFA.

Validation of alamarBlue-based assay as anti-*Chlamydia* compound screening tool

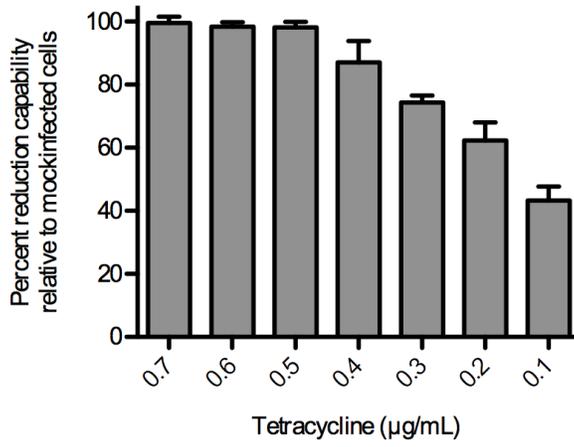
To begin validating this method as an anti-*Chlamydia* compound screening tool, inhibition of *C. trachomatis* infection by two well-established inhibitors; tetracycline hydrochloride and polymyxin B, was assessed [159, 162, 184]. Tetracycline is membrane permeable and inhibits bacterial RNA translation, whereas PMB primarily effects bacterial surface molecules (LPS/LOS) and perturbs membrane integrity. *Chlamydia* infection was performed to achieve approximately 90% infection in the untreated control sample. In both tetracycline and PMB treated samples, the compound concentration and reduction capability of infected cells resulted in a positive linear correlation with R^2 value of 0.97 and 0.99, respectively (Fig. 2.7). For tetracycline, concentration range above 0.50 $\mu\text{g/mL}$ was not included in determining this linear regression since the inhibition

Fig. 2.7. Quantification of inhibition of *C. trachomatis* infection treated by alamarBlue assay (72 hpi). Reduction capability of L929 cells infected with *C. trachomatis* was measured in the presence of known *Chlamydia* inhibitors. (A) polymyxin B and (B) tetracycline. Error bars indicate standard deviation of the triplicate infections.

A



B



plateaued at 0.50 µg/mL. Importantly, the plateau of reduction capability of infected cells at this concentration is consistent with the previously reported observation by IFA that 0.51 µg/mL was sufficient to achieve 100% inhibition of *C. trachomatis* serovar L2 [160]. Together, these data demonstrate that measuring host cell viability of infected cells can be used as an indirect enumeration of *Chlamydia* infection, as it reflected the infection level determined by the conventional IFA.

Discussion

The lack of a tool for rapid and accurate quantification of *Chlamydia* infection suitable for a large-scale compound screening is a major challenge in the development of antichlamydial agents. Limited number of methods has been reported for this purpose. *Wang et al.* described an enzymatic approach using an immunofluorescence technique that is similar to ELISA. While this method offered a significant reduction in assay time compared to the traditional manual inspection, a substantial discrepancy was reported between the new method and the traditional manual counting of chlamydial inclusions due to the limited sensitivity of ImmunoSpot reader [188]. An image-based approach for identification and enumeration of *C. psittaci* infection was reported by *Beeckman et al.* [189]. This technique demonstrated automated image acquisition and automated format analysis; however, the number of parameters utilized by the program Image J were considerably fewer compared to those measured by CellProfiler. Image J identifies inclusions based solely on the number, surface area, and intensity of the fluorescently labeled spots, making it challenging to discriminate non-invasive *Chlamydia* aggregates on cell surfaces from true inclusions. In addition, these parameters require predefined threshold values set by investigators, resulting in limited flexibility in identification of subtle and complex characteristics such as those often observed among cells infected with *Chlamydia*.

In our study, the image analysis software CellProfiler and CellProfiler Analyst were adapted to an automated image-based method for analysis of cell cultures infected with *C. trachomatis*. Using a customized pipeline applied to CellProfiler followed by machine-learning training sessions performed with CellProfiler Analyst, this automated

method yielded quantification of *C. trachomatis* infection highly comparable to the conventional manual inspection (Table 2.1). The suitability of this automated method as a large-scale application was characterized in terms of sensitivity, precision, and accuracy. Enumeration of a total of 72 samples infected with a dynamic range of purified EBs demonstrated high sensitivity of the automated method (Fig. 2.3). Low standard deviations among the replicates of each sample and between the aliquots demonstrated that the automated method was able to perform quantification of large number of samples with high precision. These data supported that iterative training and supervised machine-learning, powerful features within CellProfiler Analyst, were able to accomplish highly specific identification of inclusions by distinguishing chlamydial inclusions from background staining; this has been a major challenge in previously reported automated approaches.

When contrasting the two methods, one significant advantage the automatic method exhibited over manual enumeration was the ability to rapidly analyze large datasets. Analysis of a 96-well plate by the automated method, accommodating enumeration of 1440 images consisting approximately 130,000 cells on average per plate, was completed within two hours following automated image acquisition. During these two hours, an average hands-on time was less than 30 minutes with the rest being spent on computer processing. Notably, analysis of multiple 96-well plates does not require additional time compared to a single plate analysis. This is because regardless of the number of samples, only one training session is required in order to establish a differential rule-set, which can be applied across the entire experiment. Considering that manual enumeration required up to 2 minutes per field of view, equivalent analysis would

take up to 48 hours by the manual method. Thus, the automated method achieved substantial time saving in sample analysis in addition to high sensitivity and precision.

This automated method is equally effective in analysis of small-scale (up to hundreds of images) or large-scale screens containing thousands of images acquired by automated microscopy. While analysis of hundreds of images by CellProfiler can be completed on a single computer in a few hours, assays described in this study consisted of thousands of images, and therefore more computational processing capabilities were required to achieve rapid analysis [190]. This challenge was addressed by utilizing a dedicated 'cluster' of processors (16 quad-core 2.5 GHz processors) for image processing. With addition of an extra module to the end of a pipeline, CellProfiler was able to automatically divide images into small batches and create the files containing batches of images [191]. Files were then submitted as individual jobs to be processed separately by the cluster, significantly minimizing the time required for data processing.

In addition to the ability for accurate assessment of antichlamydial activity, this method may also provide insight into a possible mechanism of antichlamydial action for tested compounds. In this study, HEC and PMB were used as a model for the excipient and active ingredient component of microbicide, respectively. As anticipated, a dose-dependent inhibitory effect was observed for both compounds. Interestingly, however, the pattern of inhibition over the tested range of compound concentration was uniquely different between the two inhibitors. The inhibitory effect of HEC exhibited direct proportionality to the concentration of the compound before reaching complete inhibition (Fig. 2.4B). In contrast, the inhibitory effect of PMB plateaued without reaching complete inhibition and was not proportional to compound concentration (Fig. 2.4C).

Therefore, it may be suggested that two compounds use different modes of inhibition mechanisms, and these observations could be used to begin elucidating the precise mechanism of inhibition (e.g. adherence, entry, etc.). It is also important to note that the automated method consistently reported datasets with remarkably low standard deviations. When compared to datasets with higher standard deviations (i.e. manually enumerated datasets), these datasets offer greater significance to relatively subtle observations such as the difference in pattern of inhibition noted between HEC and PMB.

Whereas the image-based assay allows for detailed visualization and direct quantification of *Chlamydia* infection, IFA-based assay involves a sequence of liquid-handling steps, which is a rate-limiting factor in this process. AlamarBlue cytotoxicity assay offers a simple alternative with reduced liquid handling requirements and costs associated with immunofluorescence-based assay. Although this method involves 72-hour incubation time, the high-throughput capacity of a microplate reader considerably reduces the active time required for analysis. In addition to the ease and cost-effectiveness, the use of host cell metabolic activity as a readout can serve a dual function in analysis of *Chlamydia* infection as well as cytotoxicity of compounds. This would be greatly advantageous in high-throughput screening for identification of compound with anti-*Chlamydia* activity and no host-cell toxicity.

Chapter III.

***In vitro* Assessment of Anti-*Chlamydia* Properties among Polymyxin B Analogs and Cellulose Ether Polymers**

Abstract

In the current absence of clinically approved vaccine against genital *Chlamydia* infection, vaginally delivered topical microbicides are a viable, globally applicable option as an alternative prevention strategy. Two main components of microbicides are the active ingredient with pharmacological activity against the pathogen, and the excipient, which is used for formulation and delivery of the active ingredient. Candidate active ingredients in this study are analogues of an antibiotic polymyxin B (PMB) rationally designed to target lipid A portion of Gram-negative LPS. Excipient candidates are cellulose ether polymers. These polymers are hydrophilic and have pharmaceutical properties that favor their use in the preparation of controlled release formulations for long-term administration of vaginal microbicide. Although excipients, by definition, are considered pharmacologically inactive ingredients, a number of studies have reported antimicrobial activity of excipients. In this study, anti-*Chlamydia* activity and cytotoxicity of 18 PMB analogues and 12 cellulose ether polymers were evaluated as the active ingredient and excipient of vaginally administered topical microbicide, respectively. Out of 18 PMB analogues, 14 compounds inhibited *Chlamydia* infection by greater than 60%. Interestingly, distinct structural difference was evident in the 4 compounds without inhibitory effect on *Chlamydia* infection. A wide range of anti-chlamydial activity was observed among cellulose ether polymers.

Introduction

With over 1.3 million new cases reported each year, infection due to *C. trachomatis* is the most commonly reported infection in the United States [192]. Although *C. trachomatis* infections are highly treatable with appropriate antibiotics upon detection, the vast majority (up to 70%) of infections in women are undiagnosed due to the high asymptomatic rate, and therefore progress untreated [13]. Untreated *C. trachomatis* infections can result in severe long-term consequences in women, including pelvic inflammatory disease (PID), ectopic pregnancy, and infertility [193]. In addition, *C. trachomatis* infections have been linked to increasing the risk of acquisition and transmission of HIV [194]. These concerns associated with *C. trachomatis* infection highlight the urgent need for novel preventative strategies; however, despite ongoing efforts, protective vaccines against *Chlamydia* are currently 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.

Topical microbicides consist of two components; the active ingredient and the excipient [195]. The active ingredient possesses pharmacological activity and provides protection against transmission of infectious agents through various mechanisms, such as inactivation of the pathogen or blocking specific stages of pathogenesis. The excipients are pharmacologically inert delivery vehicles that allow uniform distribution of the active ingredient while ensuring product stability and appropriate pH in vaginal mucosa [196]. Excipients can be categorized into a number of groups including gelling agents,

preservatives, solvents, antioxidants [197]. One of the key challenges in the development of topical microbicide is identifying compounds that hold the potential to be effective against the disease causing pathogens while causing minimal or no adverse effect upon long-term repeated usage.

The active ingredient candidates in this study are a series of rationally designed compounds, developed by David and colleagues, targeting LPS of Gram-negative bacteria for therapeutic options against septic shock caused by LPS (endotoxin) [198-200] (Fig. 3.1). In order to sequester and neutralize endotoxins, small molecules were developed targeting lipid A, the biologically active and toxic moiety of LPS, which is structurally highly conserved among Gram-negative bacteria [198-200] (Fig. 1.3). Lipid A is anionic and amphiphilic, and allows interaction with a variety of cationic hydrophobic ligands [201]. Polymyxin B (PMB), a cationic peptide antibiotic isolated from *Bacillus polymyxa* [202] (Fig. 3.2 A), is well known to bind lipid A and neutralize the endotoxicity *in vivo* and *in vitro* [203-205]. However, the demonstrated toxicity of PMB in eukaryotic cells limits its use in therapy or prophylaxis [206]. In search for non-toxic PMB analogues with potency comparable to that of PMB, an NMR-derived model of PMB-lipid A complex [207] (Fig. 3.2 B), as well as *in vitro* activity studies were utilized to define and validate the crucial pharmacophore for optimal recognition and neutralization of lipid A [200]. Based on these findings, small molecule analogues of PMB were rationally designed and synthesized. As reviewed in preceding chapter (Chapter I. Introduction: Chlamydia lipooligosaccharide), chlamydial LOS shares highly conserved bis-phosphorylated glucosamine backbone of lipid A with *E. coli* LPS. Given that PMB interacts with LPS through this lipid A backbone structure, it is expected that similar

Fig. 3.1. Structures of polymyxin B (PMB) analogues. In search for non-toxic PMB analogues with potency comparable to that of PMB, 18 PMB analogues were rationally designed based on the crucial pharmacophore for optimal recognition and neutralization of lipid A determined from the NMR-derived model of PMB-lipid A complex and *in vitro* activity studies.

