**The Development and Validation of a High-throughput Screening Method for *Chlamydia trachomatis***

By

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Submitted to the graduate degree program in Molecular Biosciences and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Arts

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**ABSTRACT**

High–throughput screening is a scientific method that can be used to identify small compounds with activity against a number of bacteria and other organisms, as well as diseases, such as cancer, in a cost efficient manner within reasonable time frames. The absence of an effective HTS method for large-scale compound screening in *Chlamydia* hinders the identification of anti-chlamydial compounds that could serve as novel therapeutics, model molecules for the development of specific treatments, or molecular probes to gain insight into the basic biology of the organism. The absence of a high-throughput screening method is highly influenced by the obligate, intracellular nature of the organism, making enumeration of infection a time consuming and labor-intensive task, traditionally performed using an immunofluorescence assay. Recently in our laboratory, a new method for enumerating chlamydial infection was developed using a cell viability indicator, resazurin, to measure the reducing capability of the infected, eukaryotic host cells. In this study, the resazurin-based microplate assay was adapted to a high-throughput screening format and validated by screening the Prestwick Chemical library, containing many known anti-bacterial compounds, including anti-chlamydial compounds. An orthogonal assay was performed to further confirm the novel hits identified in the screen of the Prestwick Chemical library and to begin to identify the mechanisms of inhibition exuded on *Chlamydia* by those compounds. This high-throughput screening method was further adapted to automated liquid handling, including, plating and infecting the eukaryotic host cells, and the assay quality and reproducibility were verified by the HTS Facility at The University of Kansas in Lawrence, Kansas.

This work is dedicated to my family

for their unconditional love and support and

especially to my father, who did not live to see it completed

but had the upmost faith in my abilities.

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**The Development and Validation of a High-throughput Screening Method for *Chlamydia trachomatis***

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# *I.* *CHLAMYDIA* INTRODUCTION

## A. HISTORY AND PUBLIC HEALTH IMPACT

The obligate, intracellular organism, *Chlamydia trachomatis* is a member of the phylum *Chlamydiae,* which is composed of bacterial organisms that inhabit a parasitophorous vacuole, termed inclusion, inside of a eukaryotic host (1). *Chlamydia* was discovered over a century ago and called "Chlamydozoa" but was not classified as protozoa or bacteria. At the time of discovery this organism was considered to be a virus and was not accurately identified as a bacterium until the 1960's (2). It is now called *Chlamydia trachomatis* and is the causative agent of trachoma, an ocular infection that can lead to blindness (3). The earliest reliable descriptions of trachoma date back to 1553 BC but the link between *Chlamydia* and humans likely predates civilization (4). Despite the long history of trachoma infections, it is still a major health concern and was listed as one of the top seven neglected diseases in the 21st century (5).

*Chlamydia* infections in humans are predominantly caused by two different species, *Chlamydia pneumoniae* and *Chlamydia trachomatis,* and both have a significant and global health impact. *C. pneumoniae* is most commonly a respiratory pathogen responsible for 6-22% of community acquired pneumonias and serological studies indicate that almost every individual has been exposed to *C. pneumoniae* at some point in life (6). There is also evidence of this bacteria's role in atherosclerosis and coronary artery disease, which is the leading cause of death for both men and women in the developed world (7-9). In addition to causing ocular infections that can lead to blindness, *C. trachomatis* can also cause genital infections. This species of *Chlamydia* is the leading cause of infectious blindness worldwide and the most commonly reported sexually transmitted infection in the United States, comprising the largest proportion of sexually transmitted diseases reported to the CDC

since 1994 (10). Most people with *C. trachomatis* genital infections are asymptomatic and if left untreated these infections can lead to chronic inflammation and severe health issues, particularly for women, such as pelvic inflammatory disease, life-threatening ectopic pregnancy and infertility (11, 12)

*Chlamydia trachomatis* can be further broken down into serotypes based on the composition of their highly immunogenic, major outer membrane protein (MOMP), which contains epitopes that are genus, species, subspecies and serotype specific (13). The original classification identified fifteen MOMP serovars but subsequent studies have expanded the list of chlamydial strains to over twenty genovars, serovars and serovariants (14, 15) Trachoma is caused by serotypes A, B, Ba and C, which infect the conjunctiva of the eye, and repeat infections can lead to scarring and visual impairment (16). It is estimated that approximately 40 million people have active trachoma, 1.8 million have visual impairment from infection and an additional 1.3 million people are blind from the disease worldwide (17). Serotypes D through K are responsible for oculogenital infections that are sexually transmitted (13). The World Health Organization determined that there are approximately 90 million cases of *Chlamydia trachomatis* infections reported annually. Of those cases, it is estimated that four million are reported in the United States and 5.5 million cases of infection are reported in Europe each year (18). However, these numbers may not accurately represent the annual number of infections because up to 50% of men and up to 80% of women remain asymptomatic, making diagnosis and treatment difficult (19, 20). Serotypes D through K are not limited to genital infections and can also cause the ocular diseases opthalmia neonatorum and paratrachoma (21). In addition to serotypes D through K that are capable of causing genital infections, there is the lymphogranuloma venereum (LGV) pathovar composed of serotypes L1, L2 and L3. The distinguishing difference between these serotypes is D through K are non-invasive, but L1 through L3 are capable of

infecting monocytes and disseminating infection to the local draining lymph nodes (22). LGV can have genital and extragenital manifestations and is endemic in East and West Africa, Southeast Asia, India, the Caribbean basin and South America (23).

In addition to their human hosts, members of *Chlamydiae* infect a variety of mostly mammalian animal hosts. *Chlamydia felis* and *Chlamydia caviae* both cause conjunctivitis in cats and in guinea pigs, respectively (24). *Chlamydia muridarum* infects mice and is a strain closely related to *C. trachomatis*. It is of medical importance because mice can be infected and studied as an appropriate animal model to test treatment options and obtain insight into the immunological pathology of *Chlamydia trachomatis (25)*. There are a variety chlamydial species that infect pig herds at a high prevalence, including *Chlamydia suis,* an intestinal pathogen of swine (26). *Chlamydia psittaci* is arguably the most important species of *Chlamydiae* animal pathogens because along with its common host, parrots and poultry, it is also a human zoonotic pathogen, causing potentially deadly respiratory psittacosis (27, 28). *C. psittaci* is particularly dangerous because it is highly infective and thought to be transmitted through aerosols, making it a candidate for biosubstance regulation (29). Given the global health impact, diversity of hosts and diseases caused by *Chlamydiae* species, it is evident that these bacteria are of medical importance but many key factors about these organisms' basic biology and their pathogenic mechanisms are fully understood.

## B. TREATMENT AND VACCINE

In addition to the global impact *Chlamydia* has and the high incidences of infection annually, repeat chlamydial infections are a common occurrence. One report stated that people under 25 years of age had a reinfection rate as high as 24%, which was the highest observed among all of the populations studied (30). The rate of reinfection is highly influenced by untreated partners, whom may not know they are infected due to the

common asymptomatic nature of *Chlamydia* infections. It is the silent nature of infection that causes complications in diagnosis and treatment of *Chlamydia*.

The two most commonly prescribed antibiotics used for treatment of chlamydial infections are doxycycline and azithromycin. Doxycycline is a typical tetracycline antibiotic and a known bacterial protein synthesis inhibitor (31). Azithromycin is a member of the macrolide antibiotic family and is structurally similar to erythromycin but has increased activity against Gram-negative bacteria, such as *Chlamydia trachomatis* (32). Azithromycin is given in a high, single dose and doxycycline is prescribed in a lower dose twice daily for seven days (33). Despite their differences in treatment regiments, both of these antibiotics are currently recommended as equal treatment choices for uncomplicated *Chlamydia* infections (34, 35). With these two antibiotics, most *Chlamydia* infections can be treated but tetracycline resistance has been observed in *Chlamydia suis,* an intestinal pathogen of pigs, indicating that resistance can be obtained under environmental pressures and treatment failures do occur (36). Treatment failures have been recorded for both antibiotics that are prescribed but higher incidences of failures, as high as 8%, have been reported for azithromycin if the treatment regiment is strictly followed for doxycycline (37). A third antibiotic, amoxicillin, which is a beta-lactam antibiotic and a member of the penicillin family of antibiotics, may be prescribed for treatment of *Chlamydia* if a woman is pregnant and allergic to macrolide antibiotics to avoid the dangerous side effects of doxycycline during pregnancy and to prevent transmission of *Chlamydia* to the child when it passes through the birth canal (38, 39). Fluoroquinolone antibiotics, such as ofloxacin, have also been reported to be effective against *Chlamydia* infections but are not recommended as the first treatment options due to the adverse effects on the musculoskeletal system and the CNS that this class of antibiotics can cause (40).

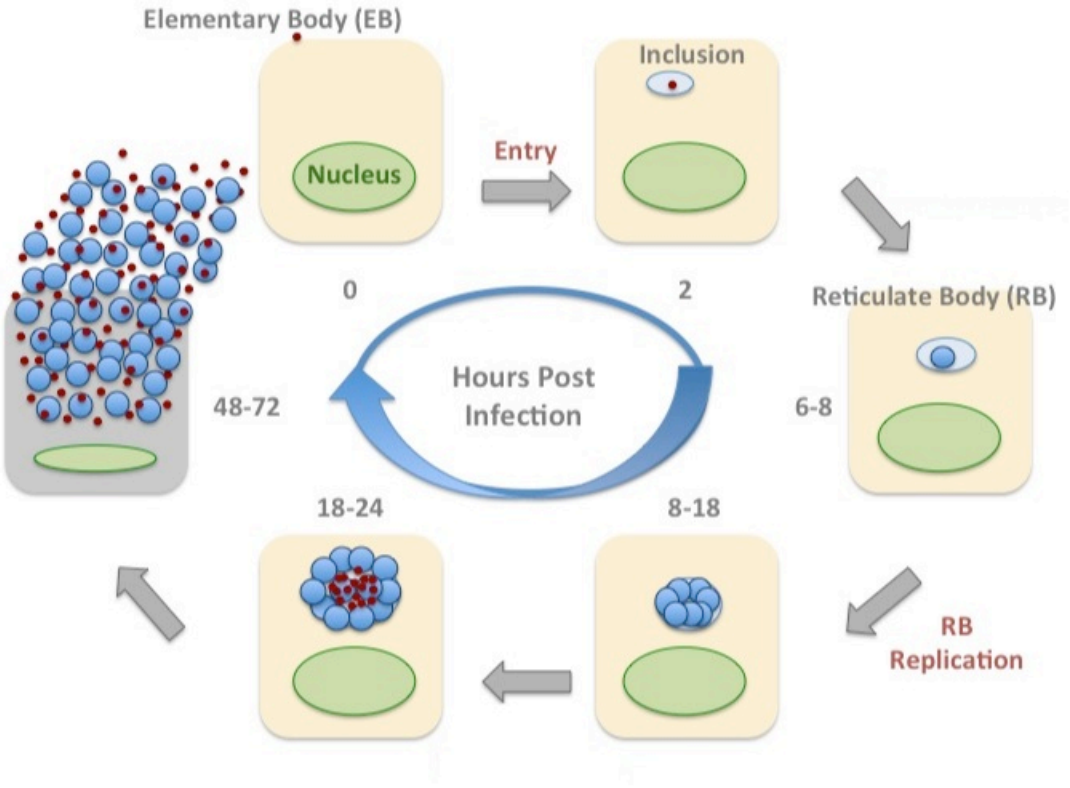
Given the high incidence of infection, reinfection and the asymptomatic nature of *Chlamydia*, prevention of infection would be preferred. Prevention could occur by two main methods for sexually active individuals; a barrier from the organism to prevent transmission or a vaccine to aid in the elimination of the organism before infection occurs. Currently, condoms serve as the primary method for prevention of infection but in the absence of regular condom use, there is not a female-controlled preventative method currently available on the market, such as vaginal microbicide. In addition to the absence of an effective microbicide and despite numerous efforts, a vaccine has also not been successfully developed for use in humans. Advancements in the understanding of the immunobiology of C. muridarum has allowed for the creation of a common model of immunity with C. trachomatis but further research is needed to develop a safe and effective vaccine candidate (41, 42).

