Mechanism of Action of ST-669 in *Chlamydia* spp.

by
Stormy Marie Scharzenberger

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Animal Science
(Honors Associate)

Presented May 18, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Stormy Scharzenberger for the degree of Honors Baccalaureate of Science in Animal Science presented on May 18, 2016. Title: Mechanism of Action of ST-669 in *Chlamydia* spp.

Abstract approved:

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Daniel Rockey

Our laboratory group is studying the molecular target of a novel, broad-spectrum antiviral compound known as ST-669. This compound has activity against a variety of different viruses and also obligate intracellular bacteria in the genera *Coxiella* and *Chlamydia.* The goal of this thesis project was to help elucidate the mechanism of action of ST-669 by evaluating the hypothesis that ST-669 acts as an inhibitor of host sphingomyelin production. In this study, we examine the salvage pathway of sphingomyelin synthesis and compared and contrasted the effects of ST-669 and the effects of myriocin, a potent sphingomyelin inhibitor, on this pathway. ST-669 and myriocin were used to examine the inclusion structure of *C. caviae*: when treated with ST-669 or myriocin, inclusions with single-lobes were observed, which is atypical of *C. caviae* growing in cell culture. It is believed that these compounds are disrupting inclusion membrane integrity resulting in fusion of vacuoles and formation of single-lobed inclusions. Treatment of infected cells with sphingosine, a sphingomyelin precursor, complemented the effects of myriocin, returning the inclusions to their typical, multi-lobed phenotype. However, treatment of infected cells with sphingosine did not alter the effects of ST-669 on *C. caviae* inclusion morphology. These results support a conclusion that the molecular target of ST-669 is

different from that of myriocin. ST-669 maybe acting on a different host protein that influences chlamydial inclusion formation. These results may lead to future proteomic studies that may help elucidate ST-669's mechanism of action or may offer a greater understanding of inclusion membrane formation in *C. caviae*.

Key Words: Chlamydia, Inclusion, ST-669, Sphingomyelin, Antimicrobial
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Brian Dolan, Committee Member, representing Biomedical Sciences

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Stormy Scharzenberger, Author

Acknowledgements

Most importantly, I would like to thank Dr. Daniel Rockey and the entirety of the Rockey Lab for their mentorship, support, and assistance for this thesis and throughout my undergraduate career. Thanks to these individuals, I plan to participate in research throughout my career. In addition to the Rockey Lab, special thanks is owed to my thesis committee members, Dr. Timothy Hazzard and Dr. Brian Dolan, who have advised and supported me over the past three years. I would also like to thank my friends and family for their continued encouragement. Lastly, I would like to acknowledge the URSA-Engage and URISC programs, as well as Dr. Ken & Paula Krane for their financial contributions.

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Introduction

Chlamydia

The chlamydiae are a family of obligate intracellular pathogens of importance in both human and veterinary medicine. *Chlamydia* spp. cause disease in a wide variety of animal species including wildlife, pets, production animals, and humans. In the United States alone, chlamydial diseases lead to billions of dollars annually in human medical expenses, and also cause major problems for the livestock industry. *C. trachomatis* causes trachoma (the leading cause of infectious blindness worldwide) and pelvic inflammatory disease in women, and is the most commonly reported sexually transmitted infection in the United States [1, 2]. Persistent or recurrent chlamydial infections in humans can lead to serious consequences including ectopic pregnancy or infertility, although infections can often be asymptomatic [3]. For these reasons, routine screening for chlamydial infection in at risk populations is very important.

Chlamydia caviae is a related species that is pathogenic in guinea pigs and serves as an excellent model for human infections. *C. caviae* was initially considered a subtype of *C. psittaci*; however, it is now classified as a species of its own. *C. psittaci* is a zoonotic respiratory pathogen in wild and farm birds and has clinical importance in veterinary medicine. Other common species include *C. abortus, C. suis,* and *C. muridarum,* which cause abortions in small ruminants, chronic disease in pigs, and mouse pneumonitis, respectively [4, 5, 6, 7].

Each of the species within the genus *Chlamydia* share certain characteristics. They are Gram-negative bacteria with a developmental cycle that alternates between dividing reticulate bodies (RBs) and infectious elementary bodies (EBs) [8]. Both RBs and EBs have lipopolysaccharide (LPS) in their outer membranes [5, 9]. Inside the host cell, *Chlamydia* spp.

form a vacuole, termed an inclusion, within which they grow. Inclusions contain chlamydial proteins, called inclusion membrane proteins (Inc) that are involved in virulence (Figure 1).

Although the function of most Incs is not known, in *C. trachomatis,* the lack of IncA is associated with non-fusogenic inclusions in cell culture [10]. In wild type *C. trachomatis* with a functional IncA, only one inclusion will form when multiple EBs enter a host cell (Figure 2). Meanwhile, in cells infected with multiple IncA-negative EBs, multiple inclusions are formed [10]. In contrast to the single-lobed inclusions typical of wild type *C. trachomatis,* multilobed inclusions are characteristic of *C. caviae*, regardless of how many bacteria (EBs) infected the host [11] (Figure 2). These lobes form early in the *C. caviae* development cycle. At this time, the inclusion membrane is tightly associated with the surface of the bacteria, and division of the bacteria and division of the inclusion are parallel [12]. Later in the chlamydial development cycle, the inclusion membrane separates from the surface of the bacteria, and the inclusions do not divide at the same rate as the bacteria [12].