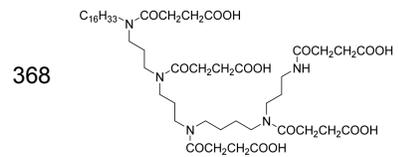
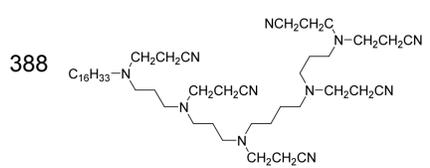
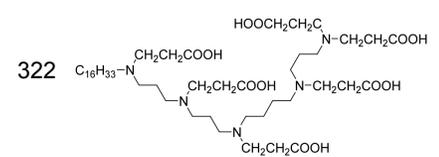
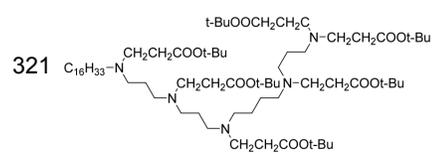
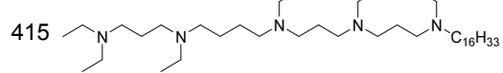
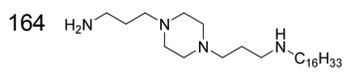
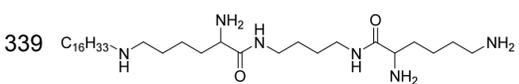
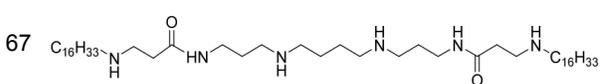
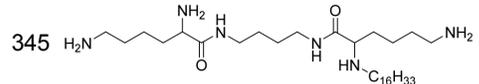
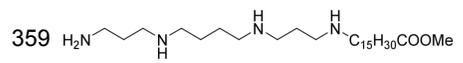
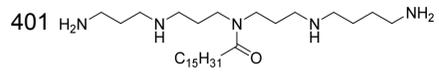
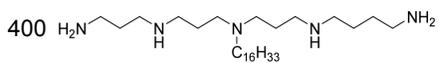
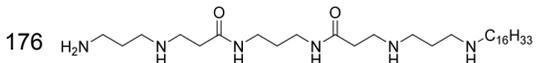
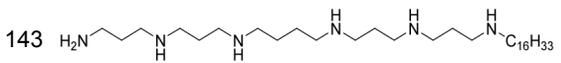
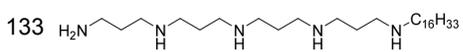
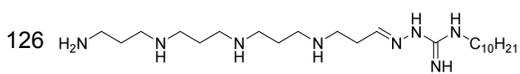
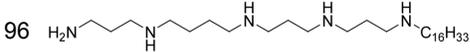
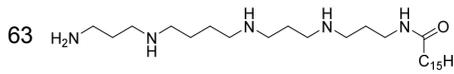
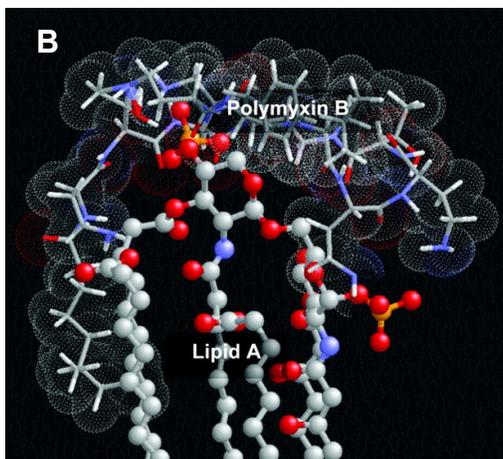
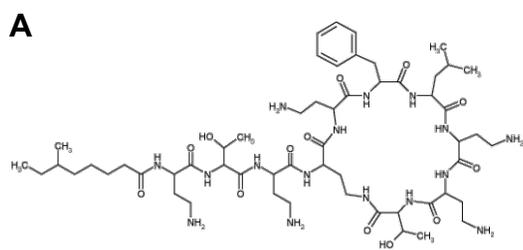


Fig. 3.2. A NMR-derived model of the PMB-lipid A complex (adapted from *Sil et al.* [198]). (A) Structure of PMB. (B) A NMR-derived model. PMB is in stick representation with van der Waals surface, and lipid A is displayed in ball-and-stick representation. Salt bridges (dotted lines) are proposed to occur between each phosphate group on lipid A and two pairs of the NH₂ groups of PMB [207].



interaction would take place between PMB and chlamydial LOS. Therefore, it was hypothesized that PMB analogues designed for neutralization of *E. coli* LPS would bind to chlamydial LOS and potentially exhibit inhibitory effects on *Chlamydia* infection by modifying the function of LOS crucial during the developmental cycle.

The excipient candidates in this study are cellulose ether polymers commonly used in vaginal gels [208] (Table 3.1). Hydroxyethyl cellulose (HEC), hydroxypropylmethyl cellulose (HPMC), methylcellulose (MC), and sodium carboxymethylcellulose (NaCMC) are cellulose ethers that differ in their substitution type, as well as degree of substitution (DS) and molar substitution (MS) (Fig. 3.3). The degree of substitution is defined as the average number of hydroxyl groups substituted per anhydroglucose unit, whereas the molar degree of substitution is defined as the average number of moles of substituent per anhydroglucose unit.

The cellulose ether polymers are hydrophilic and have pharmaceutical properties that favor their use in the preparation of controlled release formulations [209]. By definition, excipients are typically chosen from compounds with no pharmacological activity, and therefore lack any therapeutic properties. However, a number of studies have reported antichlamydial activity of excipients *in vitro* [197, 210, 211]. Specifically, HEC was demonstrated to be inhibitory against *C. trachomatis* infection of HeLa 229 cervical epithelial cells [211]. Excipient compounds that possess inhibitory effect against *C. trachomatis* would be beneficial as they can potentially enhance the overall efficacy of antichlamydial microbicide when used concomitantly with the active ingredient. As importantly, considering the use of cellulose ether polymers as “inactive” excipients in vaginal microbicides, it is critical to ensure the compounds do not contribute to increased

Table 3.1. Cellulose ether polymers

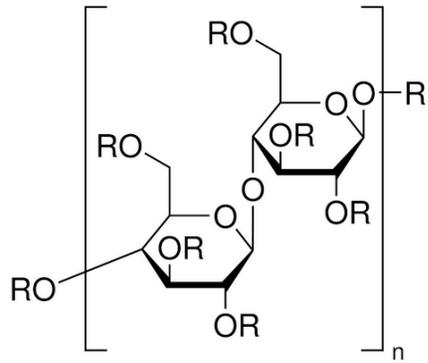
		Source	MW (kDa)	DS ^a	MS ^b
HEC	Hydroxyethyl cellulose				
	HEC-1	Sigma-Aldrich	1,300	1.5	2.5
	HEC-2	Sigma-Aldrich	90	1.5	2.5
	HEC-3	Sigma-Aldrich	250	1.0	2.0
	Natrosol	Hercules/Aqualon	1,000	1.5	2.5
NaCMC	Sodium carboxymethylcellulose				
	NaCMC-4	Sigma-Aldrich	250	0.9	NA
	NaCMC-5	Sigma-Aldrich	250	1.2	NA
	NaCMC-7HF	Hercules/Aqualon	700	0.7	NA
HPMC	Hydroxypropylmethyl cellulose				
	HPMC-7	Sigma-Aldrich	10	1.8-2.0	0.2-0.3
	HPMC-8	Sigma-Aldrich	86	1.8-2.0	0.2-0.3
	HPMC-9	Dow Chemical	86	1.8	0.13
MC	Methylcellulose				
	MC-6	Sigma-Aldrich	40	1.6-1.9	NA
	MC-A4M	Dow Chemical	86	1.8	NA

^a. Degree of substitution (DS) = the average number of hydroxyl groups replaced by the substituents per anhydroglucose ring unit of cellulose

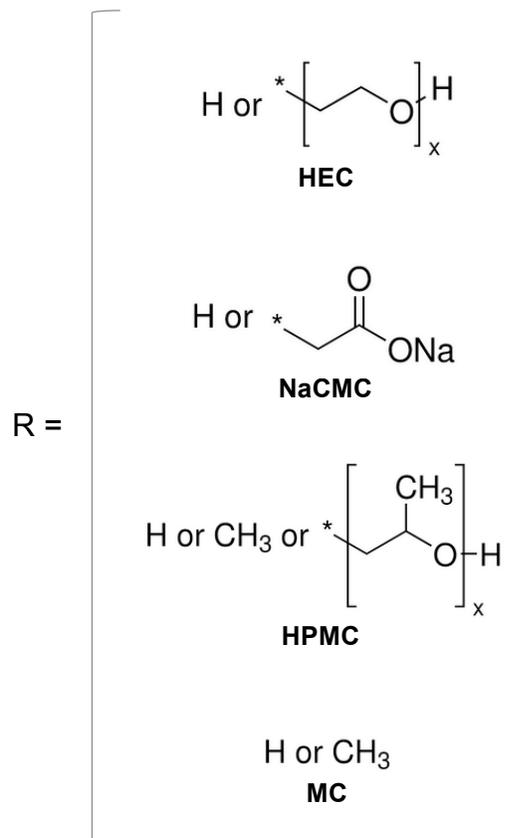
^b. Molar substitution (MS) = the average number of moles of substituents per anhydroglucose ring unit of cellulose

Fig. 3.3. Structure of cellulose ether polymers. (A) Structure of anhydroglucose units of cellulose. R denotes the substituents to the anhydroglucose unit. (B) The substituents for HEC (hydroxyethyl cellulose), NaCMC (sodium carboxymethylcellulose), HPMC (hydroxypropylmethyl cellulose), and MC (methylcellulose).

A



B



infection susceptibility by cytotoxicity or other mechanisms. *In vitro* susceptibility of *C. trachomatis* to 18 PMB analogues and 12 cellulose ether polymers were evaluated to be considered as the active ingredient and the excipient component of vaginally-delivered microbicide, respectively.

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 [177]. 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 gentamycin (MP Biomedicals, Santa Ana, CA) at 37°C in a humidified atmosphere of 5% CO₂.

Compounds preparation

PMB analogues were obtained in powder form and dissolved in 100% DMSO to 1 mM. Each compound was serially diluted to appropriate concentrations in HBSS. Hydroxyethyl cellulose (HEC), sodium carboxymethylcellulose (NaCMC), hydroxypropylmethyl cellulose (HPMC), and methylcellulose (MC) were obtained in powder. The source, molecular weight, degree of substitution (DS), and molar substitution (MS) for each compound are summarized in Table 3.1. Each cellulose ether compound was dissolved in HBSS to appropriate concentrations, vortexed to ensure homogeneity, and stored overnight at 4°C to allow hydration of the polymers. Each polymer solution was adjusted to pH 7.4.

AlamarBlue cytotoxicity assay

L929 mouse fibroblast cells were plated in a 96-well plate (Bioexpress, Kaysville, UT) at density of 7×10^4 cells/mL, 200 μ L/well, and incubated overnight. Microbicide candidate compounds were serially diluted in RPMI 1640 culture to the appropriate concentrations. Culture medium was removed and 200 μ L/well of fresh medium containing the tested compound was added to a monolayer of cells. The plates were incubated at 37 °C for 18 h. Following the incubation period, culture medium was removed from the cells and 200 μ L of RPMI 1640 culture medium without phenol red containing 10% (v/v) alamarBlue reagent (Life Technologies, Grand Island, NY) was added. The absorbance was measured in a PowerWave microplate spectrophotometer (BioTek, Winooski, VT) at 570 nm (reduced) and 600 nm (oxidized) with KC⁴ data collection and analysis software (BioTek, Winooski, VT) after 8 h. 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 manufacture: $\frac{(\epsilon_{ox})\lambda_2 A \lambda_1 - (\epsilon_{ox})\lambda_1 A \lambda_2}{(\epsilon_{ox})\lambda_2 A' \lambda_1 - (\epsilon_{ox})\lambda_1 A' \lambda_2} \times 100$, where ϵ_{ox} = molar

extinction coefficient of alamarBlue oxidized form, ϵ_{ox} = molar extinction coefficient of alamarBlue reduced form, A = absorbance of test wells, A' = absorbance of positive control well (mock-infection), λ_1 = 570 nm, and λ_2 = 600 nm.

***Chlamydia* infection and inhibition assays**

Purified *C. trachomatis* L2 EBs were diluted to the appropriate concentrations in Hank's Buffered Salt Solution (HBSS, Mediatech, Inc., Holly Hill, FL). Microbicide candidate compound or DMSO as a mock-treatment control was diluted in either HBSS

containing diluted EBs to the appropriate concentrations, according to treatment types. Bacteria and compound were incubated for an hour at room temperature for pre-treatment. Following an hour, the mixtures were added to a monolayer of cells. Cells were inoculated at room temperature for 2 h. After the inoculation period, cells were washed once with HBSS, fresh culture medium without compounds was added, and incubated at 37°C in an atmosphere of 5% CO₂. Twenty-four hours post infection (hpi), cells were washed once with HBSS and fixed with 100% methanol for 10 minutes at room temperature. Samples were kept in phosphate buffered saline (PBS) at 4°C until the time of analysis.

Immunofluorescence microscopy

Following fixation, samples were stained with 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 DAPI in PBS. DAPI was removed and 90% (v/v) glycerol in PBS was added to prevent rapid fading of fluorescence during analysis. Plates were stored in the dark at 4°C until imaging. Images were captured and analyzed using CellProfiler and CellProfiler Analyst (CP/CPA) as described in the preceding chapter (Chapter II. Development and Validation of Novel Analysis Methods for *Chlamydia* Infection).