## C. DEVELOPMENTAL CYCLE

The Gram-negative, eubacteria *Chlamydia* is characterized by its unique, biphasic developmental cycle in which it exists in two distinct, morphological forms; the small (0.3 μm), infectious, metabolically inert, electron-dense, elementary body and the larger (1 μm), non-infectious, metabolically active, reticulate body (43, 44). Despite the wide variety of hosts and tissues that each species infects, all chlamydial species have a parasitic nature and require their eukaryotic hosts for growth and replication. The existence of the biphasic developmental cycle is also conserved between species but the timing of the major events of the developmental cycle vary between species and serotypes (45).

*Chlamydia* infection is initiated by the attachment of an elementary body (EB) to the cell membrane of a terminally differentiated, non-phagocytic, epithelial cell (Fig. 1). Attachment to the host cell occurs in two stages; the first stage is reversible, electrostatic

**Figure 1: Developmental Cycle of *Chlamydia trachomatis*.** Throughout the developmental cycle, *Chlamydia* exists in two forms; the small, infectious, elementary body (EB) and the larger, non-infectious, reticulate body (RB). At 0 hours post infection (hpi) the EB attaches and induces its uptake into a terminally differentiated, non-phagocytic, epithelial cell. After approximately 2 hpi, the EB has entered the host cell and formed the parasitophorous vacuole, termed inclusion. At approximately 6-8 hpi the EB will convert into the metabolically active RB and begin cell division by binary fission, which will continue throughout the remainder of the developmental cycle. At approximately 18-24 hpi the RBs will begin to asynchronously differentiate back into newly formed, infectious EBs. The initial cycle of infection is completed after 48-72 hpi when the organisms are released into the surrounding environment by host cell lysis or the process of extrusion. Hours post infection are indicated by the grey numbers surrounding the blue center arrow.


Elementary Body (EB)
Nucleus
Entry
Inclusion
0
2
Reticulate Body (RB)
Hours Post
Infection
48-72
6-8
18-24
8-18
RB
Replication

interactions and the second is irreversible attachment (43). The EB induces its uptake into the host cell by means of its type III secretion system (TTSS) and promptly forms the inclusion that is modified to prevent fusion with the host cell lysosomes (46). Inside the inclusion, the EB converts into a reticulate body (RB) and after approximately six to eight hours it begins dividing by binary fission resulting in two daughter cells of about equal size (47, 48). At approximately 18 to 24 hours post infection, RBs begin asynchronously differentiating back into infectious EBs that gather mainly in the center of the inclusion, allowing the RBs to continue to obtain nutrients from the host by localizing at the inclusion membrane (49, 50). The initial cycle of infection is completed after 48 to 72 hours when the newly formed EBs, capable of initiating a second round of infection, are released into the surrounding environment by host cell lysis or the process of extrusion (51). Despite the characterization of the biphasic developmental cycle of *Chlamydia*, the signals that control the cycle and the mechanisms that the organism uses to modulate the host cell's function to generate an environment that is conducive to growth are not fully understood.

## *II.* ADAPTATION AND VERIFICATION OF A HTS METHOD FOR *CHLAMYDIA*

### A. INTRODUCTION

Chemical Biology and High-throughput Screening:

*Chlamydia*'s basic biology and pathogenic mechanisms are poorly understood even given this organism's broad and relevant public health impact. This deficiency in understanding is due, in part, to challenges since it is an obligate, intracellular bacterium with a biphasic developmental cycle, containing numerous essential growth stages and a deficiency in basic genetic manipulation capabilities within the organism. Until Wang *et al.* was published in 2011, a method describing the transformation of stable DNA in the form of a shuttle vector into *Chlamydia* had not been established (52). Since the introduction of a transformation system, several advances have been made in the field, such as an inducible gene expression system under the control of the Tet operator and the identification of a method allowing for transformation of *Chlamydia* growing within the host cell inclusion, but it is still not possible to successfully knockout genes in this organism *(53-55)*.

An alternative method to study *Chlamydia* and gain information about its basic biology is to use chemical biology, which is a scientific discipline that uses chemical techniques to study biological functions and processes. If a small compound with activity against an organism is identified, it can be utilized as a molecular probe to tease out information about the basic biology of what is being studied. The chemical biology approach utilizing a molecular probe has been widely used to gain insight into physiological systems, such as sodium transport into cells and tissues, and molecular probes can be conjugated to fluorescent molecules enabling localization studies (56, 57). By identifying small molecules or compounds that inhibit or stall the developmental cycle of *Chlamydia* and isolating the

targets of those compounds, we can gain insight into the basic biology of the organism and its virulence mechanisms. In addition to the paucity of genetic manipulation in *C. trachomatis*, there are currently only two antibiotics commonly prescribed for treatment of *Chlamydia trachomatis* infections; azithromycin and doxycycline (19). Most *Chlamydia* infections can be treated with doxycycline or azithromycin, but resistance to tetracycline antibiotics have been observed in other species of *Chlamydia (36)*. Not only could we gain information from molecular probes about *Chlamydia* by using a chemical biology approach, but the identified compounds could also serve as model molecules to design specific and novel therapeutics to treat infections.

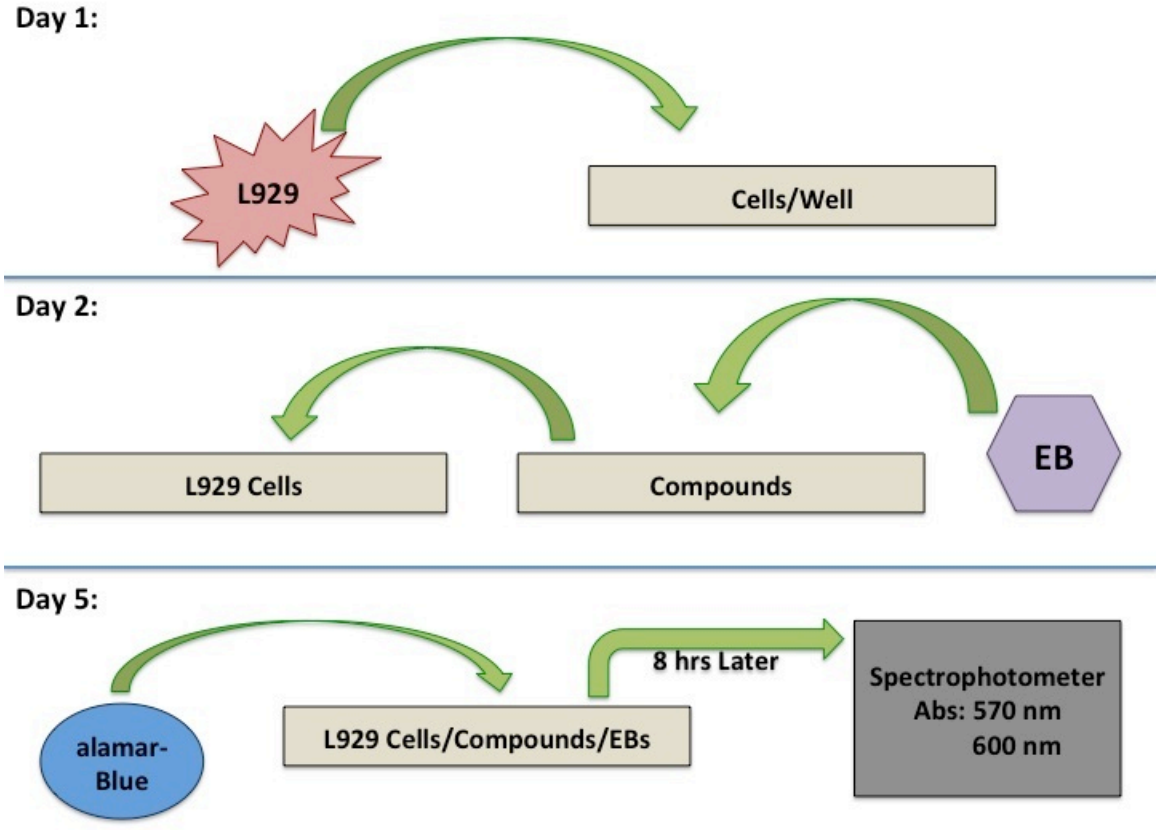
One method to identify compounds to be used as molecular probes or model molecules is by high-throughput screening of small, compounds libraries. High-throughput screening (HTS) is a scientific method that is commonly used to discover new drug treatments in an economically friendly and in a reasonable time frame. There have been several different methods that have been successfully adapted to HTS and can now be used to identify compounds for potential treatment of a wide variety of bacteria, parasites, fungi, and cancer (58-62). However, there is currently not a high-throughput screening method to identify compounds with activity against *Chlamydia*. This void is potentially due to the obligate, intracellular nature of the organism and the inability to culture it outside of a eukaryotic host cell, making enumeration of infection a labor-intensive task. The level of infection following treatment was traditionally determined by counting inclusions that are fixed, stained and imaged using an immunofluorescence assay (IFA). Even with advancements, such as image-based automated chlamydial identification and enumeration, iBAChIE, allowing for the automated enumeration of inclusions using widely available software, IFA is still not a method suited for large scale HTS of small compound libraries (63).

Resazurin-based Microplate Assay:

A new, facile method for enumeration of *Chlamydia* infection, a resazurin-based microplate assay, was recently developed in our laboratory in a 96-well format. This method drastically reduces the amount of time required for analysis and determination of the level of infection of a sample and during validation, the results of this assay strongly correlated with the results of inclusion enumeration by an immunofluorescence assay (IFA) (64). The assay utilizes the cell viability indicator known commercially as alamarBlue, and commonly as resazurin, as a colorimetric indicator of living cells. During the experiment, resazurin, a blue, non-fluorescent compound, is added to the infected and control host cells at the end of the first cycle of infection, which should correlate with host cell lysis of infected cells (Fig. 2). If the cells are viable, the resazurin is converted into the red, fluorescent, reduced form called resorufin by products of host cell metabolism. Since the resazurin and resorufin absorb at different wavelengths and given that resazurin is non-fluorescent and resorufin is highly fluorescent, measuring the absorbance or the fluorescence of each well allows for the calculation of the reducing capability of the cells. The reducing capability of the host cells corresponds to cell viability, which inversely correlates to the initial level of infection.