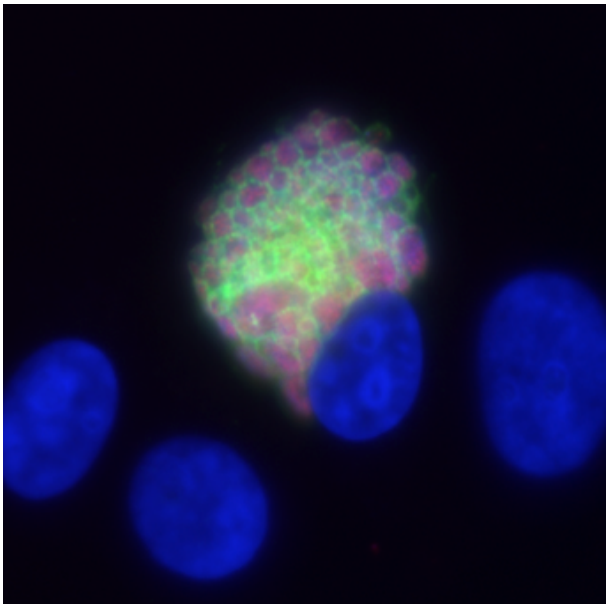

Figure 1: Fluorescence microscopy of wild-type *C. caviae*
Vero cells infected with *C. caviae* were fixed with methanol 30 hpi. Chlamydial LPS is labeled with red. *C. caviae* IncA is labeled with green. Total DNA is stained blue with DAPI.

ST-669

ST-669 is a novel, broad-spectrum antiviral compound that was developed by Siga Technologies [13]. The antiviral properties of ST-669 were determined by screening a compound library for activity against *Bunyaviridae* [13]. The compound has antiviral activity against a variety of viruses including members of the families *Orthomyxoviridae*, *Arenaviridae* and *Flaviviridae*, among others [13].

In addition to its effectiveness as an antiviral, ST-669 also has activity against bacteria in the genera *Chlamydia* and *Coxiella* [14]. Treatment of different chlamydiae with ST-669 resulted in changes in inclusion morphology as well as a decrease in genome copies [14]. This susceptibility to ST-669 was observed when the intracellular bacteria were grown in cells of primate origin (Vero cells), rather than murine origin (McCoy cells) which is consistent with the antiviral activity of the compound. Growing *Chlamydia* in the presence of cycloheximide, a eukaryotic protein synthesis inhibitor, also served to reduce the antichlamydial effects of ST-669 and partially returned the inclusions to their typical phenotype [14]. These observations led to the hypothesis that ST-669 acts on host cell processes.

Phenotypic changes in inclusion morphology were observed when *C. trachomatis* and *C. caviae* were grown in the presence of ST-669 (Figure 2). For *C. trachomatis,* inclusions were markedly smaller than wild type, and the fusogenic inclusion structure was preserved. In *C. caviae,* inclusions were markedly smaller and by 20 hours post infection (hpi) most inclusions were radically different than untreated, infected cells. The typical *C. caviae*, multi-lobed structure was present in the absence of drug; however, in the presence of ST-669, a single vacuole containing the bacteria was observed. Since the *C. caviae* inclusions undergo a

morphological change when treated with ST-669, active or inactive ST-669 treatments can be measured using fluorescence microscopy [14].

Because ST-669 results in changes in chlamydial-inclusion morphology, it is believed that ST-669 affects vesicular trafficking in chlamydia-infected cells and that ST-669 can be a useful tool in studying inclusion formation. It is also hypothesized that ST-669 may be acting on the sphingomyelin pathway and thus disrupting inclusion membrane integrity, which is similar to the action of a previously characterized sphingomyelin synthesis inhibitor, myriocin.