Results and Discussion

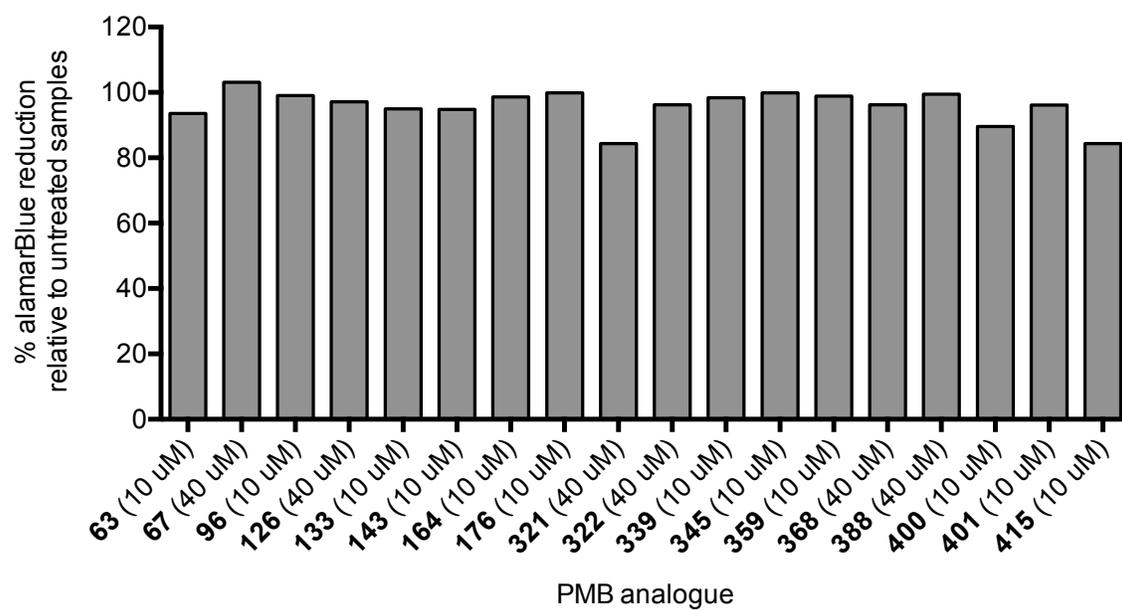
Cytotoxicity of PMB analogues in mouse L929 fibroblast cells

Due to the obligate intracellular nature of *Chlamydia*, it is also important to ensure that inhibition of *Chlamydia* growth is a direct result of disruption of chlamydial factor rather than toxicity to the host cell. Since the PMB analogues had not been studied in cell culture previously, cytotoxicity of the compounds on L929 cells was assessed prior to evaluating the inhibitory effect to determine appropriate working concentrations. The metabolic activity of the host cell was quantitatively analyzed using alamarBlue cytotoxicity assay following exposure to each compound. The standard procedure for *Chlamydia* infection of cell culture consists of 2-hour infection of the host cell monolayer followed by the removal of bacteria and incubation of infected cells at 37 °C until the time of analysis 24 hpi. Therefore, monolayers of L929 cells were incubated with serially diluted PMB analogues for 2 h, and the metabolic capability of the compounds was measured following 24 h incubation at 37 °C. A broad concentration range (0.24-100 µM) was tested, followed by several rounds of analysis to narrow the concentration range (data not shown). An appropriate working concentration, defined as the highest concentration at which the reducing capability of the cells exposed to a compound was greater than 80% relative to DMSO control, was determined for each PMB analogue compound (Fig. 3.4).

Anti-*Chlamydia* activity of PMB analogues in mouse L929 fibroblast cells

To examine the effect of PMB analogues on *Chlamydia* infection, L929 cells were infected with *C. trachomatis* in the presence of the compounds, and infection level

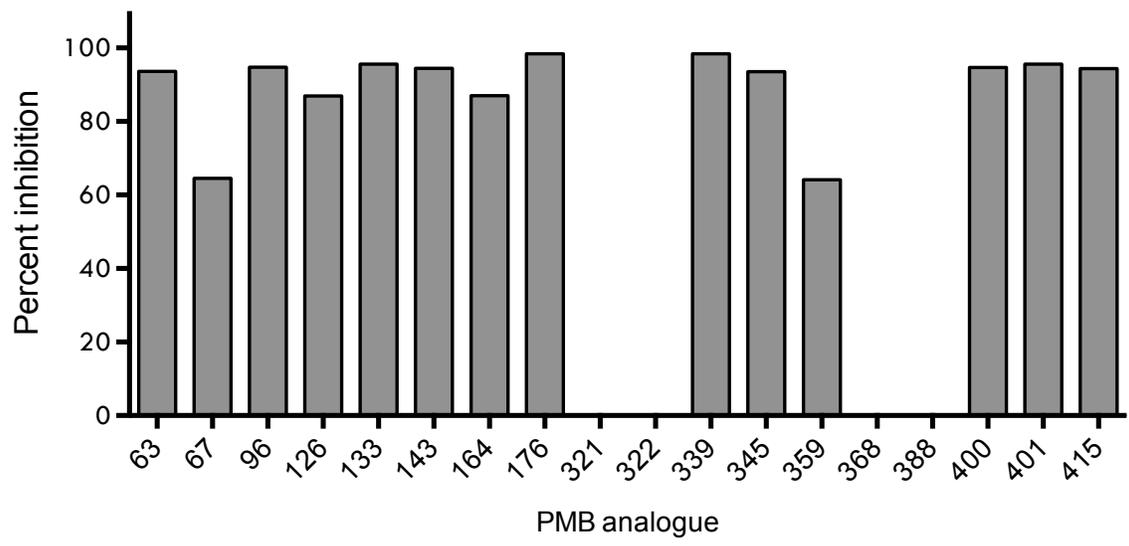
Fig. 3.4. Cytotoxicity analysis of PMB analogues. Metabolic activity of L929 cells were measured by alamarBlue redox indicator following 2h incubation with each PMB analogue. A working concentration for each compound, as defined as the highest concentration at which the metabolic activity of the host cell resulted in greater than 80% of the control sample, is indicated on the x-axis. Reducing capability of samples treated with the compounds relative to untreated control samples were determined after 24 h incubation.



was enumerated 24 hpi. Considering the rational design of PMB analogues, possible inhibitory effect is expected to be through interaction with chlamydial surface that is mediated by the affinity for LOS. For this reason, highly purified *C. trachomatis* EBs were pre-treated with each analogue for an hour prior addition to the host cell to allow sufficient time to interact with the bacterial surface. Each analogue was tested at the working concentration previously determined using alamarBlue cytotoxicity assay.

Out of 18 analogues, 14 analogues resulted in inhibition of intracellular *Chlamydia* growth by greater than 60%, and 12 of which inhibited infection by greater than 85% (Fig. 3.5). Interestingly, the four analogues with no inhibitory activity (DS-321, DS-322, DS-368, and DS-388) share apparent structural similarities (Fig. 3.1). One of the major structural features shared among these four analogues is a higher degree of substitution on amines, which is absent in the analogues with inhibitory activity. This structure-activity correlation, not surprisingly, was in agreement with the previously defined structural requirements for optimal recognition of lipid A [200]. It has been demonstrated that binding of a PMB analogue to lipid A requires the simultaneous interaction of two protonatable amine functions on the analogues with the negatively charged phosphates on lipid A [200]. Whereas most of the PMB analogues contain primary terminal amines and secondary nonterminal amines, four analogues without anti-*Chlamydia* activity contain secondary terminal amines and tertiary nonterminal amines. This suggests a possibility that bulky substitutions at these amines may sterically hinder the interaction between the analogue and lipid A, and result in the absence of inhibitory activity.

Fig. 3.5. Inhibition of *C. trachomatis* infection by PMB analogues. *C. trachomatis* EBs were treated with a PMB analogue or DMSO (mock-treatment) for an hour prior to infection. L929 cells were infected with pre-treated EBs and cultured for 24 h. Infected cultures were fixed with methanol and immunofluorescently stained 24 hpi. *Chlamydia* growth was quantified using the image-based automated enumeration method described in Chapter II. Percent inhibition was calculated based on the number of inclusion positive cells in DS-96 treated samples relative to DMSO treated samples.



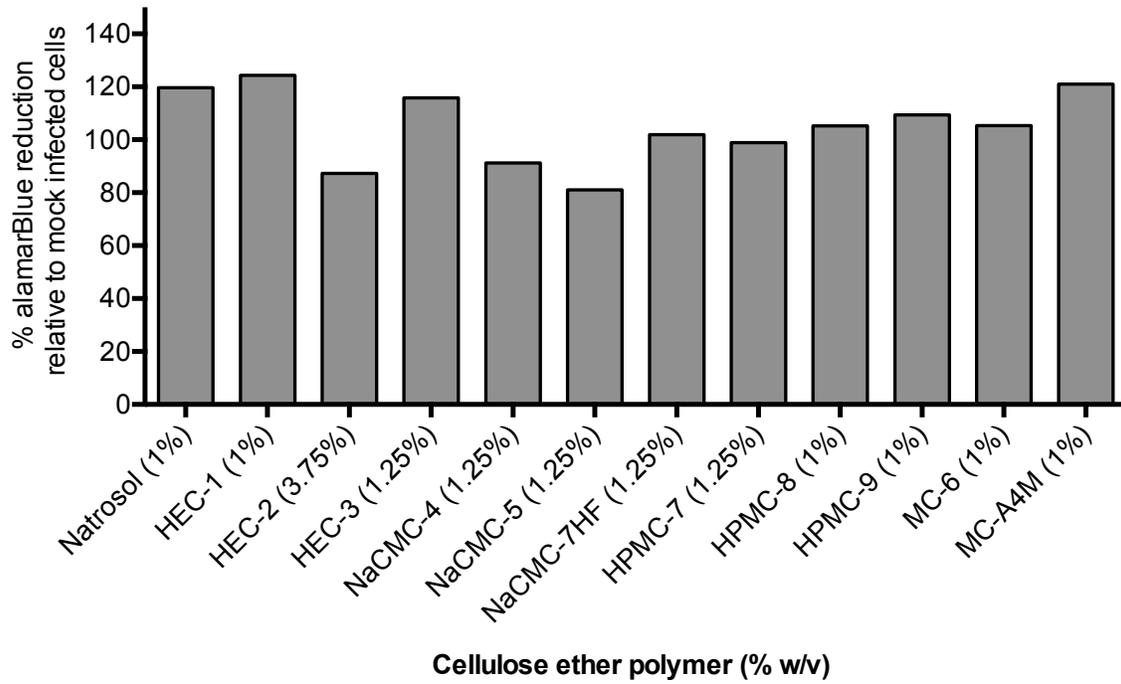
Further activity study and structural optimization will be necessary for higher potency, minimum cytotoxicity, and effectiveness in animal challenge studies. Nonetheless, it is important to note that PMB analogues appear to target *Chlamydia* LOS in a similar manner to *E. coli* LPS, and effectively inhibit *Chlamydia* infection in cell culture at relatively low concentrations without apparent cytotoxicity.

Cytotoxicity and Anti-*Chlamydia* activity of cellulose ether polymers

Water-soluble cellulose ether polymers are often used in a broad variety of industrial application including many pharmaceutical products formulated with cellulose ethers [209]. Although cellulose ethers are known to be non-toxic in animals, it is still crucial to perform cytotoxicity assay in tissue culture, as the lack of toxicity in animals may not always translate into cell culture cytotoxicity. As cellulose ether polymers readily dissolve in water to form viscous solution, one of the experimental challenges presented by these polymers is the ability for accurate pipetting. Therefore, all compounds were diluted in buffer until the viscosity of the polymer was in such that accurate pipetting could be achieved. Once working concentrations were determined, alamarBlue assay was performed to ensure that the polymers had no cytotoxic properties on the host cells at these concentrations (Fig. 3.6).

Since the cellulose ether polymers are viscous in nature, it was crucial to ensure that the inhibitory effect is not a result of the physical barrier, where the viscosity simply limits access of EBs to the host cell surface. Ideally, the working concentration of polymers should be standardized based on the factors such as viscosity and buoyant density of EBs in each polymer solution. In the absence of these studies, polymer

Fig. 3.6. Cytotoxicity analysis of cellulose ether polymers. Metabolic activity of L929 cells were measured by alamarBlue redox indicator following 2h incubation with each cellulose ether polymer diluted to the concentration with no apparent viscosity. Reducing capability of samples treated with the compounds relative to untreated control samples were determined after 24 h incubation. Error bars indicate standard deviation of the duplicate wells.