Resazurin is a colorimetric, cell viability indicator that has been used in several successfully adapted high-throughput screening methods of compounds against *Aspergillus*, trypanosomes, *Staphylococcus*, and *Mycobacterium (65-68)*. Given the effectiveness of the resazurin-based microplate assay developed for enumeration of *Chlamydia* in 96-well format, the method seemed well suited for adaptation to a smaller format more commonly used in high-throughput screening. In addition to a drastic reduction in analysis time, this method also significantly reduces the number of liquid handling steps required to perform the assay, making it more suitable to HTS compared to IFA. Once the assay is successfully

**Figure 2: 384-well Screening Method Schematic.** On the first day, L929 cells that have been routinely cultured are seeded into a clear, 384-well, tissue culture plate. On the second day, compounds to be screened are set out at RT for one hour before EBs are diluted to cell culture medium and added to each of the wells of the compound plate. The medium is aspirated off of the L929 cells seeded the previous day and medium containing EBs and the compounds to be tested are transferred from the compound plate to the designated wells containing L929 cells. Following a 72 hour incubation period, resazurin, known commercially as alamarBlue, is added to the wells and allowed to incubate for an additional 8 hours before the absorbance at 570 nm and 600 nm is read using a spectrophotometer.


Day 1:
L929
Cells/Well
Day 2:
L929 Cells
Compounds
EB
Day 5:
alamar-
Blue
L929 Cells/Compounds/EBs
8 hrs Later
Spectrophotometer
Abs: 570 nm
600 nm

adapted to high-throughput screening, it can be used to screen large libraries of small compounds with drug or predicted drug activity and would fill a niche in the *Chlamydia* field by allowing for a high-throughput screening method capable of identifying novel therapies, molecular probes or model molecules with activity against this organism.

Statistical Evaluation of High-throughput Screens:

Throughout the development and optimization of a high-throughput screening method, the functionality and effectiveness of the screen must be measured to ensure the method is well suited for high-throughput screening. The statistical parameters commonly used to evaluate HTS assays throughout optimization and screening are Z' factor and Z factor, which were originally described by Zhang *et al*. (69). Z factor is calculated using the following formula; $Z = 1 - \frac{(3\sigma_s + 3\sigma_c)}{|\mu_s - \mu_c|}$, where $\sigma_s$ is the standard deviation of the samples, $\sigma_c$ is the standard deviation of the respective control and $\mu_s$ and $\mu_c$ are the means of the sample data and the control data, respectively. The closer the resulting factor is to one, the more ideal the assay is for HTS and the more reliable the data will be. Any factor value over 0.5 is considered to be an excellent assay. Z' factor is calculated in a similar way as Z factor but instead of evaluating sample and respective control data, Z' factor is calculated using the positive and negative controls. Z' factor is primarily calculated to evaluate the assay as it is being optimized and Z factor is calculated to assess the accuracy and quality of data obtained during a screen of compounds. In any given situation, Z factor is less than or equal to Z' factor and both are very susceptible to variation in the data and require a broad separation band between the controls and samples to obtain an acceptable value. Therefore, an excellent assay that is amendable for adaptation to HTS and will generate reliable data needs to have a large dynamic range and small data variation, resulting in high Z' and Z factors.

In this study, the resazurin-based microplate assay used for enumeration of *Chlamydia* infection was successfully adapted to a smaller, 384-well format and the method was verified by screening the diverse small-compounds contained in the Prestwick Chemical Library, including many known antibiotics. Following the initial, small-scale screen, an orthogonal, immunofluorescence assay, was performed using the top non-antibacterial compounds identified in the screen of the small, chemical library. Finally, the method was further validated by adaptation to automated liquid handling and confirmation of the reproducibility of the assay was completed by the HTS facility at The University of Kansas.

## B. MATERIALS AND METHODS

### Cell Culture:

Murine, L929, fibroblast cells (ATCC CCL-1) were routinely cultured in RPMI 1640 medium (Cellgro by Mediatech; Manassas, VA 20109) with phenol red, supplemented with 0.3 mg/mL L-glutamine, 5% (vol/vol) fetal bovine serum (FBS) and 10 μg/mL gentamycin at 37°C, 5% $CO_2$.

### EB Preparation:

L929 cells were grown in RPMI with phenol red, supplemented with 0.3 mg/mL L-glutamine, 5% (vol/vol) fetal bovine serum (FBS) and 10 μg/mL gentamycin and incubated at 37°C, 5% $CO_2$ in a spinner flask on a magnetic plate that generated slight, but constant agitation. Once cells reached 8 x $10^5$ cells/mL, previously isolated *C. trachomatis* lymphogranuloma venereum (LGV) serovar L2/434/Bu seed was diluted into Hank's Balanced Salt Solution with Calcium and Magnesium (HBSS; Mediatech, Inc., Manassas, VA)

and was added to the spinner flask with a final concentration of 1 µg/mL cyclohexamide. Approximately 6 hpi, a sample was removed from the spinner flask and seeded into an 8-well Ibidi slide. At 24 hpi the approximate level of infection was determined and had to be sufficient prior to purification of the EBs. At approximately 48 hpi and after continued incubation at 37°C, 5% $CO_2$ on a magnetic plate, elementary bodies (EBs) were purified from infected cells using a 30% Renografin density gradient as previously described (70) and stored in sucrose phosphate glutamate (SPG) buffer at -80°C until needed. All of the EB preparations were combined and aliquoted in small volumes allowing for a new aliquot to be thawed for each experiment with approximately the same level of infectivity. The combined preparation's titer was determined prior to being used to infect cells for experimentation.

384-well Screening:

L929 cells were seeded 1.0 x $10^5$ cells/mL; 50 µl/well in a clear, 384-well tissue culture plate (Thermo Scientific, Nunc 164688) and incubated flat at RT to reduce edge effect. After 1 hour, cells were further incubated at 37°C, 5% $CO_2$ for approximately 24 hours. At that time, compounds aliquoted in a 384-well plate were allowed to come to RT for 1 hour and diluted to a final concentration of 2.5 µM by adding 80 µl/well of an EB cell suspension made in RPMI without phenol red, supplemented with 0.3 mg/mL L-glutamine, 5% (vol/vol) fetal bovine serum (FBS) and 10 µg/mL gentamycin. Cell culture medium was aspirated from L929 cells, seeded 24 hours previously by column, and 54 µl of treated EB cell culture medium was transferred from the compound plate to the cells in the corresponding wells. Once all cells were infected/treated, they were incubated for 72 hours at 37°C, 5% $CO_2$. Following this incubation, alamarBlue (Invitrogen) was added to each well at a final concentration of 10% (6 µl/well) and allowed to incubate with the cells for 8 hours

at 37°C, 5% $CO_2$. Each plate was then wrapped in foil and stored at 4°C. Once the initial screen was completed, all plates were allowed to come up to RT and absorbance at 570 nm and 600 nm was measured using a PowerWave microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Reducing capability was calculated, as described previously (64) using the following formula; $\frac{(\varepsilon_{OX})\lambda_2 A\lambda_{1-}(\varepsilon_{OX})\lambda_1 A\lambda_2}{(\varepsilon_{OX})\lambda_2 A\prime\lambda_{1-}(\varepsilon_{OX})\lambda_1 A\prime\lambda_2} \times 100$, where $\varepsilon_{OX}$ is the molar extinction coefficient of alamarBlue's oxidized (blue) form, A is the absorbance of the test wells, A' is the absorbance of the positive control wells (0.1% DMSO-treated, uninfected wells) and $\lambda_1$ and $\lambda_2$ are 570 nm and 600 nm, respectively. Hits were determined at 2 standard deviations above the average of the samples. The initial small compound library screened was the 2007 Prestwick Chemical Library obtained from the High-throughput Screening facility at the University of Kansas, which receives the compounds from Prestwick Chemical in Illkirch, France.

Dose-dependent Confirmation:

Dose-dependent confirmation screening was performed following the same protocol as initial screening. A list of hits from the initial screen of the Prestwick Chemical Library was sent to the High-Throughput Screening Facility at The University of Kansas and compounds were plated in different volumes to generate 8 final concentrations from approximately 20 μM to 0.156 μM. Testing the compounds at a higher concentration than 2.5 μM required additional volumes of the compound to be plated per well and therefore, increased the final concentration of DMSO on the cells. Additional DMSO controls from 0.8% to 0.1% were included to compare to the higher concentrations tested. Once the dose-dependent screen was completed, all plates were allowed to come up to RT and absorbance at 570 nm and 600 nm was measured using a PowerWave microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Reducing capability was calculated as

aforementioned using the corresponding percentage of DMSO-treated, uninfected cells as the positive control. Confirmation of the hits was determined at two standard deviations above the average of the DMSO-treated, infected controls with the corresponding final concentration of DMSO per well. $IC_{50}$ was calculated using GraphPad Prism 6.

Inhibition of Commercially Purchased Compounds:

L929 cells were seeded in a clear, flat bottom, 96-well, tissue culture plate at 1.0 x $10^5$ cells/mL; 200 µl/well and incubated, flat, at RT for 1 hour. Then, the cells were allowed to further incubate for approximately 24 hours at 37°C, 5% $CO_2$. The following day, the cell culture medium (RPMI 1640 media (Cellgro by Mediatech; Manassas, VA 20109) with phenol red, supplemented with 0.3 mg/mL L-glutamine, 5% (vol/vol) fetal bovine serum (FBS) and 10 µg/mL gentamycin) was aspirated off of the cells and EBs at a concentration of 6.0 x $10^{-5}$ in supplemented RPMI without phenol red and the desired concentration of each, individual compound was added to the designated wells. This mixture was allowed to incubate on the L929 cells for 72 hours. Following this incubation a final concentration of 10% alamarBlue was added to each well and the plate continued to incubate at 37°C, 5% $CO_2$ for an additional 8 hours. Finally, absorbance at 570 nm and 600 nm was measured using a PowerWave microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Reducing capability was calculated, as described previously. The following compounds were tested in this assay; antimycin A (Sigma Aldrich, St. Louis, MO), artemisinin (Sigma Aldrich, St. Louis, MO), trazodone hydrochloride (Sigma Aldrich, St. Louis, MO), mycophenolic acid (Santa Cruz Biotechnology), ofloxacin (Sigma Aldrich, St. Louis, MO), roxithromycin (Sigma Aldrich, St. Louis, MO), tetracycline hydrochloride (USB Corporation, Cleveland, OH) and ampicillin sodium salt (Fisher Scientific, Fair Lawn, NJ).