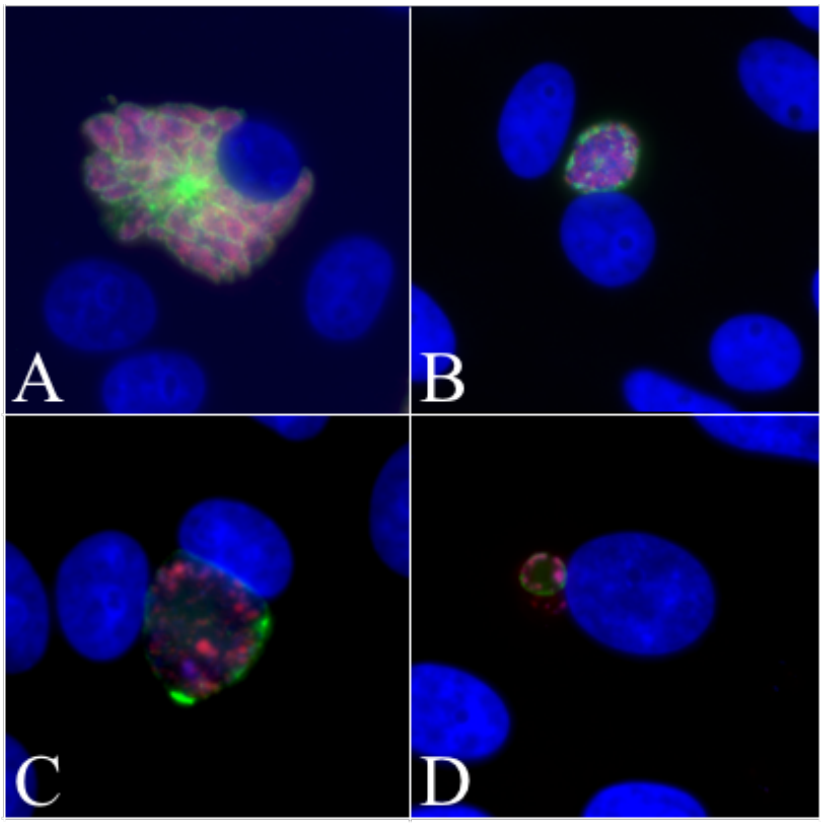

Figure 2: Fluorescence microscopy of the effects of ST-669 in *Chlamydia* spp.
Vero cells infected with: A-B) *C. caviae*, C-D) *C. trachomatis* and cultured in the presence of: A) DMSO (vehicle), B) ST-669, C) DMSO (vehicle), and D) ST-669 were fixed with methanol 30 hpi. Chlamydial LPS is labeled with red. *C. caviae* IncA is labeled with green. Total DNA is stained blue with DAPI.

Myriocin

Myriocin is an antibiotic compound produced by fungi including *Mycelia sterilia*. Myriocin acts as a very potent inhibitor of serine palmitoyltransferase (SPT), the first enzyme in sphingomyelin biosynthesis (Figure 3). This property allows myriocin to deplete cells of sphingolipids, and enables myriocin to be a useful research tool [15].

Treatment with myriocin resulted in changes in *C. trachomatis* inclusion morphology as well as a decrease in membrane integrity [15]. There were striking morphological alterations in inclusion maturation, including disruption of the inclusion and release of intracellular bacteria as early as 24 hours post infection (hpi) [15]. At 36 hpi, the myriocin-treated cells contain small multiple inclusions of heterogeneous size, rather than the large single inclusion typical of untreated *C. trachomatis*. The effects of SPT-deficiency, due to myriocin-treatment, were reversed by the addition of sphingomyelin precursors including dihydroceramide and sphingosine. These precursors are positioned downstream of SPT and allow for the restoration of sphingomyelin synthesis under conditions of SPT inactivity [15].

In *C. caviae,* treatment with myriocin results in alterations to inclusion morphology, similar to those observed when *C. caviae* infected cells are treated with ST-669 (Figure 4). Since myriocin results in changes in chlamydial inclusion morphology, it is believed that myriocin can be a useful tool in studying the importance of sphingomyelin in inclusion membrane formation and integrity in *C. caviae* as well as *C. trachomatis*.

Sphingomyelin Pathway

Sphingolipids are a class of molecules that are important to the function of the eukaryotic plasma membrane. These lipids are also involved in signaling pathways. Some of the best studied sphingolipids include sphingosine and ceramide. Ceramide is formed in mammalian

systems and is incorporated into complex sphingolipids, such as sphingomyelin, which are phospholipids that are based on a sphingosine backbone [16]. The sphingomyelin pathway consists of two forks: the *de novo* pathway and a salvage pathway. While treatment with myriocin (an inhibitor of the first enzyme along the *de novo* pathway) shuts down sphingomyelin production, addition of sphingosine (a precursor late in the salvage pathway) reverses the effects of myriocin (Figure 3).