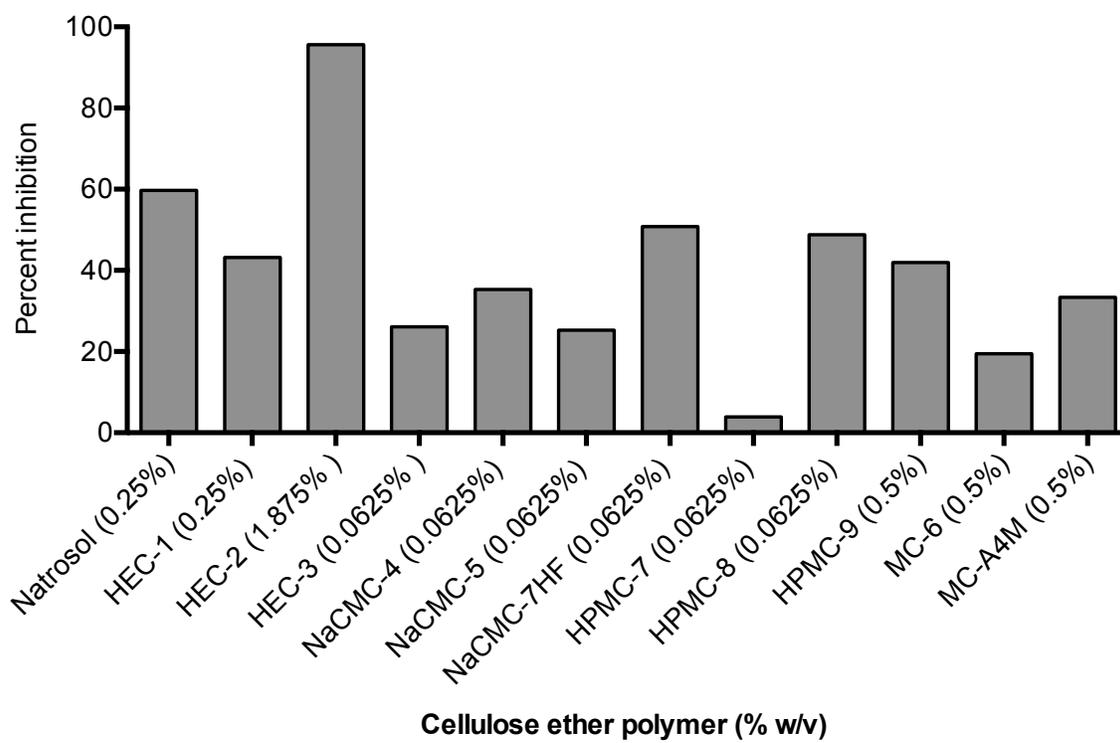
solutions were diluted until no longer viscous. *C. trachomatis* EBs were pre-treated with each of the polymers prior to infection and the growth of *Chlamydia* was analyzed 24 hpi. Although none of the polymers exhibited inhibitory activity as high as HEC-2, 90 kDa HEC previously shown to have anti-*Chlamydia* activity, all but one polymers (HPMC-7) exhibited greater than 20% inhibition in *Chlamydia* growth (Fig. 3.7). Inhibition levels were 26.1 to 95.6% for hydroxyethyl cellulose, 25.2 to 50.8% for sodium carboxymethylcellulose, 3.9 to 48.8% for hydroxypropylmethyl cellulose, and 19.5 to 33.3% for methylcellulose. No correlation was evident between the inhibitory activity and the degree of substitution, molar substitution, or molecular weight. How the cellulose-ether polymers inhibit *Chlamydia* infection remains to be determined. However, as reviewed in Chapter I, polysaccharides are thought to play a crucial role in the interaction of *Chlamydia* and the host cell surface in the initial stages of the developmental cycle. Therefore, it is possible that cellulose ether polysaccharide acts as competitive inhibitors for chlamydial adhesion.

Combined use of DS-96 with HEC results in synergistic effect

In the future animal challenge model for microbicide evaluation, the active ingredient candidates will likely require formulation into a gel suitable for topical application. When multiple compounds are applied together, the effect of one compound can depend on the presence or absence of the other compound. Such interactions between compounds are classified as additive, synergistic, or antagonistic depending on whether their combined effect is equal to, greater than, or less than expected, based on the activity of the individual compounds [212]. DS-96 and HEC-2, both of which exhibited high

Fig. 3.7. Susceptibility of *C. trachomatis* to cellulose ether polymers.

C. trachomatis EBs were treated with a cellulose ether polymer or buffer (mock-treatment) for an hour prior to infection. L929 cells were infected with pre-treated EBs and cultured for 24 h. Infected cultures were fixed with methanol and immunofluorescently stained 24 hpi. *Chlamydia* growth was quantified using the image-based automated enumeration method described in Chapter II. Percent inhibition was calculated based on the number of inclusion positive cells in DS-96 treated samples relative to DMSO treated samples.

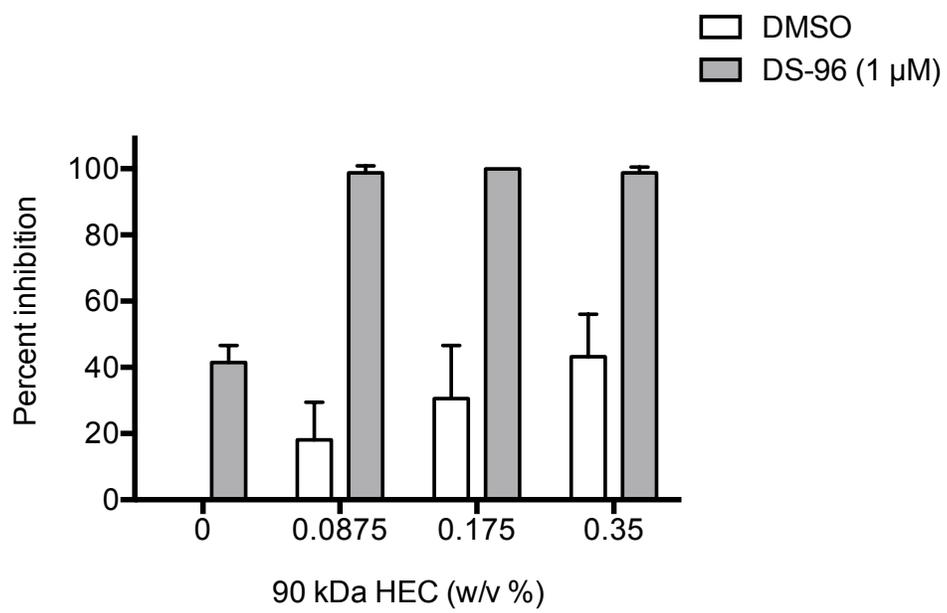


inhibitory property against *C. trachomatis*, were used to examine possible additive, synergistic, or antagonistic effects of the combined use of an active ingredient and an excipient candidate.

EBs were pre-treated with the mixture of DS-96 and HEC at concentrations where their inhibitory effects are expected to be at low level (<50%) when used individually (Fig. 3.8). When EBs were pre-treated with HEC (0.0875 to 0.35%) alone, a low level of inhibition was observed in a dose-dependent manner (18.1 to 43.2%). Similarly, DS-96 (1 μ M) alone resulted in 41.5% inhibition. Interestingly, when EBs were pre-treated with a mixture of 1 μ M DS-96 and 0.0875% HEC, *Chlamydia* growth was inhibited by 98.8%, indicating that synergistic effect exists between the two compounds because the combined use of DS-96 and HEC resulted in inhibition on *Chlamydia* growth greater than the sum of the effects of the individual compound. Whether other combinations of a PMB analogue and an excipient result in similar synergy remains to be examined. Nonetheless, this is an intriguing observation that will require further investigation to understand the underlying mechanism of synergy.

Fig. 3.8. Synergistic effect of 90 kDa HEC and DS-96 on *C. trachomatis* infection.

Effect of the combined use of 90 kDa HEC and DS-96 was examined. EBs were pre-treated with HEC (0.085, 0.175, 0.35 %) or DS-96 (1 μ M) alone, or the two compounds combined. *Chlamydia* growth was enumerated 24 hpi. Percent inhibition of *Chlamydia* growth was calculated relative to DMSO-mock treated sample. Error bars indicate standard deviation of the triplicate samples.



Chapter IV.

Lipopolysaccharide-binding Alkylpolyamine DS-96 Inhibits *Chlamydia trachomatis*

Infection by Blocking *Chlamydia* Attachment and Entry

Abstract

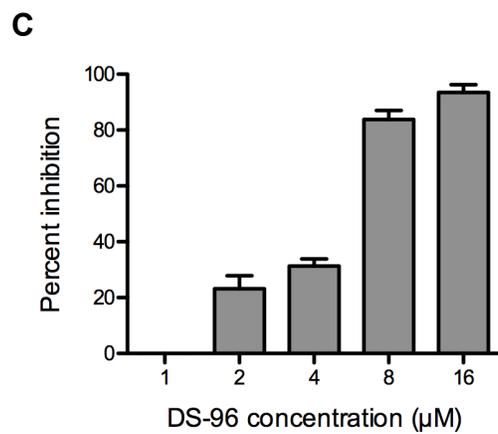
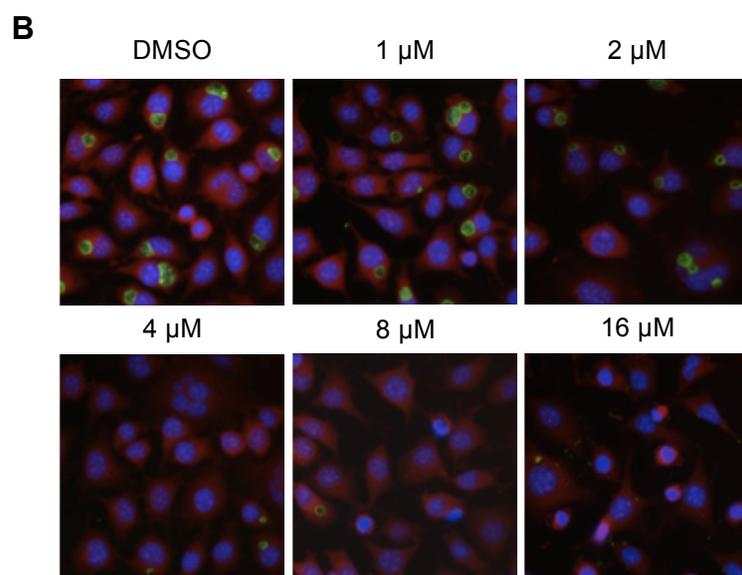
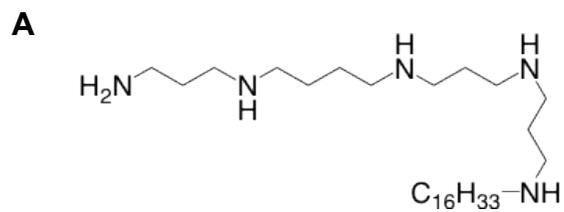
The small molecule DS-96, rationally designed for high affinity to *E. coli* lipid A, was previously demonstrated to bind and neutralize LPS from a wide variety of Gram-negative bacteria. Aside from the lack of the repeating O-antigen, chlamydial lipooligosaccharide (LOS) shares the general molecular architectures 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 a hypothesis that DS-96 inhibits *Chlamydia* infection by binding to LOS and compromising the function. In this study, anti-chlamydial 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 EBs were predominantly blocked at the attachment step, as indicated by the reduced number of EBs associated with the host cell surface following the pre-treatment. Of those EBs that were capable of attachment, the vast majority was unable to gain entry into the host cell. Inhibition of EBs attachment and entry by DS-96 suggests that *Chlamydia* LOS may be critical to these processes during the developmental cycle. Importantly, given the low associated host toxicity, DS-96 is expected to perform well in animal studies as an active compound in a vaginal microbicide.

Introduction

Blocking infection in an early stage of pathogenicity (i.e. adherence and entry) before bacteria gain access to the host is a desirable preventive 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 generally 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 the membrane integrity and providing a permeability barrier [140, 213, 214]. 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 thus may be a potential target molecule in the development of antimicrobial agents [141-144].

The small molecule DS-96, a synthetic alkylpolyamine, was previously developed to neutralize endotoxicity of LPS in the pathogenesis of Gram-negative septic shock (Fig. 4.1A) [198]. The molecule was rationally designed based on an NMR-derived model of the interaction between *E. coli* LPS and the antibiotic polymyxin B (PMB) [198, 200, 215, 216]. The cationic polypeptide antibiotic PMB is well known for the ability to bind to and neutralize the anionic lipid A moiety, which elicits strong immune response when present in systemic circulation [182]. 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 led to the discovery of a novel alkylpolyamine DS-96 [198]. In their study, DS-96 was

Fig. 4.1. DS-96 exhibits inhibition of *C. trachomatis* infection in a dose-dependent manner. (A) Structure of DS-96. (B) *C. trachomatis* EBs were treated with varying concentrations of DS-96 (0-16 μ M) or DMSO (mock-treatment) for an hour prior to infection. L929 cells were infected with pretreated EBs and cultured for 24 h. Infected cultures were fixed with methanol 24 hpi. *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 concentration of DS-96 was quantified 24 hpi using the automated image-based enumeration method (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 deviation of the triplicate samples.



demonstrated to bind to LPS with high affinity and neutralize LPS endotoxicity of a variety of Gram-negative bacteria with potency indistinguishable from that of PMB [198]. Importantly, daily administration of DS-96 at concentrations ten-fold higher than the fully protective dose resulted in no detectable toxicity in a mouse study [198]. In *Chlamydia*, PMB treatment of the organism has been demonstrated to significantly decrease infectivity in cell culture infection [152, 217, 218], and also to impair the bacterial membrane integrity [219]. Despite its effectiveness, clinical use of unmodified PMB is limited due to the associated side effects on the eukaryotic hosts [220].