Toxicity Assay:

L929 cells were seeded in a clear, flat bottom, 96-well, tissue culture plate at 1.0 x $10^5$ cells/mL; 200 µl/well and incubated, flat, at RT to reduce edge effect for 1 hour. Following this time, the cells were allowed to further incubate for approximately 24 hours at 37°C, 5% $CO_2$. The following day, the cell culture medium (RPMI 1640 media (Cellgro by Mediatech; Manassas, VA 20109) with phenol red, supplemented with 0.3 mg/mL L-glutamine, 5% (vol/vol) fetal bovine serum (FBS) and 10 µg/mL gentamycin) was aspirated off and the desired concentration of each, individual compound was added to the designated wells after being diluted in RPMI 1640 medium (Cellgro by Mediatech; Manassas, VA 20109) without phenol red, supplemented with 0.3 mg/mL L-glutamine, 5% (vol/vol) fetal bovine serum (FBS) and 10 µg/mL gentamycin. The compounds were allowed to incubate on the uninfected cells for either 24 or 72 hours at 37°C, 5% $CO_2$ prior to addition of alamarBlue at a final concentration of 10%. After an additional 8 hour incubation at 37°C, 5% $CO_2$, absorbance at 570 nm and 600 nm was measured using a PowerWave microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Reducing capability was calculated, as described previously (64) using the following formula; $\frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2}{(\varepsilon_{OX})\lambda_2 A'\lambda_1 - (\varepsilon_{OX})\lambda_1 A'\lambda_2} \times 100$, where $\varepsilon_{OX}$ is the molar extinction coefficient of alamarBlue's oxidized (blue) form, A is the absorbance of the test wells, A' is the absorbance of the positive control wells (0.1% DMSO-treated, uninfected wells) and $\lambda_1$ is 570 nm and $\lambda_2$ is 600 nm. The following compounds were tested; antimycin A (Sigma Aldrich, St. Louis, MO), artemisinin (Sigma Aldrich, St. Louis, MO), trazodone hydrochloride (Sigma Aldrich, St. Louis, MO), mycophenolic acid (Santa Cruz Biotechnology), ofloxacin (Sigma Aldrich, St. Louis, MO), roxithromycin (Sigma Aldrich, St. Louis, MO), tetracycline hydrochloride (USB Corporation, Cleveland, OH) and ampicillin sodium salt (Fisher Scientific, Fair Lawn, NJ).

Immunofluorescence Assay:

L929 cells were seeded in a glass bottom 96-well plate at $7.5 \times 10^4$ cells/mL; 200 µl/well and incubated, flat, at RT to reduce edge effect. After 1 hour, cells were incubated at 37°C, 5% $CO_2$ for approximately 24 hours. *C. trachomatis* EB preparations, stored in SPG buffer at -80°C, were brought to room temperature immediately prior to infection and diluted to a final concentration of $8 \times 10^{-5}$ in Hank's Balanced Salt Solution, (HBSS; Mediatech, Inc., Manassas, VA). The cell culture medium was aspirated from the L929 cell cultures and 90 µl/well of the diluted *C. trachomatis* cell suspension was added. Following a two-hour incubation, flat, at room temperature, the infection inoculum was aspirated off and cell culture medium containing 1 µg/mL of cyclohexamide and the desired concentration of each, individual compound was added to the designated wells in triplicate. The cultures were then incubated at 37°C, 5% $CO_2$. Cells were fixed at 24 hours post infection (hpi) using 100% methanol for 10 minutes at room temperature and washed once with PBS. Cells were stained using the MicroTrack *C. trachomatis* culture confirmation test (Syva Co., Palo Alto, CA) diluted 1:40 in PBS (65 µl/well) for 110 min in the dark. In addition to the stain, 1 µg/mL 4-,6-diamidino-2-phenylindole (DAPI) in PBS further diluted 1:100 in PBS (7.5 µl/well) was added to each well and allowed to incubate in the dark for an additional 15 minutes. After staining, cells were washed three times for 10 minutes with 100 µl/well of PBS. Prior to storage, approximately 200 µl/well of 90% glycerol, 0.1% Tris-HCl, pH 8 was added upon the removal of PBS. The plate was stored in the dark at 4°C until visualization. The following compounds were tested in this assay; antimycin A (Sigma Aldrich, St. Louis, MO), artemisinin (Sigma Aldrich, St. Louis, MO), trazodone hydrochloride (Sigma Aldrich, St. Louis, MO), mycophenolic acid (Santa Cruz Biotechnology), ofloxacin (Sigma Aldrich, St. Louis, MO), roxithromycin (Sigma Aldrich, St. Louis, MO), tetracycline hydrochloride (USB Corporation, Cleveland, OH) and ampicillin sodium salt (Fisher

Scientific, Fair Lawn, NJ). Images were acquired and then analyzed using Cell Profiler and Cell Profiler Analyst as previously described (63).

Automated Screening:

L929 cells were seeded at 1.0 x $10^5$ cells/mL; 50 µl/well in a clear, 384-well tissue culture plate (Thermo Scientific, Nunc 164688) and incubated flat at RT for 1 hour. Cells were further incubated at 37°C, 5% $CO_2$ for approximately 24 hours. Cell culture medium was aspirated from L929 cells, seeded 24 hours previously, and 20 µl of RPMI without phenol red, supplemented with 0.3 mg/mL L-glutamine, 5% (vol/vol) fetal bovine serum (FBS) and 10 µg/mL gentamycin was added per well. An EB cell suspension (5.6 x $10^{-5}$) generated in RPMI without phenol red, supplemented with 0.3 mg/mL L-glutamine, 5% (vol/vol) fetal bovine serum (FBS) and 10 µg/mL gentamycin is added to each well and the appropriate controls were added to the designated control wells. Following a 72 hour incubation at 37°C, 5% $CO_2$, alamarBlue (Invitrogen) was added to each well at a final concentration of 10% and allowed to incubate with the cells for 8 hours at 37°C, 5% $CO_2$. Absorbance at 570 nm and 600 nm was measured using a microplate spectrophotometer. Reducing capability was calculated, as described above.

## C. RESULTS

### 1. Adapting the Assay to 384-well Formation:

#### a. Adjusting Cell Concentrations and EB Dilutions:

To adapt the assay to a 384-well format, several factors, including cell concentration and EB dilution, needed to be adjusted. The first factor to be adjusted to

adapt the resazurin-based assay from 96-well format to 384-well format was the proper concentration of L929 cells per well. Two concentrations of host cells per well were tested and the respective Z' factors were calculated using infected cells treated with a high concentration of a known inhibitor of *Chlamydia*, 1 μg/mL tetracycline, as the positive control and the DMSO treated/infected cells as the negative control. The wells with 5,000 cells/well had a resulting Z' factor of 0.52, indicating this is an excellent assay (Table 1). This was higher than the resulting Z' factor of 0.35 for 15,000 cells/well when infected with the same concentration of purified EBs and thus, 5,000 cells/well was the cell concentration used moving forward.

To develop an assay amendable for HTS a large dynamic range with low sample variability is required. Therefore, an EB dilution that results in a high level of infection, without over-infecting the cells, and with low sample variability was desired. To identify a dilution of EBs that satisfied those requirements, four different dilutions of purified EBs were tested on 5,000 cells/well. The respective Z' scores were calculated, using 1 μg/mL tetracycline, a concentration that should provide 100% inhibition of infection, as the positive control and DMSO-treated infected cells as the negative control. The dilution $3.5 \times 10^{-5}$, which generates approximately a 75% level of infection, resulted in the highest Z' factor of 0.74 and was the EB dilution utilized in the following screens (Table 1).

b. Effect of DMSO on L929 Cells:

The compounds to be screened for activity against *Chlamydia* are dissolved in 100% dimethyl sulfoxide (DMSO), an organic solvent that helps to prevent compound degradation and to aid in enhancing the shelf life of the compound (71). Given that the resazurin-based assay tests cell viability, the final concentration of DMSO could not be cytotoxic to the host

**Table 1: Cell Concentrations and EB Dilutions Tested in 384-well Format.**

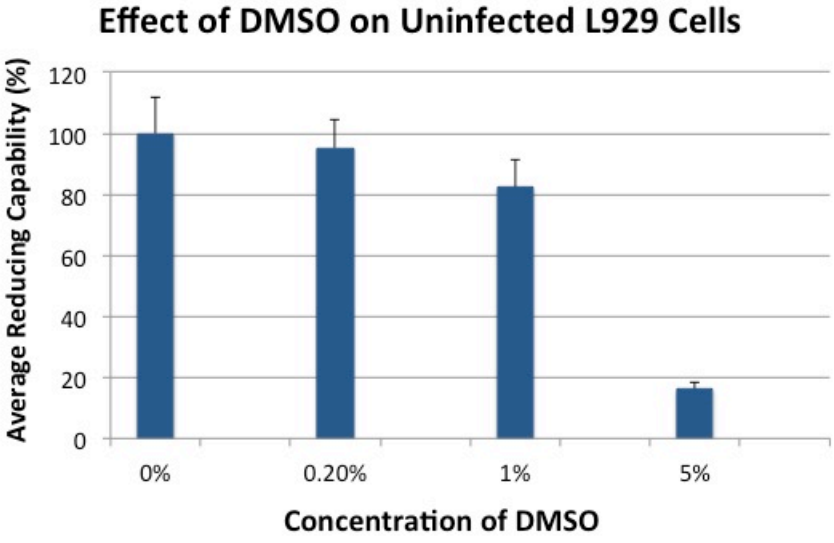
| Cells/Well: | | EB Dilution on 5,000 cells/well: | |
|---|---|---|---|
| **Tested Value (cells/well):** | **Z' Factor** | **Tested Value:** | **Z' Factor:** |
| | | $3.5 \times 10^{-5}$ | 0.74 |
| 5,000 | 0.52 | $4.5 \times 10^{-5}$ | 0.65 |
| | | $5.5 \times 10^{-5}$ | 0.63 |
| 15,000 | 0.35 | $6.5 \times 10^{-5}$ | 0.56 |

cells or the assay would not be functional. To test the cytotoxic effect of DMSO on uninfected L929 cells, three concentrations of DMSO diluted in cell culture medium were allowed to incubate on L929 cells for 72 hours prior to the addition of resazurin. It was found that 5% DMSO decreased the reducing capability, which corresponds to cell viability, of uninfected L929 cells by approximately 85%, indicating that 5% DMSO exposure over 72 hours was cytotoxic (Fig. 3). Cells exposed to 1% DMSO demonstrated approximately a 20% decrease in reducing capability but cells exposed to 0.2% DMSO had a slight to negligible decrease in reducing capability compared to the control cells. The final concentration of DMSO on the cells during the initial screen was approximately 0.1% DMSO, which was deemed an acceptable concentration during screening and had no obvious cytotoxic effects on the host cells or the *Chlamydia*.

c. Identification of Positive Controls:

Reference controls were also needed for the screen to finish adapting and optimizing the screen to 384-well format. Since this is a colorimetric-based assay, a mid-range inhibitor that exhibited approximately 50% inhibition of *Chlamydia* infection and a complete inhibitor of infection were ideal. Tetracycline is a known inhibitor of *Chlamydia* and was therefore a likely candidate to utilize as a reference control. To identify concentrations of tetracycline that fulfilled the desired reference controls, various concentrations were tested. As previously reported, we observed concentrations higher than 0.5 μg/mL resulted in complete inhibition of *Chlamydia* infection and dose dependent inhibition for concentrations lower than 0.5 μg/mL (72). The two concentrations chosen were 0.25 μg/mL and 1 μg/mL, which resulted in approximately 50% inhibition and complete inhibition of infection, respectively (Fig. 4A). Additional controls for the screen were chosen to ensure there were no measurable effects of DMSO on the eukaryotic host

**Figure 3: The Effect of DMSO on Uninfected, L929 Cells.** The effect of DMSO on uninfected, L929 cells was determined by incubating the tested concentrations of DMSO for 72 hours on the cells prior to the addition of alamarBlue (resazurin). Eight hours later the absorbance at 570 nm and 600 nm was measured using a spectrophotometer and the reducing capability was calculated relative to the control containing 0% DMSO. 5% DMSO was determined to be cytotoxic to the cells for this period of time and decreasing toxicity as the concentration of DMSO decreased was observed. Error bars represent the standard deviation of the samples.