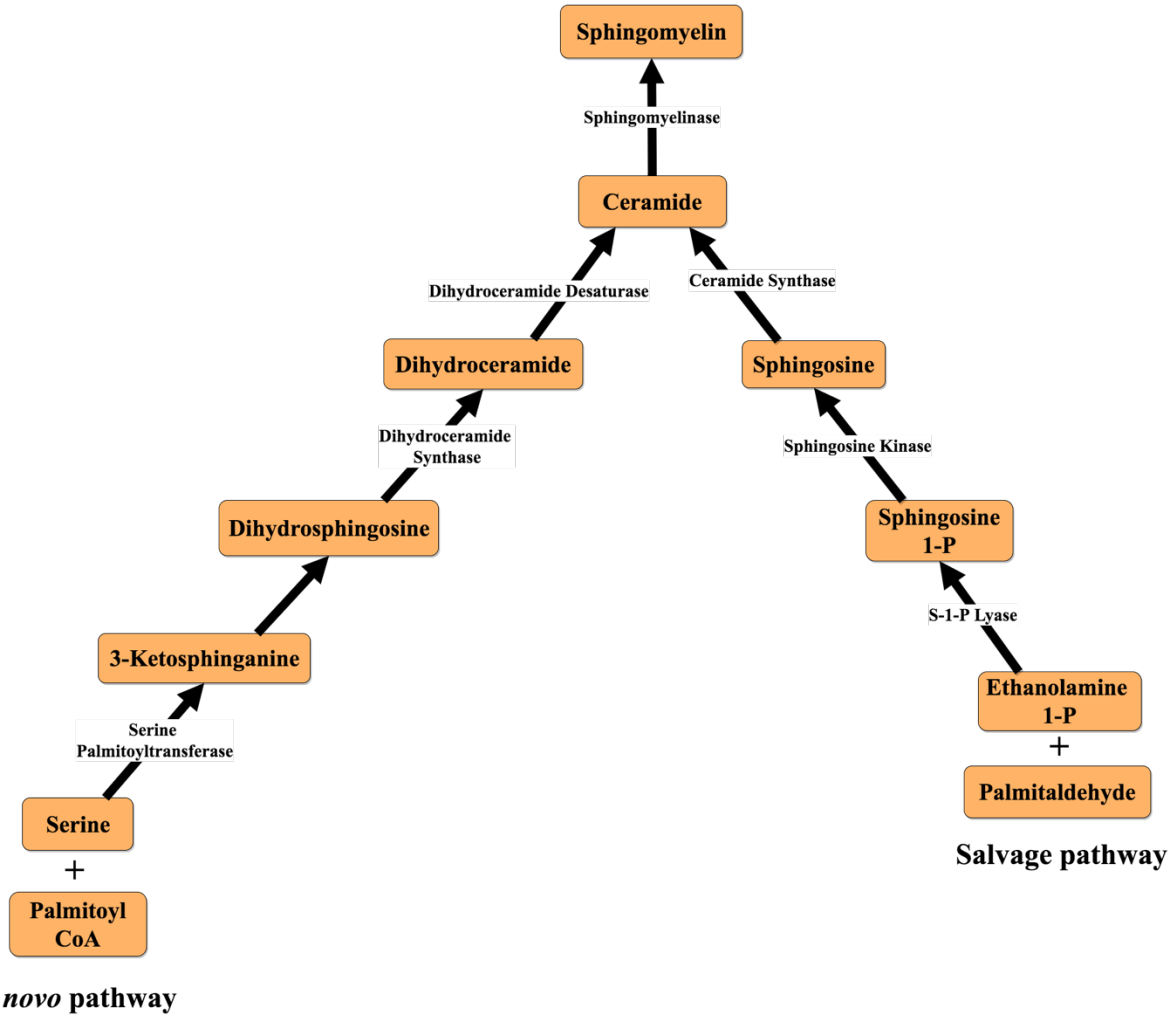

Figure 3: *De novo* and salvage pathways of sphingomyelin synthesis.
The sphingomyelin pathway consists of two forks: 1) *de novo* pathway and 2) salvage pathway. Myriocin acts as a potent inhibitor of serine palmitoyltransferase, the first enzyme in the *de novo* pathway. Addition of sphingosine, a precursor along the salvage pathway, reverses the inhibitory effects of myriocin.

While sphingomyelin constitutes a significant portion of the eukaryotic plasma membrane, many prokaryotes, including *C. trachomatis* and *C. caviae*, cannot synthesize sphingolipids. These bacteria have developed mechanisms that allow them to facilitate their growth and virulence through the scavenging of host cell sphingolipids. The chlamydiae are able to incorporate these host cell sphingolipids into the bacterial membrane by fusing their inclusion bodies with vesicles containing host sphingomyelin. In this way, production of sphingomyelin by the host cell is important for chlamydial inclusion formation [16].

Studying the sphingomyelin pathway and the acquisition of sphingomyelin by *Chlamydia* spp. may help elucidate ST-669's molecular target and may become important in understanding the utility of this and related candidate therapeutics. A greater understanding of this pathway and related processes may also give insight into inclusion membrane formation in *C. caviae* relative to other species of *Chlamydia*, including *C. trachomatis.*

Thesis Statement

The goal of this thesis is to help elucidate the mechanism of action of ST-669 by evaluating the possibility of ST-669 acting as an inhibitor of host sphingomyelin production. We hypothesize that ST-669 acts on the same molecular target as myriocin and that ST-669 may become a useful tool in investigating inclusion formation in *C. caviae*.

Methods

Summary of Experimental Design

In preparation for this project, *C. caviae* and *C. trachomatis* EBs were grown in Vero cells. Reagents and sphingomyelin precursors and inhibitors were purchased and prepared as specified. Three experiments were set-up comparing the effects of: 1) ST-669 and myriocin, 2) myriocin and sphingosine, and 3) ST-669 and sphingosine on *C. trachomatis* inclusion structure. Images were collected via fluorescence microscopy. These experiments were repeated in *C. caviae*. These experiments were replicated in *C. caviae* for three trials, and both qualitative and quantitative data were collected simultaneously.

Chlamydial Strains

Experiments were conducted with *Chlamydia caviae* strain GPIC and *Chlamydia trachomatis* strain L2 RFP (red fluorescent protein). Frozen EBs were thawed, titered to determine an appropriate multiplicity of infection (MOI), aliquoted into microcentrifuge tubes, and stored at -80°C.

Preparing Stock Inhibitors and Sphingomyelin Precursors

10μM ST-669

ST-669 was purchased from Siga Technologies. The dry chemical was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10mM. The stock ST-669 was stored at -20°C. For a working concentration of 10μM, 1μL of the 10mM ST-669 was diluted in 1mL of Minimum Essential Medium (MEM) when used on infected cells.

25μM Myriocin

Myriocin from *Mycelia sterilia* was purchased from Sigma Technologies. The powder was dissolved in methanol for a stock concentration of 2.5mM. The stock myriocin was stored between 2-8°C. For a working concentration of 25μM, 10μL of the 2.5mM sphingosine was diluted in 1mL of MEM.

5μM Sphingosine

D-Sphingosine (synthetic) was purchased from Sigma Technologies in a white, powder form. The powder was dissolved in chloroform for a stock concentration of 5mM. The stock sphingosine was stored at -20°C. For a working concentration of 5μM, 1μL of the 5mM sphingosine was diluted in 1mL of MEM.