C. trachomatis, like many other nonenteric mucosal pathogens, possesses lipooligosaccharide (LOS) rather than LPS in their 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 lacks the repeating O-antigen polysaccharide side chain, which is similar to a rough enterobacterial LPS [146]. Nonetheless, LOS and LPS share a conserved bis-phosphorylated glucosamine backbone of lipid A at which interaction with PMB takes place. In contrast to enteric bacteria, chlamydial lipid A has been shown to exhibit low endotoxicity, reportedly due to the fewer number of hydrocarbon and the longer chain length [147, 148]. 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. This rigid outer membrane is expected to provide structural rigidity and stability [149, 150]. To date, relatively few studies have been reported addressing the biological significance of chlamydial LOS in the unique bi-phasic developmental cycle of *Chlamydia*.

The developmental cycle of *Chlamydia* begins with the attachment of the infectious but metabolically inert elementary body (EB) to the surface of non-phagocytic epithelial cells. This attachment involves a two-step process with the initial reversible electrostatic interaction with glycosaminoglycan, followed by a more specific irreversible binding to putative cell surface receptors [221]. Following the 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 [222]. 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 transform back to EBs and are released by host cell lysis or extrusion [186].

Recently, *Nguyen et al.* demonstrated the crucial role of chlamydial LOS in proper conversion of RBs into EBs, using small molecule inhibitors of the lipid A biosynthesis pathway [151]. In contrast, little effect on the organism's replication was observed in the presence of the inhibitor. However, since the inhibitors of LOS biosynthesis do not have inhibitory effect on the pre-formed LOS, the possible role of LOS during early stages of the developmental cycle of *Chlamydia* remains to be examined.

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

Materials and Methods

Bacteria, cell culture, and compound

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 [177]. 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 gentamycin (MP Biomedicals, Santa Ana, CA) at 37°C in a humidified atmosphere of 5% CO₂. DS-96 was a kind gift from Dr. Sunil David (University of Kansas). DS-96 was obtained in powder form, dissolved in DMSO to the stock concentration of 1 mM, and stored at 4°C.

AlamarBlue cytotoxicity assay

L929 mouse fibroblast cells were plated in a 96-well plate (Bioexpress, Kaysville, UT) at density of 7×10^4 cells/mL, 200 µL/well, and incubated overnight. DS-96 was serially diluted in RPMI 1640 culture medium containing. Culture medium was removed from the 96-well plate culture and 200 µL/well of fresh medium containing various concentration (0.5-128 µM) of DS-96 was added. The plates were incubated at 37 °C for 18 h. Following the incubation period, culture medium was removed from the cells and 200 µL of RPMI 1640 culture medium without phenol red containing 10% (v/v) alamarBlue reagent (Life Technologies, Grand Island, NY) was added. The absorbance was measured in a PowerWave microplate spectrophotometer (BioTek, Winooski, VT) at 570 nm (reduced) and 600 nm (oxidized) with KC⁴ data collection and analysis software

(BioTek, Winooski, VT) after 8 h. 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 manufacture: $\frac{(\epsilon_{ox})\lambda_2 A \lambda_1 - (\epsilon_{ox})\lambda_1 A \lambda_2}{(\epsilon_{ox})\lambda_2 A' \lambda_1 - (\epsilon_{ox})\lambda_1 A' \lambda_2} \times 100$, where ϵ_{ox} = molar extinction coefficient of alamarBlue oxidized form, ϵ_{ox} = molar extinction coefficient of alamarBlue reduced form, A = absorbance of test wells, A' = absorbance of positive control well (mock-infection), $\lambda_1 = 570$ nm, and $\lambda_2 = 600$ nm.

***Chlamydia* infection and inhibition assays**

Purified *C. trachomatis* L2 EBs were diluted to the appropriate concentrations in Hank's Buffered Salt Solution (HBSS, Mediatech, Inc., Holly Hill, FL). 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 types. The mixtures of bacteria and compound were incubated for an hour at room temperature. Following an hour, the mixtures were added to a monolayer of cells. Cells were inoculated at room temperature for 2 h. For the centrifugation-assisted inoculation, the cells were centrifuged at 900 x g for 1 h. After the inoculation period, cells were washed once with HBSS, fresh culture medium was added, and incubated at 37°C in an atmosphere of 5% CO₂. For the treatment of host cells, diluted DS-96 or DMSO in HBSS containing no bacteria were transferred to ~70% confluent L929 cell monolayers and incubated for an hour at room temperature. Following the incubation period, the compound was removed, cells were washed and inoculated with the diluted bacteria. For the time-course treatment, bacteria were pre-treated, or DS-96 was added to culture medium at the indicated time period.

Twenty-four hours post infection (hpi), cells were washed once with HBSS and fixed with 100% methanol for 10 minutes at room temperature. Samples were kept in phosphate buffered saline (PBS) at 4°C until the time of analysis.

Immunofluorescence microscopy

Following fixation, samples were stained with 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 DAPI in PBS. DAPI was removed and 90% (v/v) glycerol in PBS was added to prevent rapid fading of fluorescence during analysis. Plates were stored in the dark at 4°C until imaging. Samples were visualized using Olympus ix71 inverted fluorescence microscope (Center Valley, PA) with a 40X objective with 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 CellProfiler and CellProfiler Analyst (CP/CPA) was performed as described previously [223].

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 for control at room temperature for pre-treatment. After 1 h, 300 µL of the treated bacteria were 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 were stained with monoclonal anti-LOS antibody (ViroStat, Cat# 1683) diluted 1:500 in PBS at room temperature for 1 h 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 (ViroStat, Cat# 1621) diluted 1:500 in PBS for an hour, followed by a 5 min stain with 1 µg/mL DAPI in PBS. Samples were viewed with Olympus ix71 inverted fluorescence microscope (Center Valley, PA) with a 40X objective with 0.55 numerical aperture. Images were captured using 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 pre-treatment 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 heparan sulfate (HS) was used as a positive control for chlamydial attachment. L929 cells were inoculated with compound-treated EBs and incubated at 4 °C for an hour. Following inoculation, cells were washed three times with cold HBSS to remove nonspecifically bound EBs, fixed with 100% methanol, and stained with MicroTrack *C. trachomatis* culture confirmation kit. Images were captured using SlideBook (Intelligent Imaging Innovations, Inc., Denver, CO) software and Olympus IX81 inverted microscope (Center Valley, PA) equipped with a 1.45 numerical aperture oil-immersion 100X objective and Hamamatsu EM-CCD camera.

Entry assay

Purified *C. trachomatis* L2 EBs were diluted in HBSS and incubated with 8 μ M DS-96 at room temperature for an hour for pre-treatment. 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 diluted 1:500 in PBS (ViroStat, Cat# 1621) 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 1:1000 dilution for an hour. Following three washes with PBS, the cells were incubated with primary mouse anti-*Chlamydia* antibody (Abcam, Cat# ab21019) 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 μ g/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 Olympus IX81 inverted

microscope (Center Valley, PA) equipped with a 1.45 numerical aperture oil-immersion 100X objective and 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 pre-treated with various concentrations of the compound ranging 0 to 16 μM . The standard procedure for *Chlamydia* infection of cell culture consists of 2-hour infection of eukaryotic the cell monolayer at room temperature to facilitate attachment. This is followed by washing to remove unbound bacteria and placement at 37 °C to fully enable chlamydial entry. In the inhibition assay, EBs were pre-incubated with DS-96 for an hour before addition to cell monolayer. 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 the host cell. Exposure of infectious EBs to concentration of DS-96 greater than 1 μM resulted in inhibition of chlamydial growth as indicated by a decrease in the percentage of the host cells containing inclusions after 24 h (Fig. 4.1B and C). Over a concentration range of 0-16 μM , a dose-dependent inhibition of infection was observed with greater than 80% inhibition at 8 μM relative to DMSO mock-treated culture. More than a doubling in the number of infected cells was observed as DS-96 concentration was reduced from 8 to 4 μM (83.9% and 31.3% inhibition, respectively). Interestingly, the size of individual inclusions after 4 μM DS-96 treatment appeared much smaller in comparison to those of DMSO mock-treated sample (Fig. 4.1B), 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 DS-96 treatment at 4 μM . In concert with this hypothesis,

inhibition levels observed between treatments with 2 and 4 μM of DS-96 are similar; however, the inclusions in 2 μM are larger and similar in size to 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 [198], 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. 4.2). 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. 4.1B). 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

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 the bacterial growth was observed (Fig. 4.3). This suggests a possibility that DS-96 is

Fig. 4.2. AlamarBlue cytotoxicity assay. Cytotoxic effect of DS-96 was examined using a redox indicator alamarBlue. Host cells were incubated with various concentrations of DS-96 for 2 h and metabolic activity of the host cells was measured after 24 h using spectrophotometric readings at 570 nm and 600 nm.

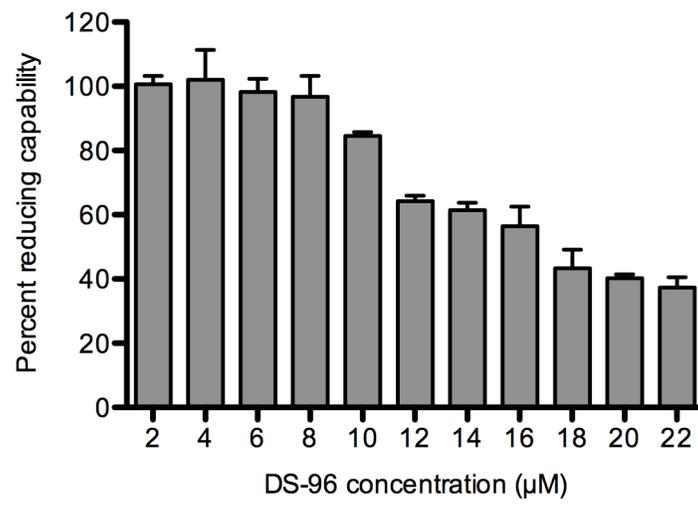
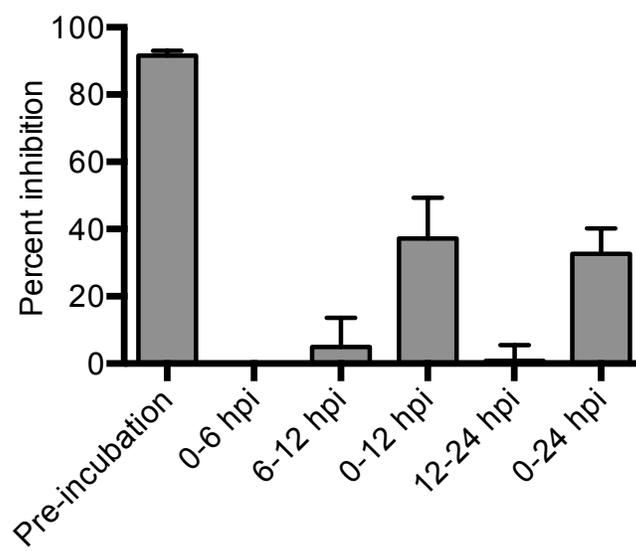


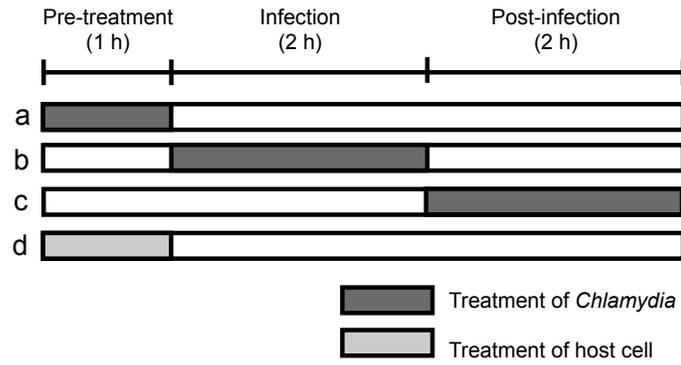
Fig. 4.3. Comparison of *Chlamydia* infection levels following various DS-96 pre-incubation conditions. Infection levels were compared among the samples treated with DS-96 in various treatment conditions during the developmental cycle. Percent inhibition of infection level in the presence of DS-96 relative to DMSO treatment was quantified using the automated image-based method. Error bars indicate standard deviation of the triplicate samples.