Effect of DMSO on Uninfected L929 Cells
Average Reducing Capability (%)
120
100
80
60
40
20
0
0%
0.20%
1%
5%
Concentration of DMSO

cells or the organism and represent the dynamic range of the assay by showing the colorimetric scale from dark blue to fluorescent red (Fig. 4B).

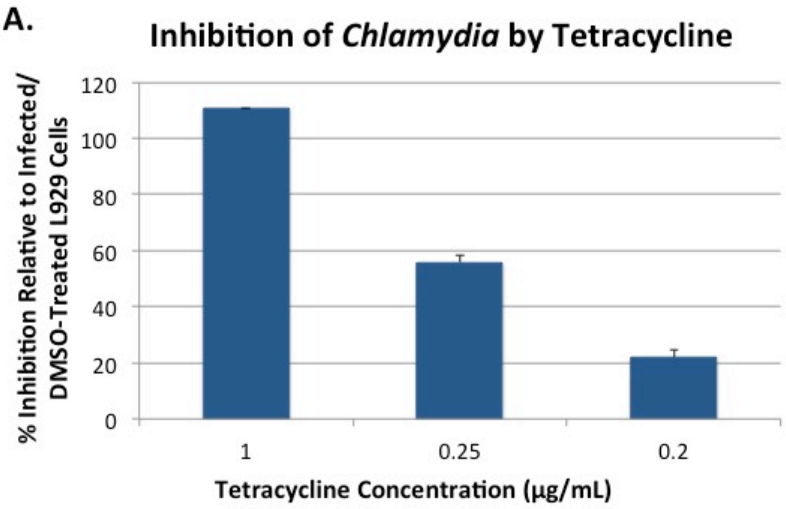
2. Initial Screen of Prestwick Chemical Library:

To verify the utility and functionality of the assay the Prestwick Chemical library, a small compound library containing many known antibiotics and agency approved, off-patent drugs, was screened for inhibitors of *Chlamydia* after adapting the assay to 384-well format. In all, the Prestwick Chemical library is composed of 1120 compounds and contains a variety of compounds with diverse medical purposes, mechanisms of action and, origins of discovery. Following the initial, small-scale screen 52 hits were identified at 2 standard deviations above the sample average (Table 2). Of those hits, 41 compounds were known to have anti-bacterial activity. The additional eleven compounds identified as hits were of diverse medical uses and activities, including organ transplant rejection medications, anti-depressants, natural products, and anti-malarial drugs.

Four separate 384-well plates were required for the library screen to test all 1120 of the compounds. The resulting Z' factor for the plates were calculated individually using the DMSO-treated, uninfected and DMSO-treated, infected cells as the positive and negative controls, respectively and resulting factors were 0.58, 0.56, 0.49, 0.37. The resulting Z factors for the plates individually were 0.06, 0.21, 0.12 and 0.24. These Z factors were much lower than anticipated based on the Z' factors calculated for the individual plates but due to the high hit rate of over 4.6%, and the identification of several highly inhibitory compounds, the standard deviation of the sample averages was higher than expected, resulting in lower Z factors. To illustrate this observation, the Z factors were re-calculated using the sample average and standard deviation in the absence of the identified hit values. The resulting Z factors for the plates following those calculations were 0.49, 0.54, 0.44 and 0.37, indicating

**Figure 4: Identification of Controls for Assay.** Tetracycline is a known inhibitor of Chlamydia, making it a good candidate for reference controls in this assay. Since this assay is a colorimetric assay, a concentration of tetracycline that resulted in approximately 50% inhibition of *Chlamydia* and a concentration that resulted in complete inhibition were desired. Of the tested concentrations, 0.25 $\mu$g/mL and 1 $\mu$g/mL fulfilled those requirements, respectively, and were used as the reference controls for the resulting screens. Error bars represent the standard deviation of the samples (A). In addition to the two concentrations of tetracycline, there were four more controls used during screening, for a total of six different controls; UU, uninfected/untreated cells, UT, infected/untreated cells, $T_L$, 0.25 µg/mL tetracycline, DUI, DMSO-treated/uninfected cells, DI, DMSO-treated/infected cells, and $T_H$, 1 µg/mL tetracycline (B). The cells that were not viable at the time alamarBlue (resazurin) was added were not capable of converting the resazurin to resorufin and appear dark blue. As the cell viability increases, the red color of the well increases due to an increased amount of resorufin.

A.


Inhibition of *Chlamydia* by Tetracycline
% Inhibition Relative to Infected/ DMSO-Treated L929 Cells
120
100
80
60
40
20
0
1
0.25
0.2
Tetracycline Concentration (µg/mL)

B.

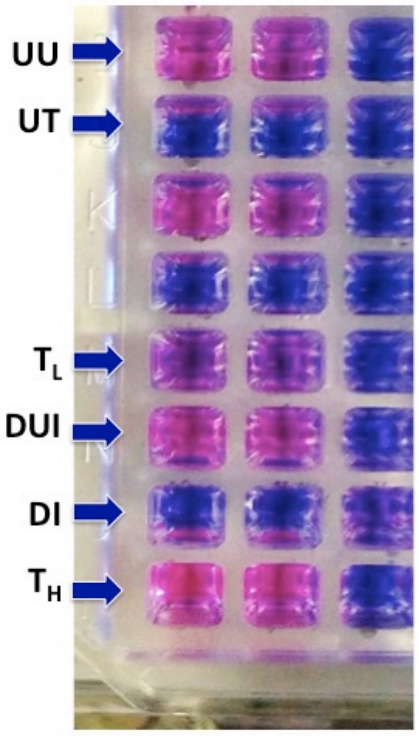

UU
UT
$T_L$
DUI
DI
$T_H$

**Table 2: Prestwick Chemical Library Initial Hits.**

| Class: | Compound: | Percent Inhibition: |
|---|---|---|
| **Anti-bacterial:** | | |
| **Beta-lactams:** | | |
| | Cyclacillin | 107 |
| | Benzylpenicillin sodium | 95.1 |
| | Metampicillin sodium salt | 94.1 |
| | Phenethicillin potassium salt | 92.8 |
| | Talampicillin hydrochloride | 90.4 |
| | Benzathine benzylpenicillin | 88.4 |
| | Bacampicillin hydrochloride | 85.8 |
| | Ticarcillin sodium | 84.2 |
| | Nafcillin sodium salt monohydrate | 83.2 |
| | Pivmecillinam hydrochloride | 81.6 |
| | Ampicillin trihydrate | 77.6 |
| | Amoxicillin | 73.4 |
| **Fluoroquinolones:** | | |
| | Ofloxacin | 99.9 |
| | Ciprofloxacin hydrochloride | 76.4 |
| | Lomefloxacin hydrochloride | 55.3 |
| **Macrolides:** | | |
| | Erythromycin | 93.4 |
| | Josamycin | 92.4 |
| | Midecamycin | 92.1 |
| | Roxithromycin | 86.1 |
| | Troleandomycin | 71.8 |
| **Tetracyclines:** | | |
| | Minocycline hydrochloride | 110 |
| | Methacycline hydrochloride | 104 |
| | Oxytetracycline dihydrate | 92.6 |
| | Doxycycline hydrochloride | 86.3 |
| | Demeclocycline hydrochloride | 79.6 |
| | Meclocycline sulfoxalicylate | 75.7 |
| | Tetracycline hydrochloride | 70.1 |
| | Chlortetracycline hydrochloride | 69.5 |
| **Other:** | | |
| | Florfenicol | 94.9 |
| | *Nifuroxazide | 89.1 |
| | Rifabutin | 87.5 |
| | Clindamycin hydrochloride | 81.2 |
| | *Thiostrepton | 80.8 |
| | Chloramphenicol | 79.0 |
| | Rifampicin | 75.7 |
| | Thiamphenicol | 75.5 |
| | Ceforanide | 62.5 |
| | Trimethoprim | 59.9 |
| | *Monensin sodium salt | 46.2 |
| | *Clofazimine | 34.9 |
| | *Nitrofural | 33.4 |
| **Non-antibacterial:** | | |
| | *Lysergol | 79.1 |
| | *Mebhydroline 1,5-naphtalenedisulfonate | 79.0 |
| | *Piperlongumine | 77.7 |
| | *Methiothepin maleate | 75.9 |
| | *Mycophenolic acid | 50.3 |
| | *Trazodone hydrochloride | 43.8 |
| | *Antimycin A | 38.6 |
| | *Artemisinin | 38.3 |
| | *Dacarbazine | 31.9 |
| | *Halofantrine hydrochloride | 31.7 |
| | *Ribavirin | 16.3 |

Asterisks (*) indicate novel compounds without previously reported anti-chlamydial activity.

the effect the high hit rate and resulting data variation had on the calculated Z factors for this library screen.

3. Dose-Dependent Confirmation of Hits:

To confirm the hits identified in the initial screen, eight concentrations of each compound from 20 μM to 0.156 μM were screened for dose-dependent analysis. Following this subsequent screen, all of the compounds with known anti-bacterial properties were confirmed and accounted for approximately 88% of the hits obtained (Table 3). Of the four classes of antibiotics that can be used to treat *Chlamydia* infections, the majority of these compounds were beta-lactam antibiotics, followed by tetracyclines, macrolides, and fluoroquinolones. The remaining hits belonged to other families of antibiotics such as chloramphenicol antibiotics and derivatives. The high percentage of hits with known anti-bacterial properties identified from screening the Prestwick Chemical library verified the effectiveness of this method to identify compounds with activity against *Chlamydia* and of the additional eleven compounds identified in the initial screen, six were confirmed by dose-dependent analyses. Since these compounds did not have previously reported anti-bacterial properties their mechanism of inhibition of *Chlamydia* was not known and they were interesting and unexpected hits. The compounds with the top four percentages of inhibition of *Chlamydia* were chosen for further investigation.