Cell Culture and Chlamydial Infection

Vero cells were grown at 37°C in 5% CO_2 in MEM plus 10% FBS, and 5mM L-glutamine (MEM-10). At 0 hours, Vero cells were set into 24-well trays at 30% confluence, and cultured in wells of 24-well trays, either with or without 12 mm coverslips. For treatments using sphingosine, drug was added to media at 0 hours (Table 3). Vero cells were allowed to grow overnight, and at 19 hours, the media were removed from the wells and the cells were washed once with 1x Dulbecco's phosphate-buffered saline (DPBS). Chlamydial EBs were diluted in 1x DPBS and inoculated onto cells at a MOI of 0.3, meaning that approximately 30% of host cells were infected. The trays were centrifuged at 930 x g at room temperature for 1 hour. The DPBS was removed and media were added to the wells containing the specified treatments (Tables 1-3). At 30 hpi, the cells were methanol-fixed and prepared for microscopy. To fix the cells, the

media was aspirated and ~500µL 100% methanol was added to each well, and cells were incubated at room temperature for 10 min. After the removal of the methanol, 1x DPBS was added into each well.

Fluorescence Microscopy

Methanol-fixed cells were washed 2X with 1x DPBS. For *C. caviae* coverslips, monoclonal antibodies against chlamydial LPS and *C. caviae* IncA were diluted 1:1 and 1:250, respectively, in FA Block (2% BSA in PBS). For *C. trachomatis* coverslips, monoclonal antibodies against chlamydial heat shock protein 60 (Hsp60) and *C. trachomatis* IncA were diluted 1:1 and 1:250, respectively, in FA Block. Diluted primary antibody was added to the cells and incubated at room temperature for 1 hour. The primary antibody was removed and the cells were washed two times with 1x DPBS. Secondary antibodies conjugated to either rhodamine or fluorescein were diluted 1:1000 in FA Block and then added to the cells and incubated at room temperature for 1 hour in the dark. Secondary antibodies were removed and the cells were washed two times with 1x DPBS. The coverslips were inverted and placed onto microscope slides using mounting media (4µg/mL 4′,6′-diamidino-2-phenylindole (DAPI) in Vectashield; Vector Laboratories). Images were collected at 100x magnification with a Leica fluorescence microscope and a PC-based digital camera imaging system (Qimaging Co, Surrey BC, Canada).

Quantifying Lobes per Inclusion

Since treatment with ST-669 results in changes in the lobed-structure of *C. caviae* inclusion membranes, our results could be quantified by counting the number of lobes per inclusion across treatments. Coverslips were prepared for microscopy and affixed to microscope slides using DAPI and Vectashield, as previously described. For each treatment, the number of

host cells and infected cells were counted for at least 10 fields of view. Next, 50 randomly-selected inclusions were observed for each coverslip, and the number of lobes per inclusion recorded. Categories were broken down as follows: 1-2 lobes per inclusion, 3-5 lobes per inclusion, 6-8 lobes/inclusion, or 9+ lobes per inclusion. Results were plotted and error bar values were calculated as standard deviation.

Table 1: Contents of Media for ST-669 Treatments		
Treatment	Contents of media before infection	Contents of media after infection
ST-669	Minimum essential medium (MEM), 10% FBS, and 5mM L-Glutamine	Minimum essential medium (MEM), 10% FBS, 5mM L-Glutamine and 10uM ST-669 in DMSO
Control: ST-669 vehicle	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine and equivalent volume DMSO (1:1000)
ST-669 and Myriocin	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine, 10uM ST-669 in DMSO and 25uM myriocin in methanol
Control: ST-669 and Myriocin vehicle	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine and equivalent volume DMSO (1:1000) and methanol (1:100)
ST-669 and Sphingosine	MEM, 10% FBS, 5mM L-Glutamine and 5uM sphingosine in chloroform	MEM, 10% FBS, 5mM L-Glutamine, 10uM ST-669 in DMSO and 5uM sphingosine in chloroform
Control: ST-669 and Sphingosine vehicle	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine and equivalent volume DMSO (1:1000) and chloroform (1:1000)

Table 2: Contents of Media for Myriocin Treatments		
Treatment	Contents of media before infection	Contents of media after infection
Myriocin	Minimum essential medium (MEM), 10% FBS, and 5mM L-Glutamine	Minimum essential medium (MEM), 10% FBS, 5mM L-Glutamine and 25uM myriocin in methanol
Control: Myriocin vehicle	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine and equivalent volume methanol (1:100)
ST-669 and Myriocin	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine, 10uM ST-669 in DMSO and 25uM myriocin in methanol
Control: ST-669 and Myriocin vehicle	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine and equivalent volume DMSO (1:1000) and methanol (1:100)
Myriocin and Sphingosine	MEM, 10% FBS, 5mM L-Glutamine and 5uM sphingosine in chloroform	MEM, 10% FBS, 5mM L-Glutamine, 25uM myriocin in methanol and 5uM sphingosine in chloroform
Control: Myriocin and Sphingosine vehicle	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine and equivalent volume methanol (1:100) and chloroform (1:1000)