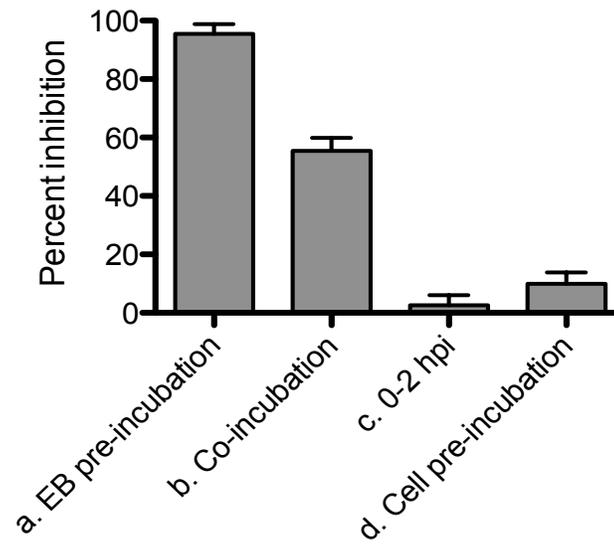
membrane impermeable, and consequently, does not have access to the bacteria once they are internalized into host cells. It could also indicate that LOS is not essential once *Chlamydia* has gained access to the cell and begun replicating. Therefore, the effect of DS-96 treatment prior to the internalization of EBs was examined further (Fig. 4.4). The treatment conditions included pre-treatment of EBs for an hour prior to addition to host cells, co-incubation of DS-96 and EBs with the host cell, and addition of the compound after EBs were allowed to incubate with host cells (Fig. 4.4A). 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 pre-treated with DS-96 for an hour prior to incubation with the bacteria. As expected, the compound was most effective in inhibiting *Chlamydia* infection when the bacteria were pre-treated prior to infection (95.5%, Fig. 4.4B). When DS-96 and the bacteria were simultaneously added to the cells (co-incubation), 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 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. Pre-treatment 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.

Fig. 4.4. DS-96 inhibits initial stages of *Chlamydia* infection through interaction with EBs rather than host cell. Infection levels were compared among various treatment conditions of DS-96 (8 μ M). (A) Treatment conditions; a. pre-treatment of bacteria, b. co-incubation of bacteria and host cell, c. addition of DS-96 to the infected culture 0-2 hpi, d. pre-treatment of the host cell. (B) Inhibition of inclusion formation relative to mock-treated samples was quantified for each treatment condition 24 hpi using the automated image-based method (32). 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 deviation of triplicate samples.

A



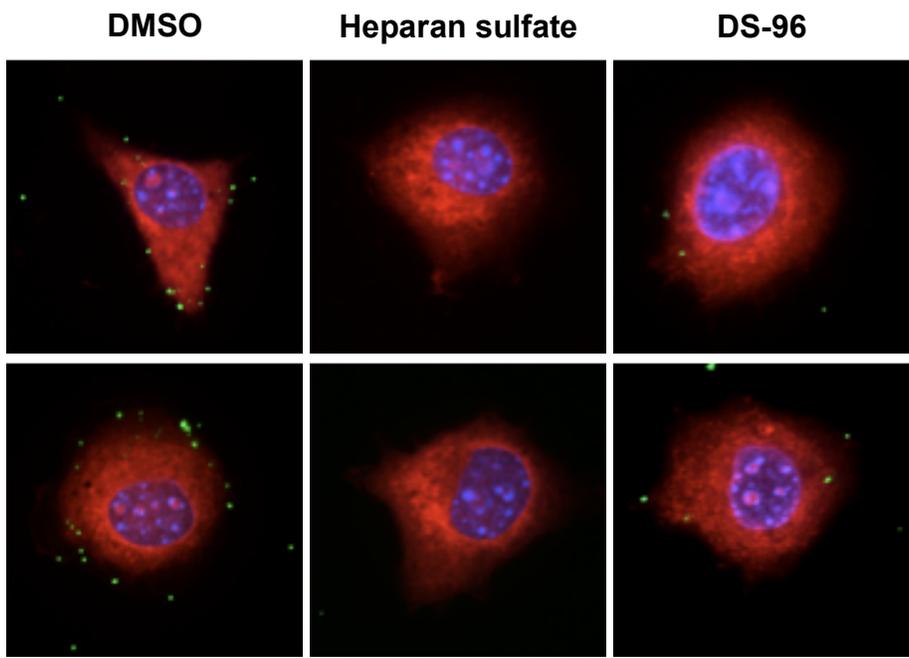
B



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 pre-treated with DS-96 for an hour prior to infection, and then given sufficient time (1h) 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 immunofluorescent microscopy (Fig. 4.5). Heparan sulfate, as a highly negatively charged polysaccharide, is known to block *Chlamydia* adhesion to the target cell *in vitro* by charge repulsion [105, 108, 110, 112, 224], 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. 4.5, DMSO). As expected, the bacterial adhesion was blocked when EBs were pre-treated with heparan sulfate prior to infection (Fig. 4.5, Heparan sulfate). In samples pre-treated with DS-96, inhibition was observed as indicated by a decrease in the number of EBs associated with host cell. However, unlike heparan sulfate, DS-96 treatment did not result in complete prevention of bacterial adhesion, and rather, the treatment decreased the number of bacteria bound to a single cell by approximately with 70%, with 2 to 5 EBs per cell remained bound to the cell surface (Fig. 4.5, DS-96). This observation was striking given that 8 μ M of DS-96 consistently resulted in high level of inhibition (greater than 85%) of inclusion formation throughout our study (Fig. 4.1C, 4.3, 4.4). This result indicated that the majority of EBs that remained bound to the host cell in the presence of the compound was still unable to form productive inclusions.

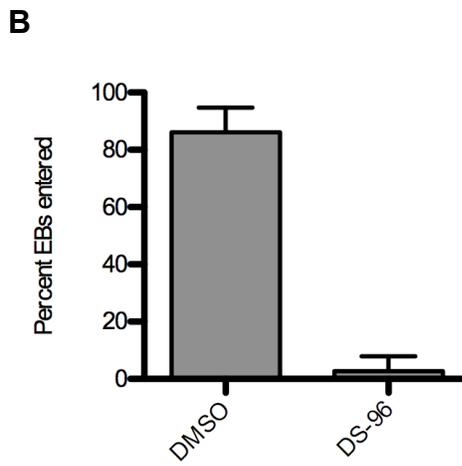
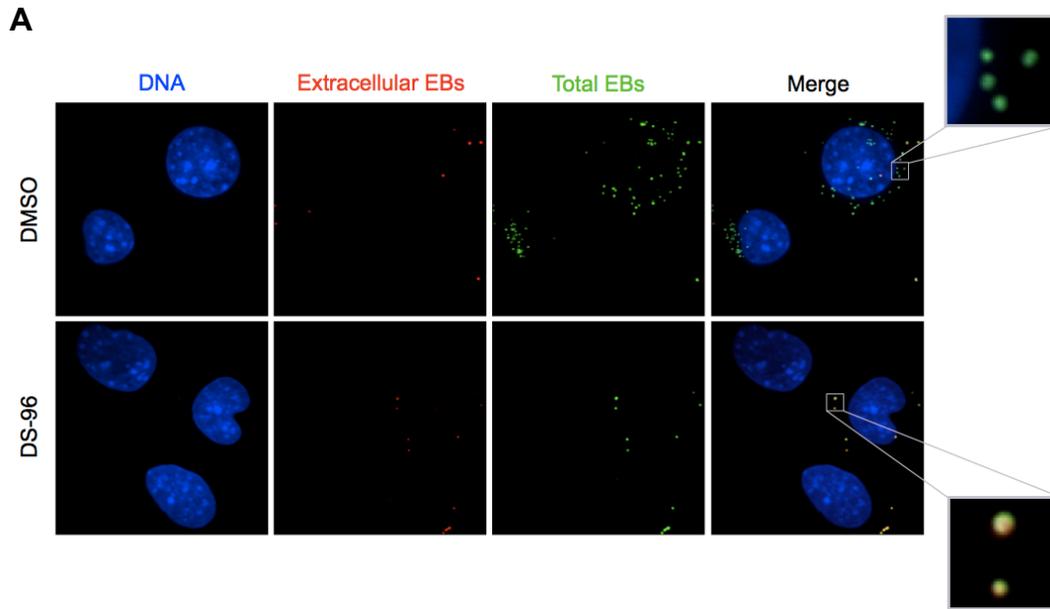
Fig. 4.5. DS-96 blocks attachment of *C. trachomatis* EBs host cell surface. L929 cells were incubated with highly purified EBs pre-treated with DS-96 (8 μ M), heparan sulfate, or mock-treated (DMSO) for an hour at 4 °C. Infected cultures were thoroughly washed to remove unbound bacteria, fixed with methanol, and immunofluorescently stained.



One possible explanation for this observation is that DS-96 blocked post-attachment events, in addition to the bacterial adhesion. To investigate this possibility, entry of *Chlamydia* into the host cell was examined. Similar to the attachment assay, EBs pre-treated with DS-96 or mock-treated (DMSO) were allowed sufficient time (4h) to attach and enter the host cell. Differential immunofluorescent staining was performed to discriminate between the internalized bacteria and those that remained on the surface of the host cell, and the samples were assessed by confocal microscopy (Fig. 4.6A). 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. 4.6A, the majority of EBs in mock-treated sample appeared in green, indicating that these EBs had been internalized. When pre-treated 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 mock-treated sample, EBs associated with the host cell in DS-96 treated samples were co-stained and appeared in yellow, indicating that these EBs remained extracellular. Quantification of the number of the extracellular and total EBs revealed that pre-treatment of the bacteria with DS-96 resulted in a drastic decrease in the percentage of internalized EBs from 86.3% in mock-treated sample to 2.6% in DS-96 treated sample (Fig. 4.6B). These findings demonstrate that DS-96 blocks entry of *C. trachomatis* into host cells, in addition to adherence.

Fig. 4.6. 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 + extracellular) were labeled with anti-*Chlamydia* antibody (green) after permeabilization. The inset shows the internalized bacteria (green) in mock-treated sample and the extracellular bacteria (yellow) in DS-96 treated sample. (B) Percent of EBs internalized relative to total EBs was quantified.

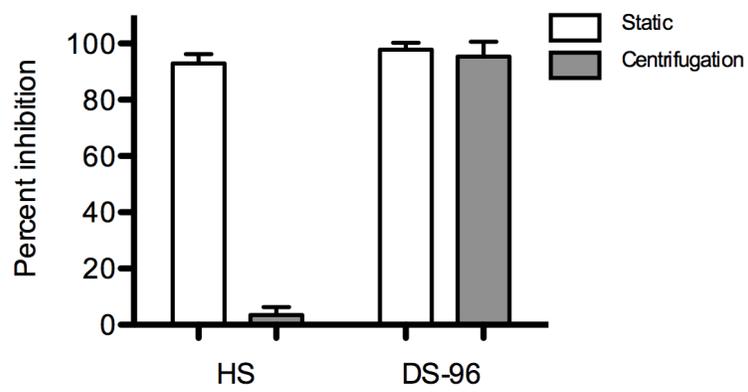


The effect of DS-96 on centrifugation-assisted infection

Since the first report by *Weiss and Dressler* [225], it has been long 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 [224]. Interestingly, it has been reported that the inhibitory effect of neutralizing antibody and heparan sulfate against *Chlamydia* is overcome by centrifugation [226]. To examine whether DS-96 was merely inhibiting binding and entry or also having further effect, such as inactivation of EBs, the inhibition assay was performed with static and centrifugation-assisted infection (Fig. 4.7). 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 x g for 1 h). In comparison, when *C. trachomatis* EBs were treated with DS-96, a high level of inhibition (95.3%) was maintained through centrifugation-assisted infection. Further studies are needed to determine what post-entry event(s) is perturbed by DS-96, or if infectious EBs are inactivated after exposure to the compound. Nevertheless, it is of interest to note that the bacteria are unable to progress through the developmental cycle even when the absorption is facilitated by centrifugation.

While *C. trachomatis* LGV biovar 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 relative ease of cultivation and shorter developmental cycle. However, trachoma

Fig. 4.7. Inhibition by DS-96 is maintained after centrifugation. Following pretreatment of EBs with DS-96 (8 μ M) or heparan sulfate (HS) for 1 h, infection was performed with or without centrifugation (900 x g for 1 h). Infected cultures were fixed with methanol, immunofluorescently stained, and analyzed for inhibition of inclusion formation 24 hpi using the image-based automated assay. Error bars indicate standard deviation of the mean of triplicate samples.



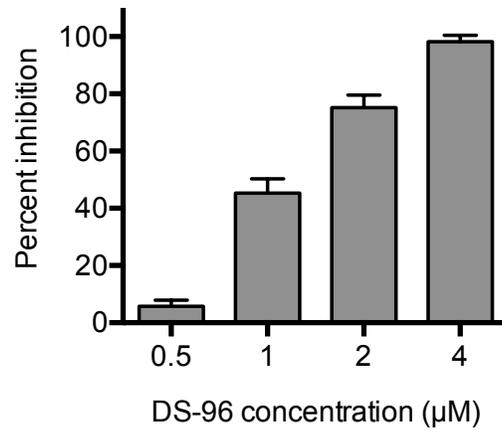
serovars D, E, and F are more clinically relevant strains since they are reported to be the most prevalent urogenital serovars worldwide [227]. With the data revealing that the effect of DS-96 is maintained through centrifugation-assisted inoculation, an inhibition experiment was performed with *C. trachomatis* serovar D and the mouse pathogen *C. muridarum*, both of which are typically infected using the 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 needed for equivalent inhibition of *C. trachomatis* LGV strain (Fig. 4.8).