4. Inhibition of Commercially Purchased Compounds:

To begin to delineate the mechanisms of inhibition exuded on *Chlamydia* by the top four inhibitory, non-antibacterial compounds identified in the initial screen of the Prestwick Chemical Library, the following compounds, including positive controls were purchased commercially; antimycin A, artemisinin, trazodone hydrochloride, mycophenolic acid,

**Table 3: Prestwick Chemical Library Confirmed by Dose-dependent Analysis.**

| Class: | Compound: | $IC_{50}$ (μM): |
|---|---|---|
| **Anti-bacterial:** | | |
| **Beta-lactams:** | | |
| | Benzylpenicillin sodium | <0.15 |
| | Cyclacillin | <0.15 |
| | Ticarcillin sodium | <0.15 |
| | Benzathine benzylpenicillin | 0.27 |
| | Bacampicillin hydrochloride | 0.30 |
| | Metampicillin sodium salt | 0.30 |
| | Phenethicillin potassium salt | 0.60 |
| | Ampicillin trihydrate | 0.75 |
| | Talampicillin hydrochloride | 0.83 |
| | Pivmecillinam hydrochloride | 0.87 |
| | Nafcillin sodium salt monohydrate | 1.16 |
| | Amoxicillin | 1.23 |
| **Fluoroquinolones:** | | |
| | Ciprofloxacin hydrochloride | 0.57 |
| | Ofloxacin | 1.88 |
| | Lomefloxacin hydrochloride | 3.26 |
| **Macrolides:** | | |
| | Josamycin | 0.49 |
| | Roxithromycin | 0.59 |
| | Erythromycin | 0.98 |
| | Midecamycin | 1.23 |
| | Troleandomycin | 2.02 |
| **Tetracyclines:** | | |
| | Demeclocycline hydrochloride | <0.15 |
| | Doxycycline hydrochloride | <0.15 |
| | Meclocycline sulfoxalicylate | <0.15 |
| | Methacycline hydrochloride | <0.15 |
| | Tetracycline hydrochloride | <0.15 |
| | Oxytetracycline dihydrate | 0.30 |
| | Minocycline hydrochloride | 1.22 |
| | Chlortetracycline hydrochloride | 1.86 |
| **Other:** | | |
| | *Monensin sodium salt | <0.15 |
| | Rifabutin | <0.15 |
| | Rifampicin | <0.15 |
| | *Thiostrepton | 0.50 |
| | Florfenicol | 0.53 |
| | Chloramphenicol | 0.81 |
| | *Nifuroxazide | 0.86 |
| | Thiamphenicol | 1.02 |
| | Clindamycin hydrochloride | 2.13 |
| | Ceforanide | 2.20 |
| | *Nitrofural | 2.50 |
| | Trimethoprim | 3.50 |
| | *Clofazimine | >20.0 |
| **Non-antibacterial:** | | |
| | *Mycophenolic acid | 0.56 |
| | *Trazodone hydrochloride | 3.73 |
| | *Artemisinin | 3.74 |
| | *Antimycin A | 4.85 |
| | *Dacarbazine | 7.91 |
| | *Ribavirin | 13.8 |

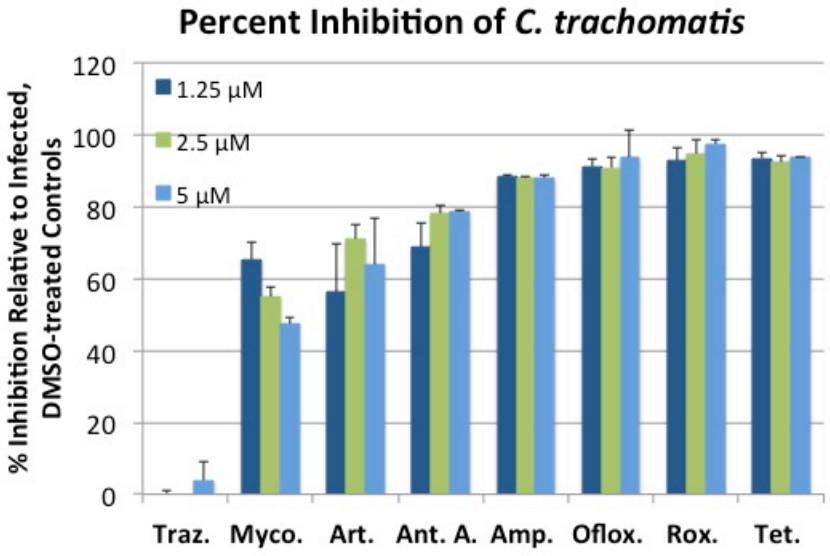
Asterisks (*) indicate novel compounds without previously reported anti-chlamydial activity.

ofloxacin, roxithromycin, ampicillin sodium salt, and tetracycline hydrochloride. The first four compounds listed are compounds with miscellaneous drug purposes but were identified and confirmed as hits in the screen of the Prestwick chemical library. Antimycin A is a secondary metabolite produced by *Streptomyces* and is commercially used as a fish poison. Artemisinin is an ancient, naturally derived drug used in combination to treat fever and malaria. Trazodone hydrochloride is serotonin reuptake inhibitor medically used as an anti-depressant and mycophenolic acid was originally utilized as an immunosuppressant drug to prevent organ transplant rejection. The final four compounds listed above were used as positive controls in the assay because each one is a member of a different family of antibiotics, all with varying mechanisms of action against *Chlamydia*. Ofloxacin is a fluoroquinolone antibiotic and roxithromycin is a macrolide antibiotic, which is the same family of antibiotics as azithromycin. Azithromycin is used to treat *Chlamydia* infections but is not a compound included in the Prestwick Chemical Library and was therefore, not obtained as a hit or used in further studies. Ampicillin is a beta-lactam antibiotic, which is the same family that includes amoxicillin, another anti-biotic that can be used for treatment of *Chlamydia* infection. Finally, tetracycline is a typical tetracycline with a similar mechanism of action as doxycycline, the other commonly prescribed antibiotic used to treat *Chlamydia*.

Once the compounds were received and solubilized they were tested in three concentrations, 5 μM, 2.5 μM, and 1.25 μM. All of the compounds exuded measurable levels of inhibition of *Chlamydia* as seen in the initial and dose-dependent screens of the Prestwick Chemical Library except trazodone hydrochloride, which did not exude any significant inhibition (Fig. 5). It is possible that the stock of trazodone hydrochloride that was purchased commercially was inactive for an undetermined reason or there could have been an error on the part of the HTS facility that plated the compounds for the initial and dose-

**Figure 5: Inhibition of *C. trachomatis* with Commercially Purchased, Hit Compounds**.

The four compounds without known anti-bacterial properties that resulted in the highest percent of inhibition of *Chlamydia* were purchased commercially for further investigation. In addition to those four compounds, four positive controls, ampicillin, ofloxacin, roxithromycin and tetracycline, were also included. To validate the screening results and the activity of the compounds, dose-dependent studies were performed. All of the compounds resulted in measurable inhibition compared to the initial Prestwick Chemical Library screen except trazodone hydrochloride, which did not result in measureable inhibition of *Chlamydia*. Error bars represent the standard deviations of the samples.


Percent Inhibition of C. trachomatis
% Inhibition Relative to Infected, DMSO-treated Controls
120
100
80
60
40
20
0
1.25 µM
2.5 µM
5 µM
Traz.
Myco.
Art.
Ant. A.
Amp.
Oflox.
Rox.
Tet.

dependent screens of the Prestwick Chemical Library. Further analysis is required to address this contrasting observation.

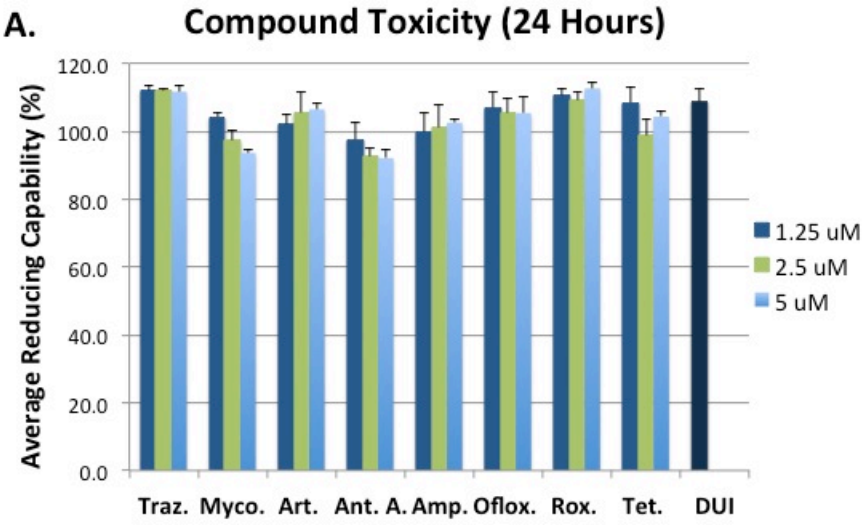
In addition to the absence of detectable inhibition by trazodone hydrochloride, the resulting inhibition for mycophenolic acid was not dose-dependent as would be expected. Generally, when a compound is inhibitory, increasing the concentration will increase inhibition until a saturation point is reached but increasing concentrations of mycophenolic acid resulted in an observed decrease of inhibition. Given that resazurin is a cell viability indicator, the health of the host cell will influence the results of the assay. In this case, the reverse, dose-dependent response may indicate that mycophenolic acid is cytotoxic to the host cells for the 72 hour incubation period of the experiment.

5. Toxicity of Compounds on L929 Cells:

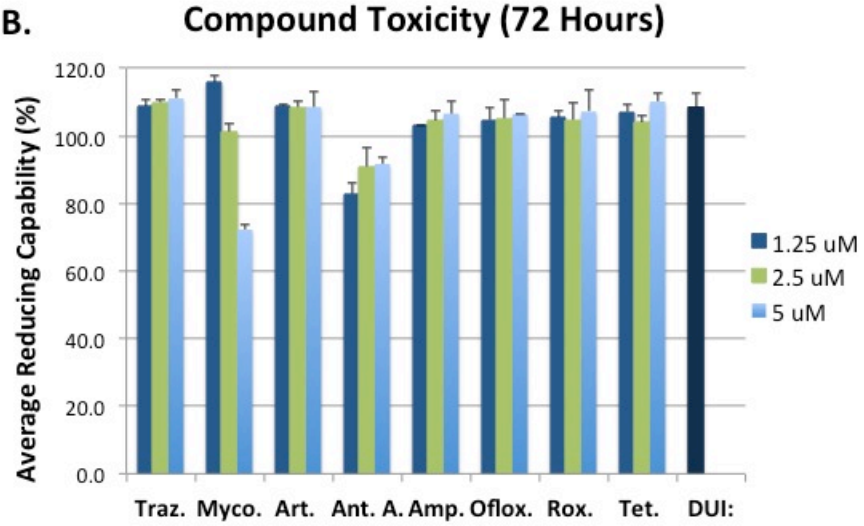
To test the cytotoxicity of the compounds on the L929 cells, uninfected cells were treated for either 24 hours or 72 hours with the compounds and their viability was measured using resazurin. After a 24 hour incubation period, the reducing capability, which corresponds with the cell viability, of the host cells treated with mycophenolic acid decreased as the concentration of the compound increased (Fig. 6A). This trend was even more striking after a 72 hour incubation period with the reducing capability of the treated host cells decreasing by over 30% when treated with 5 μM and compared to the DMSO-treated, uninfected controls (Fig. 6B). Interestingly, a decrease in the host cell reducing capability for cells treated with antimycin A was also observed but was not as striking as the resulting reducing capability for cells treated with mycophenolic acid (Fig. 6). Following the completion of these toxicity assays, it was apparent that mycophenolic acid is cytotoxic in higher concentrations to uninfected L929 cells for 72 hours and antimycin A also has measureable, toxic effects on the uninfected, host cells. The observable cytotoxic effects by

**Figure 6: Toxicity of Compounds on Uninfected, L929 Cells.** For a compound to be a suitable candidate to be used for treatment or a molecular probe, the compound must not be cytotoxic to the host cells. To test the cytotoxicity of these compounds, the compounds was diluted to the desired concentration in tissue culture medium and incubated on uninfected, L929 cells for 24 hours (A) and 72 hours (B). The two compounds that caused the highest amount of host cell cytotoxicity were mycophenolic acid and antimycin A. The observed cytotoxicity increased for both compounds as the incubation time increased. Error bars indicate the standard deviation of the samples.