Table 3: Contents of Media for Sphingosine Treatments		
Treatment	Contents of media before infection	Contents of media after infection
Sphingosine	Minimum essential medium (MEM), 10% FBS, 5mM L-Glutamine and 5uM sphingosine in chloroform	Minimum essential medium (MEM), 10% FBS, 5mM L-Glutamine and 5uM sphingosine in chloroform
Control: Sphingosine vehicle	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine and equivalent volume chloroform (1:1000)
ST-669 and Sphingosine	MEM, 10% FBS, 5mM L-Glutamine and 5uM sphingosine in chloroform	MEM, 10% FBS, 5mM L-Glutamine, 10uM ST-669 in DMSO and 5uM sphingosine in chloroform
Control: ST-669 and Sphingosine vehicle	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine and equivalent volume DMSO (1:1000) and chloroform (1:1000)
Myriocin and Sphingosine	MEM, 10% FBS, 5mM L-Glutamine and 5uM sphingosine in chloroform	MEM, 10% FBS, 5mM L-Glutamine, 25uM myriocin in methanol and 5uM sphingosine in chloroform
Control: Myriocin and Sphingosine vehicle	MEM, 10% FBS, and 5mM L-Glutamine	MEM, 10% FBS, 5mM L-Glutamine and equivalent volume methanol (1:100) and chloroform (1:1000)

Results

ST-669 treatment affects inclusion structure

Chlamydia caviae inclusions are unique to other chlamydial inclusions because they maintain their multilobed phenotype late into infection, while other species of chlamydia, including *C. trachomatis,* exhibit fusogenic inclusions (Figure 2). When *C. caviae* cells are grown in the presence of ST-669, fusogenic inclusions with a single lobe are observed late in infection (30+ hpi), and few wild type inclusions, with greater than 8 lobes, are observed (Figure 4). Quantitative data on the number of lobes per inclusion served to expand qualitative results observed via fluorescence microscopy. On average, ~24.5% of the inclusions had 1-2 lobes/inclusions, exhibiting a fusogenic phenotype. In wild type *C. caviae,* ~90% of inclusions exhibited over 8 lobes while only ~17.5% of the ST-669-treated inclusions had greater than 8 lobes/inclusions (Figure 5). In addition to the change in lobed structure, these inclusions are also markedly smaller than typical wild type *C. caviae* inclusions.

While an obvious change in morphology is observed in *C. caviae*, which allows us to easily observe the effect of ST-669 on the *C. caviae* inclusion structure and to quantify these results, the change is more subtle in *C. trachomatis*. In *C. trachomatis*, the fusogenic inclusion structure is preserved under ST-669-treatment, however the inclusions are markedly smaller than typical wild type *C. trachomatis* inclusions (Figure 2). This is similar to the effect observed in *C. caviae*, but the lack of a morphological change (from multilobed to fusogenic) makes these effects less easily observed and quantified.

Myriocin treatment affects inclusion structure

We observed similar changes in inclusion morphology when infected cells were treated with myriocin and ST-669 at 30 hpi (Figure 4). When cells are grown in myriocin, *C. caviae* inclusion with a single lobe are observed, and fewer multilobed inclusions are found in this treatment than in wild type *C. caviae* (Figure 4). On average, ~33.0% of inclusions had 1-2 lobes, exhibiting a fusogenic phenotype. Only ~25.0% of inclusions had greater than 8 lobes/inclusion (Figure 5). When *C. caviae* were grown in the presence of myriocin and ST-669 together, fusogenic inclusions were still observed. As observed in cells treated with ST-669, myriocin-treated *C. caviae* inclusions are smaller than wild type *C. caviae* inclusions (Figure 4). This led us to believe that ST-669 and myriocin may have the same effects on inclusion structure by similar mechanisms.

Addition of sphingosine complements effects of myriocin

The addition of sphingosine influenced the phenotype of myriocin-treated *C. caviae.* When cells were grown in the presence of myriocin and sphingosine, *C. caviae* inclusions appeared as wild type and 96% of inclusions had greater than 8 lobes/inclusion (Figure 6). The addition of sphingosine restored the cells to their wild type phenotype, with 98% of inclusions exhibiting greater than 8 lobes/inclusion (Figure 7).

Addition of sphingosine does not complement effects of ST-669

The addition of sphingosine did not influence the phenotype of ST-669-treated *C. caviae.* When cells were grown in the presence of ST-669 and sphingosine, *C. caviae* inclusions resembled ST-669-treated *C. caviae* (Figure 8). On average, 18% exhibited fusogenic inclusions with only 1-2 lobes, while only 10% had greater than 8 lobes (Figure 9). Single-lobed, fusogenic

inclusions were observed in the presence of ST-669 and sphingosine, and fewer multilobed inclusions were found than typical of *C. caviae* (Figure 8).