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 immunofluorescence microscopy (Fig. 4.9). It was anticipated that binding of DS-96 to LOS could possibly alter the antibody-binding epitope on LOS, and consequently results in the absence of LOS staining. To perform this study, purified *C. trachomatis* EBs were pre-treated with DS-96 or mock-treated with DMSO for 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 for control, and counterstained with DAPI. When visualized by immunofluorescence microscopy, anti-MOMP antibody stained the bacteria similarly to DAPI staining in both

Fig. 4.8. 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 automated enumeration method. 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 deviation of the triplicate samples.

A



B

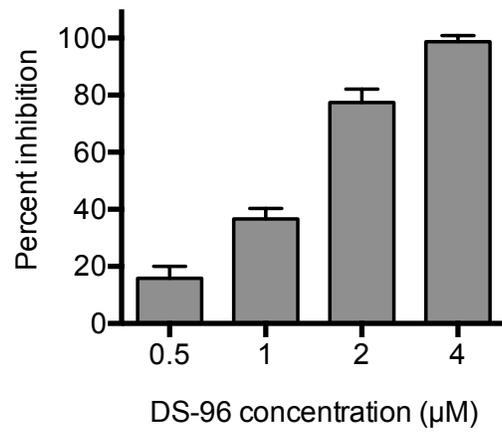
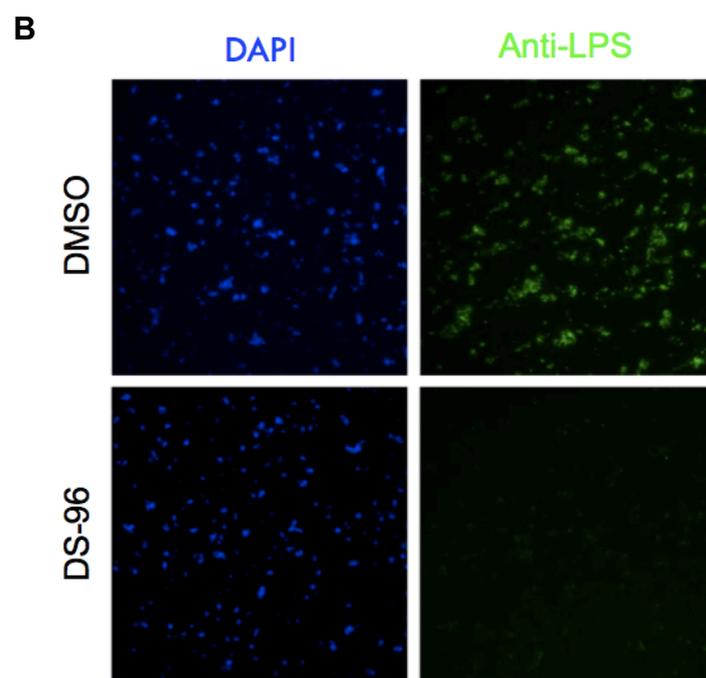
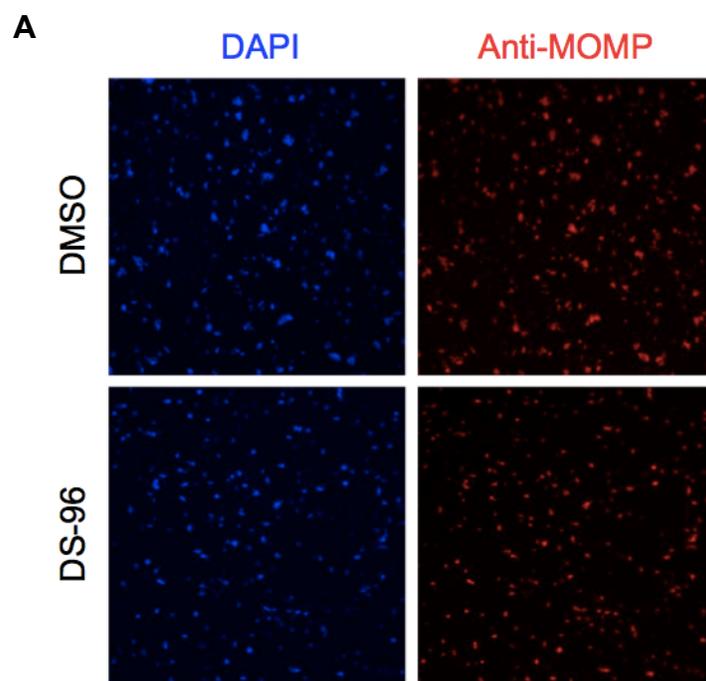


Fig. 4.9. Pre-treatment of EBs with DS-96 results in reduction of anti-LOS staining. Purified EBs were treated with DS-96 (8 μ M) or DMSO and stained for MOMP (A) or LOS (B) following methanol fixation. Samples were counterstained with DAPI to visualize the total number of bacteria.

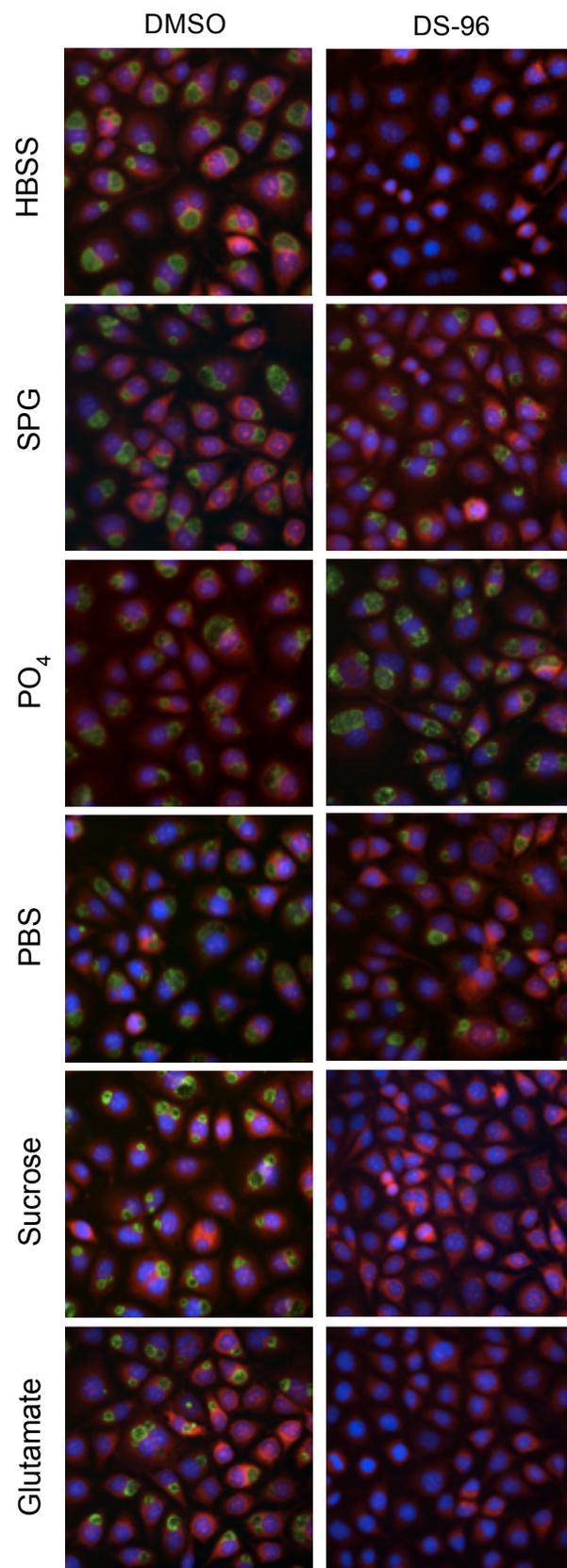


control and DS-96 treated samples. In contrast, while anti-LOS staining was consistent with DAPI staining in the control sample, staining by anti-LOS was completely absent in the sample treated with DS-96. Although this does not provide a direct evidence for an interaction of DS-96 with LOS, it suggests 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 was used throughout this study. However, sucrose-phosphate-glutamate (SPG) has been generally 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 μ M) 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 Na₂HPO₄, 3.7 mM KH₂PO₄, 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. 4.10). In the presence of sucrose or glutamic acid, the inhibitory activity of DS-96 remained comparable to the control (HBSS alone), resulting in a high level of inhibition. In contrast, when Na₂HPO₄ and KH₂PO₄ were added together to HBSS, the level of infection was similar to the untreated sample, indicating that the activity of DS-96 against *Chlamydia* infection was impaired in the

Fig. 4.10. Phosphate impairs activity of DS-96. Inhibition assay was performed with HBSS. Inhibition assay was performed with HBSS supplementing with each constituent of SPG. Samples were fixed with methanol 24 hpi. *Chlamydia* was stained with anti-MOMP antibody (green) and host cells were counterstained with DAPI (nuclei, blue) and Evan's Blue (cytoplasm, red).



presence of these components. Considering that the molecular interaction of 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 [198, 215], it is reasonable to speculate that free phosphate from Na_2HPO_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 present in also HBSS, the concentration of phosphate is approximately 15 times higher in SPG than in HBSS, which suggests the possibility that 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 the phosphate concentration comparable to SPG. The data revealed that PBS blocked the activity of DS-96 similarly to SPG, supporting that phosphate is the responsible component in 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 similar manner 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 μ M (Fig. 4.1B and C). Attachment to the host cell appears to be the stage of infection where EBs were predominantly inhibited by DS-96 treatment (Fig. 4.5), although entry and possibly post-entry events were also affected by the treatment (Fig. 4.6 and 4.7). The possible effect on post-entry 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. 4.9), as well as the effect of high phosphate concentration on DS-96 activity (Fig. 4.10), which is discussed in further depth below. Together, these data support that DS-96 is an effective inhibitor of *Chlamydia* infection, albeit within tissue culture cells.

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. 4.8), as one of the most clinically prevalent serovars [228-230] and a mouse pathogen, respectively. Interestingly, lower doses of DS-96 were equally effective against these strains as compared to LGV, indicating higher susceptibility. Although further studies will be required to provide more evidence, these observations may suggest a possibility

that serovar D and *C. muridarum* have greater requirement for LOS in their infectivity compared to serovar LGV. Considering that the structure of lipid A glucosamine backbone is well conserved throughout Gram-negative bacteria [231], it is highly unlikely that the affinity of DS-96 towards 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 difference in their tissue tropism. 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 the bacterial attachment and entry [221]. 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* [152].

DS-96 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 [232-237], 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 possible role of LOS in the 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 [151, 152]. *Fadel and Eley* postulated a possible general role of LOS in *Chlamydia* infectivity [152]. 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 [152]. Similar to our study, a role of *Chlamydia* LOS in adherence was also suggested [152]. 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 [151]. Their study revealed that a decrease in the number of progeny EBs was generated in the presence of LpxC inhibitors [151]. They also reported that OmcB, a membrane protein specific to EB surface, was poorly expressed in the absence of LOS [151]. 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 be disrupting 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 T3S effector, Tarp [238, 239]. Considering the findings by *Nguyen et al.*, it is possible that DS-96 treatment of LOS results in functional disruptions of other outer membrane surface molecules such as T3SS.

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 model. 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. 4.10). The observation was further confirmed when PBS, a buffer containing phosphate at a concentration similar to SPG,

also blocked DS-96 activity. This observation was particularly intriguing considering the molecular design of DS-96. *Sil et al.* reported the central pharmacophore, which has been experimentally tested and defined for optimal recognition and neutralization of lipid A [198, 240]. These pharmacophore properties include: (i) two protonatable cationic functions ≈ 14 Å apart are necessary for simultaneous ionic interactions with the negatively charged phosphates on lipid A (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, albeit indirectly, supports 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 under 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. Nonetheless, considering the effectiveness against *C. trachomatis* infection *in vitro* along with the demonstrated low toxicity in a mouse model, it is expected that DS-96 will performed well as a microbicide candidate compound in future animal model studies.

Chapter V.

Discussion

In the effort to develop a vaginally-delivered microbicide for prevention of genital *C. trachomatis* infections, our first task was to address the lack of effective method for rapid enumeration of *Chlamydia* in cell culture, which has greatly hampered the possibility for large-scale *in vitro* studies of the organism, including identification of anti-*Chlamydia* agents. Successful development of the automated image-based method enabled us to evaluate the susceptibility of *Chlamydia* to microbicide candidates throughout this work in a relatively large-scale assay, and is expected to make an important contribution to the *Chlamydia* research field by providing a powerful analysis tool.

As further described below, LOS was rationally selected as a molecular target for prevention of *C. trachomatis* infection in our study as a Gram-negative specific molecule that predominates on chlamydial surface. Whereas LOS is not generally considered important as a protective antigen in *C. trachomatis* infections for the purpose of vaccine [241, 242], our study demonstrated that targeting chlamydial LOS by a small molecule is highly effective in blocking infection prior to the bacterial entry *in vitro*, and therefore, has a high potential to be a suitable approach for prevention of *Chlamydia* infection. In addition, utilizing DS-96 as a molecular probe, our study revealed the possible role of chlamydial LOS in the bacterial attachment and entry steps, and expanded upon the current understanding of the biological significance of chlamydial LOS.