A.


Compound Toxicity (24 Hours)
Average Reducing Capability (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Traz.
Myco.
Art.
Ant. A.
Amp.
Oflox.
Rox.
Tet.
DUI
1.25 uM
2.5 uM
5 uM

B.


Compound Toxicity (72 Hours)
Average Reducing Capability (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Traz.
Myco.
Art.
Ant. A.
Amp.
Oflox.
Rox.
Tet.
DUI:
1.25 uM
2.5 uM
5 uM

the compounds to the host cells could have resulted in the inversely related dose-dependent inhibition of *Chlamydia* by the resazurin-based assay.

6. Orthogonal Assay:

    a. Immunofluorescence Assay:

To observe how antimycin A, artemisinin, mycophenolic acid and trazodone hydrochloride may be inhibiting *Chlamydia*, L929 cells were infected with purified EBs and each compound was tested at three concentrations (5 μM, 2.5 μM, 1.25 μM). At 24 hpi, cells were fixed and stained and images were acquired. Inclusions were visible in all of the samples except the DMSO-treated, uninfected control wells, indicating that none of the tested compounds at these concentrations were able to completely inhibit inclusion formation (Fig. 7). However, three of the four positive control compounds, ofloxacin, roxithromycin, and tetracycline treatment resulted in a striking decrease of inclusion formation and inclusion size, as expected (Fig. 7H-7J).

    b. Inhibition of Inclusion Formation:

To quantify the effect on inclusion formation by each compound, the images were analyzed using Cell Profiler and Cell Profiler Analyst software, which utilizes an algorithm that classifies infected cells and uninfected cells from each sample. Cells are classified as infected by the presence of an inclusion in the cell. Although Cell Profiler/Cell Profiler Analyst can measure inclusion size, it is not a factor that is included in determining whether or not a host cell is infected. This is important to consider when examining the results from this assay. It would appear antimycin A, artemisinin and mycophenolic acid do not significantly inhibit *Chlamydia* (Fig. 8), but upon closer examination of the images, it is evident that the inclusions in the cells treated with these compounds are smaller than the

**Figure 7: Immunofluorescence Assay to Observe the Effect of Tested Compounds on Chlamydia.** L929 cells were infected with *C. trachomatis* and treated with compounds at 0 hpi. At 24 hpi, cells were fixed and stained to observe the individual effects of each compound on Chlamydia. Images were captured at 40x magnification. The 0.1% DMSO-treated uninfected (DUI; A.) and infected (DI; B.) controls are represented in the top row of images. Cells treated with the four non-antibacterial compounds, trazodone hydrochloride (Traz.; C.), mycophenolic acid (Myco.; D.), artemisinin (Art.; E.), and antimycin A (Ant. A; F.), are also displayed in rows two and three. The final two rows of images include L929 cells treated with the four anti-bacterial compounds, ampicillin (Amp.;G.), ofloxacin (Oflox.; H.), roxithromycin (Rox.; I.) and tetracycline (Tet.; J.).

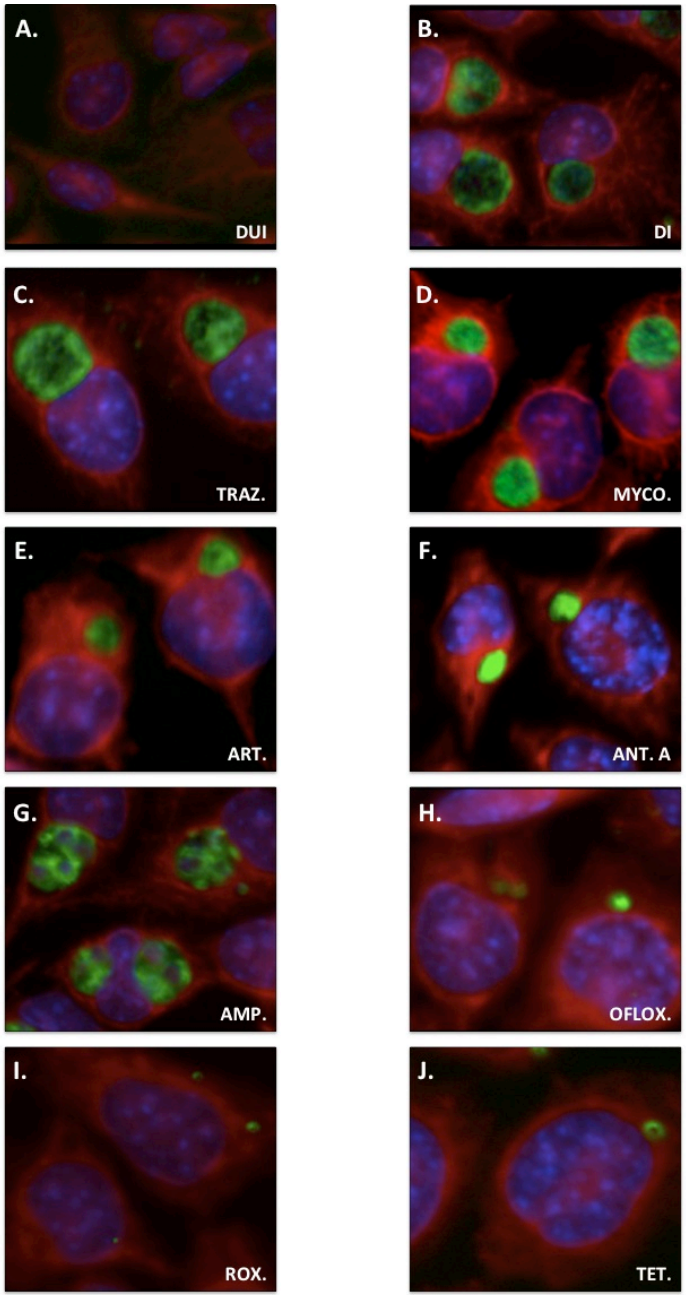
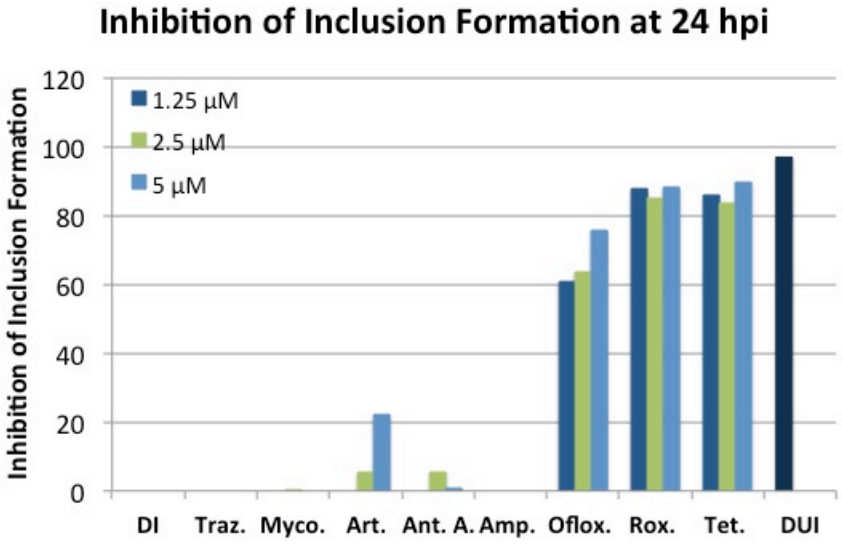

A.
DUI
B.
DI
C.
TRAZ.
D.
MYCO.
E.
ART.
F.
ANT. A
G.
AMP.
H.
OFLOX.
I.
ROX.
J.
TET.

**Figure 8: Inhibition of Inclusion Formation.** Cells were infected with purified EBs and compounds were added at 0 hpi. The cells were fixed and stained at 24 hpi and images were captured. After acquiring images, Cell Profiler and Cell Profiler Analyst software was used to determine the percent inhibition of inclusion formation relative to the infected control, DI (DMSO-treated, infected L929 cells). Artemisinin and antimycin A were the only non-antibacterial compounds that resulted in measureable inhibition with artemisinin being the only one of those two that resulted in a dose-dependent increase in inhibition of inclusion formation.


Inhibition of Inclusion Formation at 24 hpi
Inhibition of Inclusion Formation
120
100
80
60
40
20
0
1.25 µM
2.5 µM
5 µM
DI
Traz.
Myco.
Art.
Ant. A.
Amp.
Oflox.
Rox.
Tet.
DUI

DMSO-treated, infected control cells (Fig. 7B, 7D-7F). Similar to the results of the resazurin-based microplate assay performed on these commercially purchased compounds, trazodone hydrochloride did not exude measurable inhibition of *Chlamydia* inclusion formation and the inclusions do not appear significantly smaller compared to the DMSO-treated, infected control inclusions.

7. Automation of Screening Method:

To confirm the assay is well suited for large scale screening of chemical libraries, the HTS Facility at The University of Kansas, which is an independent, state-of-the-art drug-discovery facility, automated the liquid handling steps of the assay, and verified the assay's reproducibility (73, 74). The automated screening of this method confirmed a large separation band of an approximate 76-fold difference between the DMSO-treated, uninfected cells and the DMSO-treated, infected cells. The Z' factors using these two controls were approximately 0.86 and 0.80 for two, separate, 384-well plates, indicating that the assay is statistically acceptable for large-scale screening of chemical libraries. In addition to being found statistically acceptable, the assay was found to be highly reproducible and well adapted for automated screening by the HTS Facility.

## D. DISCUSSION

The aim of this study was to develop a high-throughput screening method for *Chlamydia trachomatis* by adapting a previously developed resazurin-based microplate assay to 384-well format, and automating the liquid handling steps, allowing for large scale screening of compound libraries. One of the important advantages of using the cell viability indicator, resazurin, in the high-throughput screening method is the dual purpose of the

indicator. In several high-throughput screening methods, an additional, counter-screen must be performed on any hits identified to test eukaryotic cell cytotoxicity (75), but using this method, the anti-chlamydial compounds that may be identified during screening should not be cytotoxic to the eukaryotic host cells. Even if a compound were a strong inhibitor of *Chlamydia*, it would not be identified as a hit from the screen due to its cytotoxic nature, making the host cells incapable of converting resazurin to resorufin. This dual function not only makes the method more cost efficient by eliminating the need for an additional, counter-screen but it also reduces the time required to identify a strong, anti-chlamydial, lead compound.