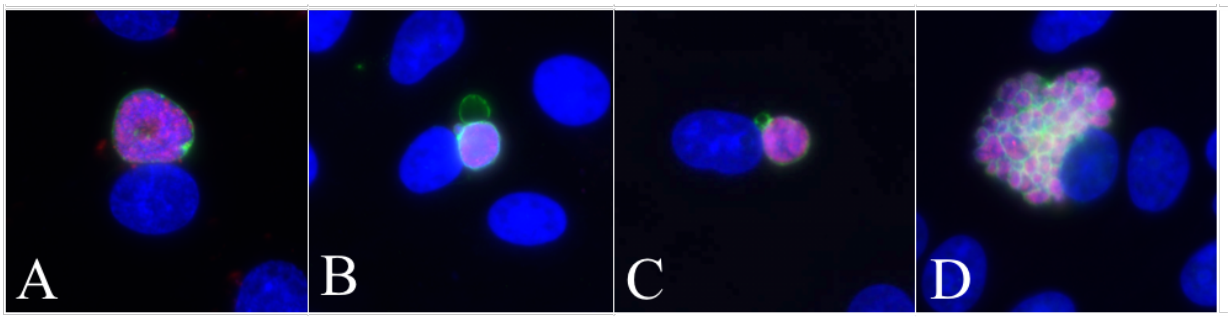

Figure 4: Fluorescence microscopy of the effects of ST-669 and myriocin in *Chlamydia caviae*. Vero cells infected with *C. caviae* and cultured in the presence of: A) ST-669 and myriocin, B) myriocin, C) ST-669, and D) DMSO and methanol (vehicles) were fixed with methanol 30 hpi. Chlamydial LPS is labeled with red. *C. caviae* IncA is labeled with green. Total DNA is stained blue with DAPI.

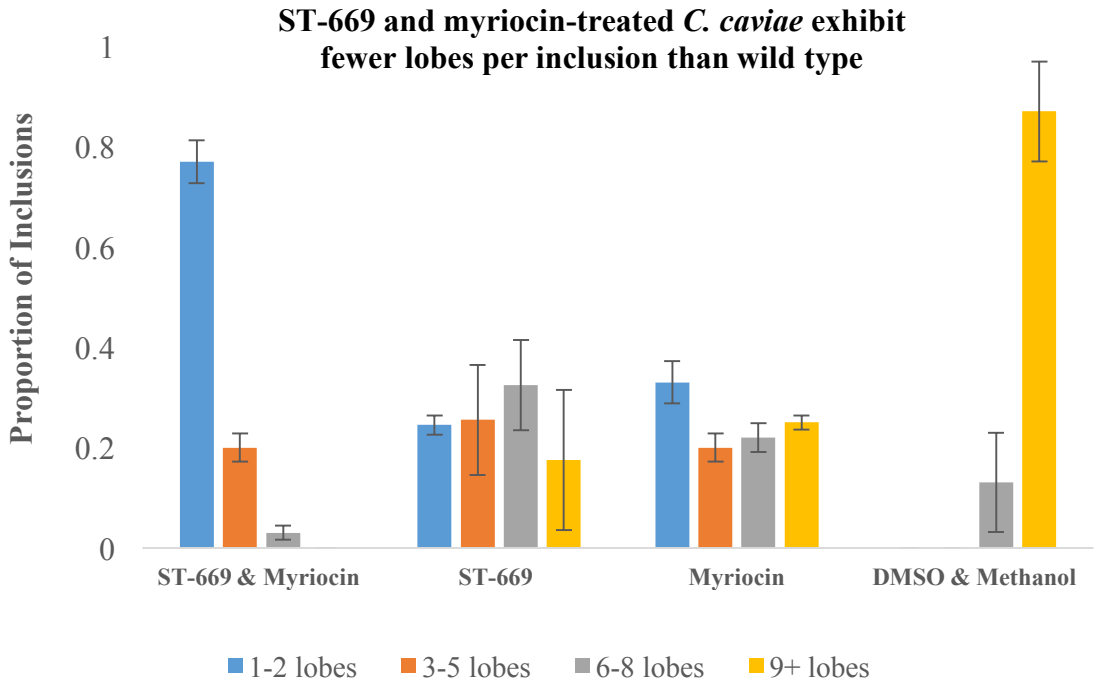

Figure 5: ST-669 and myriocin treated *C. caviae* exhibit fewer lobes/inclusions than wild type. Vero cells infected with *C. caviae* and cultured in the presence of: ST-669 and myriocin, myriocin, ST-669, and DMSO and methanol (vehicles) were fixed with methanol 30 hpi. 50 randomly selected inclusions were observed per coverslip and the number of lobes per inclusions were counted and recorded categorically: 1-2 lobes, 3-5 lobes, 6-8 lobes, or >8 lobes per inclusion.

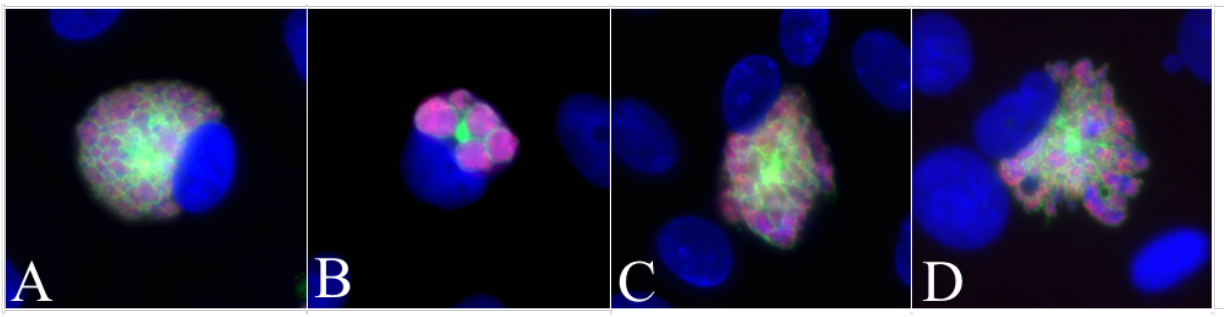

Figure 6: Fluorescence microscopy of the effects of myriocin and sphingosine in *Chlamydia caviae*. Vero cells infected with *C. caviae* and cultured in the presence of: A) myriocin and sphingosine, B) myriocin, C) sphingosine, and D) methanol and chloroform (vehicles) were fixed with methanol 30 hpi. Chlamydial LPS is labeled with red. *C. caviae* IncA is labeled with green. Total DNA is stained blue with DAPI.