Utility of the automated assays developed

The automated image-based analysis method developed in our study significantly improved the efficiency of fluorescence microscopy analysis of *Chlamydia* infection in cell culture, for which the traditional method is manual and not amenable to a large-scale assay. This method was successfully utilized throughout the studies described in Chapter III and Chapter IV, as well as in recently published reports [155, 237]. The use of this assay, however, is not limited in application for simple quantification of chlamydial inclusions. With fluorescent labeling of various molecules of interest, along with minor modifications to CellProfiler pipeline, this method offers a powerful tool for a wide variety of *Chlamydia* research that currently relies on manual analysis of fluorescence microscopy images. For instance, the method was used in a recently published study to measure inclusion size [237]. Quantification of inclusion size allows us to observe subtle alterations due to delayed or halted developmental cycle, which is beyond the simple presence or absence of inclusion. Whereas a simple binary classification of infected and uninfected host cells was used in our study, minor modifications to the pipeline within CellProfiler would allow classification of analytic subjects into more than two classes for identification of complex and subtle phenotypes. For example, translocation of chlamydial proteins into host cell cytosol (e.g. T3S effector proteins), recruitment of host cell proteins to chlamydial inclusion (e.g. GTPase, lipid transferase), *Chlamydia*-induced modification of host cell processes (e.g. actin remodeling, Golgi fragmentation), or other subcellular localization of host or chlamydial proteins can be analyzed in large-scale. In addition, the recent advent of a method for transformation of *Chlamydia* enabling the development of essential molecular tools [232] is likely to extend application of this

image-based method in studies utilizing transformed *Chlamydia* expressing fluorescent proteins of interest.

While the image-based method developed in our study offers a tool for the detailed studies of *in vitro* *Chlamydia* infection, our adaptation of alamarBlue assay is perhaps more amenable to a true high-throughput format anti-*Chlamydia* compound screening, owing to the reduced liquid-handling requirements, cost-effectiveness, and the overall hands-on time involved in the assay. Additionally, a high-throughput drug screening process often requires a separate counter-screen to eliminate cytotoxic compounds [243]. AlamarBlue assay detects a decrease of host cell viability regardless of the underlying mechanisms, which can be either progression of *Chlamydia* infection in the absence of anti-*Chlamydia* activity, or cytotoxicity of compounds. And therefore, alamarBlue-based assay serves dual functions in screening of inhibitory compounds. A large-scale anti-*Chlamydia* compound screening using the alamarBlue-based automated assay has been initiated in our laboratory in collaboration with the University of Kansas High-Throughput Screening (KU HTS) facility [244, 245]. Automation of liquid transfer, including plating of eukaryotic host cells and *Chlamydia* infection, is also in progress and expected to enable high-throughput screening of anti-*Chlamydia* compounds in a fully automated fashion.

Limitations of Cell Culture Model for studies of *Chlamydia*

In this study, identification of anti-*Chlamydia* compounds and elucidation of biological function of chlamydial LOS were performed based on microscopic observations of infected cell culture. Largely due to the obligate intracellular nature of the

organism, *Chlamydia* research heavily relies on cell culture model. As reviewed in Chapter I, various and sometimes conflicting studies have been reported regarding the host cell receptors and chlamydial ligands involved in attachment and entry processes. A possible explanation for the discrepancy among various studies on the interaction of *Chlamydia* with the host cell may partially be due to the limitations of cell culture models.

Cell lines commonly used for infection of *Chlamydia* include McCoy and L929 fibroblast lines, both of which *C. trachomatis* may not infect *in vivo* [246, 247], or HeLa cells, human papilloma virus-transformed cervical epithelial cells. During natural infection, *C. trachomatis* urogenital serovars predominantly infect the polarized epithelial cells lining the reproductive tract. These polarized cells are quite different in organization compared to non-polarized cell culture lines mentioned above. The plasma membrane of polarized epithelial cells is divided into apical and basolateral domains, which are biochemically and physiologically unique, and characterized by markedly different protein and lipid compositions as well as distinct receptors [248, 249]. The inability for clinically relevant strains of *C. trachomatis* to achieve adequate infectivity *in vitro* without the aid of centrifugation may suggest that the tissue culture cells lack host cell receptors or other factors utilized by *Chlamydia* in natural infection. Supporting this idea, numerous studies have reported intriguing differences in *in vitro Chlamydia* infections of polarized versus non-polarized cells, including infectivity, mechanisms of entry, the duration and synchronization of the developmental cycle, persistent state, innate inflammatory response, and antibiotic susceptibility [250].

The pH of the culture is another key parameter to be considered in cell culture system as it influences both chemical and biological properties within the cell culture. For instance, the culture pH can influence the surface charge of *Chlamydia* and the host cells, which are involved in the electrostatic interaction mediating the initial association of the bacteria and the host cell. While the pH of cell culture medium is typically around 7, the vaginal pH of healthy women typically ranges 3.6 to 5.0 [251]. A study reported the effect of pH on *in vitro* anti-*Chlamydia* activity of cellulose-ether polymers, where the inhibitory effect was polymer concentration-dependent at pH 5, and inversely concentration-dependent at pH 7 [211]. Our attempt in testing microbicide candidates at lower pH, however, was unsuccessful due to obvious adverse effects observed in host cells in buffer mimicking vaginal pH, which is considerably lower than the pH used typically for L929 cells (i.e. around pH 7). Together, these observations highlight the limitations of studies in cell culture model and importance of translating the basic observations from cell culture system into a more natural context such as a small animal model.

Potential Roles of *Chlamydia* Lipooligosaccharide

While numerous studies have been reported on the antigenic and immunogenic properties of *Chlamydia* LOS, the knowledge concerning the biological functions of LOS in viability or the pathogenesis of *Chlamydia* is surprisingly limited. As with all Gram-negative bacteria, LOS is a major component of the outer leaflet of chlamydial outer membrane [252]. The inner leaflet of the outer membrane and both leaflets of the inner membrane are made up with phospholipids, which are either synthesized by

Chlamydia or acquired and modified from host cell sources [253]. Interestingly, it has been reported that *Chlamydia* tolerates alteration of the final phospholipid composition and remain viable [253]. In contrast, *C. trachomatis* contains all of the genes necessary for *de novo* synthesis of LOS [254], suggesting the vital roles of LOS in *Chlamydia* pathogenesis and viability.

Using DS-96 targeting lipid A as a chemical probe, we examined inhibition of *Chlamydia* growth at specific stages during the developmental cycle. We demonstrated that ability of EBs to attach and enter the host cell was greatly impaired in the presence of DS-96 (Fig. 4.5 and 4.6), and proposed that *Chlamydia* LOS plays an important role in these processes. The scope of this study, however, did not extend to exploring the mechanisms as to how LOS may be involved in these key steps of *Chlamydia* pathogenesis. And therefore, we cannot eliminate the possibility that LOS may not be the chlamydial component directly involved in the interaction with the host cell surface, and rather plays an indirect role by providing structural support for other molecules that are critical in these processes. A study has been reported to show that treatment of *C. trachomatis* EB with cross-linking agent disuccinimidyl selenodipropionate (SSP) resulted in cross-linking of LOS and MOMP [255], indicating the close association of LOS with MOMP on the surface of EBs. Considering the high abundance of LOS and MOMP on the surface of *Chlamydia*, it is reasonable to speculate that when bound by DS-96, LOS, either alone or with its association with MOMP, interferes with chlamydial protein/structure that plays a direct role in attachment and entry. The evidence for the role of MOMP in adherence of *C. trachomatis* has been presented by several studies [107, 256, 257].

It is also possible that binding of DS-96 to lipid A of LOS results in a compromise of the chlamydial surface, such as loss of the outer membrane integrity, rather than disruption of specific ligand-receptor interaction. Perhaps supporting this possibility is the observation that inhibition by DS-96 was maintained through centrifugation-assisted infections (Fig. 4.7), where it was concluded that EBs may have been “inactivated” by DS-96. If this “inactivation” was a reflection of the outer membrane disruption, an alteration of the outer membrane morphology would be expected. We performed scanning electron microscopy (SEM) to examine the effect of DS-96 on the surface morphology of EBs. This preliminary study did not show an apparent alteration of EB surface by the treatment; however, the lack of observable effects does not eliminate the above possibility, since effects could be too subtle for visualization by SEM.

While our study primarily focused on the function of chlamydial LOS in the infectious EB form, LOS is shown to be present in both EBs and RBs. The differences in the exposure and arrangement of LOS between the surface of EBs and RBs have been reported by several studies [255, 258-260], suggesting distinct roles LOS plays in these two forms of the organism. The conversion of EB to RB occurs approximately 2 to 8 hpi and replication continues until about 18 hpi for *C. trachomatis* L2. When DS-96 was added during 0-6 and 6-12 hpi to examine the effect of the compound during these stages of the developmental cycle, no inhibition of chlamydial growth was observed (Fig 4.3). However, since membrane permeability of DS-96 has not been determined, the absence of effect may be as a result of the inability of DS-96 to have access to the bacteria once they are internalized by the host cell.

Although our chemical biology approach was unable to address the role of LOS after chlamydial entry, a recent report by *Nguyen et al.* used inhibitors of the LOS biosynthesis pathway to characterize the function of LOS post-chlamydial entry. It was demonstrated that RBs were able to replicate normally, and no alteration in the ultrastructure of the outer and inner membrane of RBs was observed when LOS biosynthesis was inhibited. However, these RBs were unable to undergo morphological transition into EBs in the absence of LOS, suggesting that chlamydial LOS in RB is essential for proper formation of infectious EBs [151]. These observations revealed an intriguing function of chlamydia LOS in the non-infectious RBs. Their study, however, did not address the role of LOS in the infectious EBs since the inhibitors have no effect on pre-formed LOS, and progeny EBs were not formed in the absence of LOS biosynthesis. Therefore, our research effort provided valuable new insights into the role of LOS in infectious EBs.

DS-96 as a novel anti-*Chlamydia* compound

The need for continued discovery and development of novel anti-chlamydial agents is highlighted by the limitations of the current strategy for prevention and treatment of *C. trachomatis* infection. To date, a limited number of small molecule inhibitors has been identified for *Chlamydia*. Several studies have been reported on the identification of compounds targeting *Chlamydia* type III secretion system (T3SS), including small molecule inhibitors of *Yersinia* T3SS [261-264] and a peptide mimetic targeting protein interaction essential for T3SS [265]. Recently, *Chlamydia* serine

protease CtHtrA was found to inhibit the viability of *Chlamydia* both in cell culture and mouse model [266].

For the purpose of vaginal microbicide, the candidate inhibitors must have the ability to specifically target vaginal pathogens while having no deleterious effect on the normal vaginal flora. Vaginal microbiota of healthy women is typically dominated by the hydrogen peroxide-producing Gram-positive *Lactobacillus* species that are essential in maintaining vaginal homeostasis and providing the natural defense against pathogenic organisms [267-269]. The activity of DS-96 against Gram-positive bacteria species has not been examined. However, considering that DS-96 is designed to specifically target lipid A, a virulence factor unique to Gram-negative bacteria, it is highly anticipated that DS-96 would not have a major adverse effect on the *Lactobacillus*-dominated normal vaginal microbiota.

On the other hand, DS-96 is likely to have activity against another common pathogen of genital mucosa, *Neisseria gonorrhoeae*. Like *Chlamydia*, the Gram-negative *N. gonorrhoeae* possesses LOS. Whereas the terminal oligosaccharides of gonococcal LOS are known for their ability to undergo phase variation at high rate [270, 271], the glucosamine backbone of lipid A is conserved. Similar to our study, *Neisseria* LOS has been implicated in the adhesion and invasion of host epithelial cells [272]. Which component of gonococcal pathogenesis would be inhibited by DS-96 remains to be elucidated; however, it is reasonable to expect that DS-96 binds to gonococcal lipid A, which in turn inhibits *Neisseria* infection. Therefore, our future studies must include *in vitro* susceptibility studies of *N. gonorrhoeae* and *Lactobacillus* species to DS-96.

Another obvious future work would be to examine if *in vitro* inhibitory effects of DS-96 against *Chlamydia* infection would translate *in vivo*. In collaboration with Dr. Luis de la Maza (University of California, Irvine), investigation of DS-96 activity in a mouse challenge model has been initiated. Although no definitive conclusions could be made from the initial study due to the lack of statistically sufficient number of test animals involved, two out of five animals showed nearly complete protection when EBs were pre-treated with DS-96 prior to injection into the mouse (data not shown). These animal challenges will be repeated with larger test groups to obtain statistically significant and reproducible results.

While this study focused on the application of DS-96 as a candidate for microbicide, the application of DS-96 may not necessarily be limited to prevention. DS-96 may also be effective as a therapeutic by blocking spread of *Chlamydia*. A plaque assay can be performed to examine if cell-to-cell transmission of a single EB over several successive developmental cycles can be blocked in the presence of DS-96.

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