The first steps in adapting the method from 96-well to 384-well format were to identify the proper concentration of cells per well and to identify a dilution of EBs to provide a consistent and sufficient level of infection in the chosen concentration of cells. It was initially thought that a higher number of cells per well would provide more consistent results by helping to reduce well-to-well variation. However, it was determined that 5,000 cells per well was an optimum concentration capable of forming a confluent monolayer, and growing over a 72 hour incubation period without severe cell death due to lack of sufficient reagents. The chosen cell concentration also resulted in a Z' factor higher than 0.5, which is indicative of an excellent high-throughput screening assay as originally described by Zhang *et al*. (69).

After determining an appropriate concentration of cells, the next task was to identify an appropriate dilution of EBs and it was critical to prevent over-infecting the host cells. If the host cells were over infected, cell lysis could occur earlier during the developmental cycle, adding another variable into the assay, and potentially decreasing the likelihood of identifying true hits. If more than one EB enters a cell, the separate inclusions will eventually fuse into a larger, single inclusion and following the cell division of RBs and

the asynchronous conversion of EBs, the inclusion will grow faster due to a higher number of organisms. The larger inclusion will lead to earlier and less uniform host cell lysis, increasing the variability of the end point of the assay. The chosen dilution with the highest Z' score generated approximately a 75% level of infection, which provided a large separation band and was highly reproducible with low standard deviations between samples.

It was critical to identify concentrations that gave consistent readings from well to well to reduce the standard deviations of the samples. Z factor is significantly influenced by the variation and dynamic range and is calculated using the following formula; $Z = 1 - \frac{(3\sigma_s + 3\sigma_c)}{|\mu_s - \mu_c|}$, where $\sigma_s$ and $\mu_s$ are the standard deviation and mean, respectively, of the sample data and $\sigma_c$ and $\mu_c$ indicate the standard deviation and the mean of the control data, respectively. The standard deviations highly influence the Z' and Z factors for an assay or a screen because the data variations of the sample and the respective control data are multiplied by three and added to one another during the calculation of Z factor. If a screen has high variability the resulting Z factor will be a lower than desired value, because even in the presence of a wide separation band, the dynamic range is not capable of compensating for the variability of the assay. High variability in the assay may also decrease the accuracy of the assay, causing an increase in observed false positives.

The final step to complete the adaptation of the micro-plate assay to 384-well format and to prepare for the initial small-scale screen was to identify positive controls. Tetracycline was chosen as the positive control compound because it has been extensively used as a chlamydial inhibitor and it's mechanism of action is well studied. The goal was to identify two concentrations of tetracycline that provided approximately 50% inhibition of *Chlamydia* and a concentration that fully inhibited the organisms' growth. The lower concentration was intended to serve as a reference, colorimetric control allowing for the

visual identification of mid-level inhibitors, given that not all of the expected hits would exude complete inhibition of *Chlamydia*. Throughout the initial, small screen and during the identification of the lower concentration control, the amount of inhibition obtained by 0.25 μg/mL tetracycline was not consistent. This observed variability could be due to the potency of tetracycline as a *Chlamydia* inhibitor, which follows a steep dose-dependent response curve. Osaka *et al.* reported a 60% increase in observable inhibition between 0.1 and 0.5 μg/mL tetracycline, indicating that a small increase in the concentration of this inhibitor can result in a significant decrease in *Chlamydia* infection. The small error obtained during diluting tetracycline by manual pipetting could skew the actual final concentration of the compound and therefore, effect the amount inhibition recorded. Even given its inconsistency, it was still included as a control in the screen for visualization purposes but was not included in any final calculations of Z' or Z factor scores to evaluate the quality of the assay or the screen.

The Prestwick Chemical Library was screened following the adaptation to 384-well format to verify the utility and functionality of the assay. The library is composed of 1120 compounds in total and 156 of those compounds have diverse, anti-bacterial activities. Following the initial screen, 41 compounds identified had known anti-bacterial properties and belonged to a variety of anti-bacterial classes. The identification of a large number of hits with known anti-bacterial properties, many of which belong to the four classes of antibiotics known to be effective against Chlamydia, strongly suggests that the assay is functional and capable of identifying anti-chlamydial compounds from small, compound libraries. The remaining compounds not identified in the library as hits but having known anti-bacterial properties could have remained unidentified for several reasons. The compound, despite its drug uses, may have been cytotoxic to the L929 host cells for the assay's required incubation period or the tested concentration may have been too low to

exude the level of inhibition of *Chlamydia* to be identified as a hit. There are also a variety of anti-bacterials that are incapable of crossing the eukaryotic cell membrane, preventing their mechanism of action from being effective against *Chlamydia* since it is an obligate, intracellular organism that inhabits a parasitophorous vacuole. Of the anti-bacterial compounds that were identified in the initial screen, all 41 were confirmed during the dose-dependent confirmation, as expected, further validating that the hits obtained were compounds with anti-chlamydial activity and were not false positives. This confirmation additionally verified the functionality of the assay and its potential utility as a true, HTS method suitable for large scale screening of compounds.

In addition to the expected, large number of anti-bacterial hits in the initial and dose-dependent screens, six compounds were identified that did not have previously reported anti-bacterial properties. Three of the four compounds with the highest percent of inhibition of *Chlamydia*, antimycin A, artemisinin and mycophenolic acid, still exuded measurable inhibition, as reported by the resazurin-based assay, after being obtained from a commercial sources for further investigation (Fig. 5). Upon examination of the images captured during IFA, none of these three compounds were capable of preventing the formation of chlamydial inclusions, indicating that they may not have an effect on *Chlamydia's* entry into the eukaryotic host cell (Fig. 7, Fig. 8). However, each of the compounds resulted in decreased inclusion size when compared to the DMSO-treated, infected control inclusions (Fig. 7).

The resulting smaller inclusions and differences in measured inhibition between the resazurin-based assay and the observations from the immunofluorescence assay could be caused by an effect of the compounds on *Chlamydia's* developmental cycle. If the developmental cycle is delayed, the host cells may not lyse by 72 hours post infection, when the cell viability indicator is added for the resazurin-based assay, and therefore, they would

still be capable of converting resazurin, the form that is reduced by products of cell metabolism, to resorufin. One of the positive controls, ampicillin, is an example of such an event. This compound allows for the formation of inclusions, which are recognized by Cell Profiler/Cell Profiler Analyst; however, the inclusions are not phenotypically normal inclusions (Fig. 7G). Beta-lactam antibiotics stall the developmental cycle by disrupting the division of RBs, generating enlarged RBs and what is termed aberrant inclusions (76). These cells do not lyse by 72 hpi and are therefore, capable of converting the viability indicator. This disruption in the developmental cycle is read by the resazurin-based assay as viable cells and is indicative of inhibition of *Chlamydia* in that assay, even though the removal of the antibiotic allows *Chlamydia* to come out of its persistent state and resume normal development (Fig 5).

There are several essential growth stages throughout the chlamydial developmental cycle that the compounds could be effecting if their mechanism of action is not lethal to the bacterial organism. Two of the tested compounds, mycophenolic acid and antimycin A both have measurable cytotoxic effects on the eukaryotic host cells, which could be indirectly causing the observed effect on *Chlamydia*. Mycophenolic acid is known to act on inosine monophosphate dehydrogenase, which controls the rate of GMP formation in the path of purine synthesis and was used and recommended as an immunosuppressant drug for the treatment of autoimmune diseases and to prevent organ transplant rejection (77). Antimycin A is known to inhibit the oxidation of ubiquinol, disrupting the formation of a proton gradient and the ATP production by oxidative phosphorylation (78, 79). It is well known that *Chlamydia* has a reduced genome and as an obligate, intracellular organism requires a variety of nutrients and growth factors from the its eukaryotic host, including sphingomyelin, and cholesterol, which can be used for inclusion membrane expansion and other species of *Chlamydia*, including *C. psittaci*, have mechanisms of taking ATP from the

host in exchange for ADP (50, 80). If these compounds are having negative effects on the host and preventing the organism from obtaining the nutrients it requires for growth, those negative effects may be responsible for the observable effects on the organism following treatment with each of these compounds, individually.

Unlike, antimycin A and mycophenolic acid, artemisinin does not cause any observable cytotoxic effects on the host cells. However, it still exudes the most noticeable effects on inclusion size and inhibition of inclusion formation, indicating it has a mechanism of action unrelated to host cell cytotoxicity. Artemisinin, also known as qinghaosu has been used to treat fever and malaria for many centuries in China and is a natural product derived from the herb *Artemisia annua (81)*. Recently, it has been used around the world in combination with other antimalarials to treat malarial infections but *Plasmodium* resistance has been observed (82). It is thought to kill the parasite by means of free radicals produced by the presence of its endo-peroxide bridge, leading to modification of malarial target proteins. It is also known that artmisinin is hydrophobic and it has been shown to partition into biological membranes and to localize to specific parasite membranes, vacuole membranes and mitochondria (83). It is possible that if the compound is capable of crossing eukaryotic membranes to get to the inclusion membrane and have exposure to *Chlamydia*, it could be using a similar free radical mechanism to eliminate the bacterial organisms. Further studies are required to fully investigate all three of these compounds' specific mechanism of action but given the absence of host cell cytotoxicity and the most striking effects on *Chlamydia*, artemisinin may be the best candidate to probe into the biology of this organism or to possibly serve as a model molecule for a novel therapy. Adding these compounds to infected cells at different critical points during the developmental cycle may shed some light on the growth stage effected by the addition of each compound and

incubating purified EBs prior to infection could indicate whether or not any of these compounds play a role in EB attachment prior to entry into the cell.

Following the results of the initial screen, dose-dependent confirmation, and the orthogonal assay to further confirm and validate the resulting hits, the screening method was adapted for automated liquid handling in collaboration with the HTS facility at The University of Kansas. The HTS facility also confirmed the assay reproducibility and determined that this method was statistically acceptable for high-throughput screening. Since that confirmation, the CMLD (Chemical Methodologies and Library Development) compound library containing approximately 5,200 novel compounds with predicted drug-like activity has been screened in a fully automated format using the HTS resazurin-based method. The CMLD Library is a library of compounds synthetically derived using new principles of scaffold design. The drug design principles used during the generation of the library increases the probability that these compounds will have pharmacological activity and drug-like characteristics. Preliminary data indicate an initial hit rate of approximately 1.75% and dose-dependent analysis is to soon follow. This resazurin-based high-throughput screening assay serves as the first automated, high-throughput screening method capable of identifying anti-chlamydial compounds that could be used as molecular probes, model molecules or novel *C. trachomatis* therapies. It will also lead the way for the development of HTS methods in other species of *Chlamydia*, such as *C. psittaci*, and additional obligate intracellular organisms with significant public health impacts and a greater need for identification of novel treatment therapies.

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