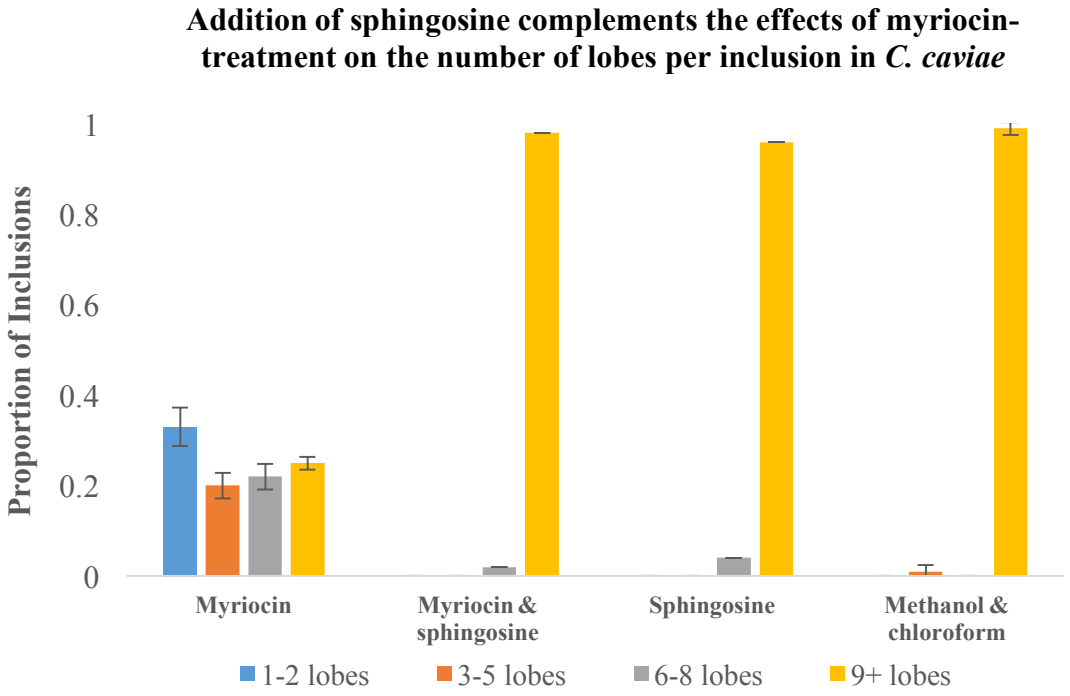

Figure 7: Sphingosine complements the effect of myriocin-treatment on the number of lobes/inclusion in *C. caviae*. Vero cells infected with *C. caviae* and cultured in the presence of: myriocin and sphingosine, myriocin, sphingosine, and methanol and chloroform (vehicles) were fixed with methanol 30 hpi. 50 randomly selected inclusions were observed per coverslip and the number of lobes per inclusions were counted and recorded categorically: 1-2 lobes, 3-5 lobes, 6-8 lobes, or >8 lobes per inclusion.

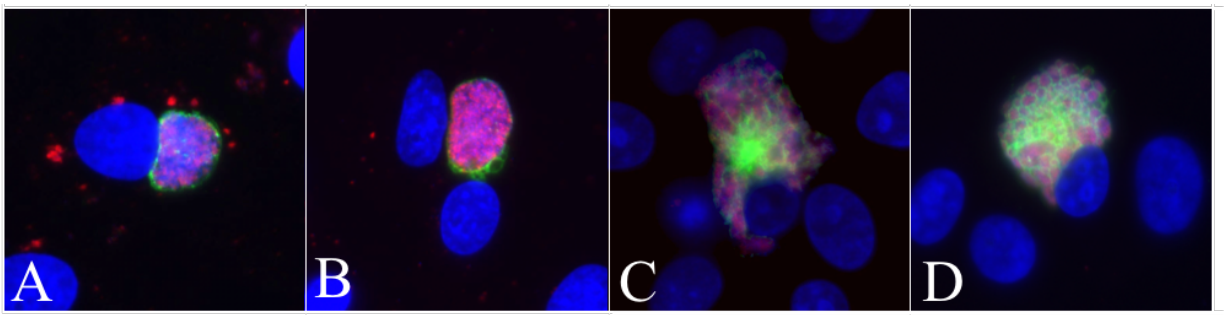

Figure 8: Fluorescence microscopy of the effects of ST-669 and sphingosine in *Chlamydia caviae*. Vero cells infected with *C. caviae* and cultured in the presence of: A) ST-669 and sphingosine, B) ST-669, C) sphingosine, and D) DMSO and chloroform (vehicles) were fixed with methanol at 30 hpi. Chlamydial LPS is labeled with red. *C. caviae* IncA is labeled with green. Total DNA is stained blue with DAPI.

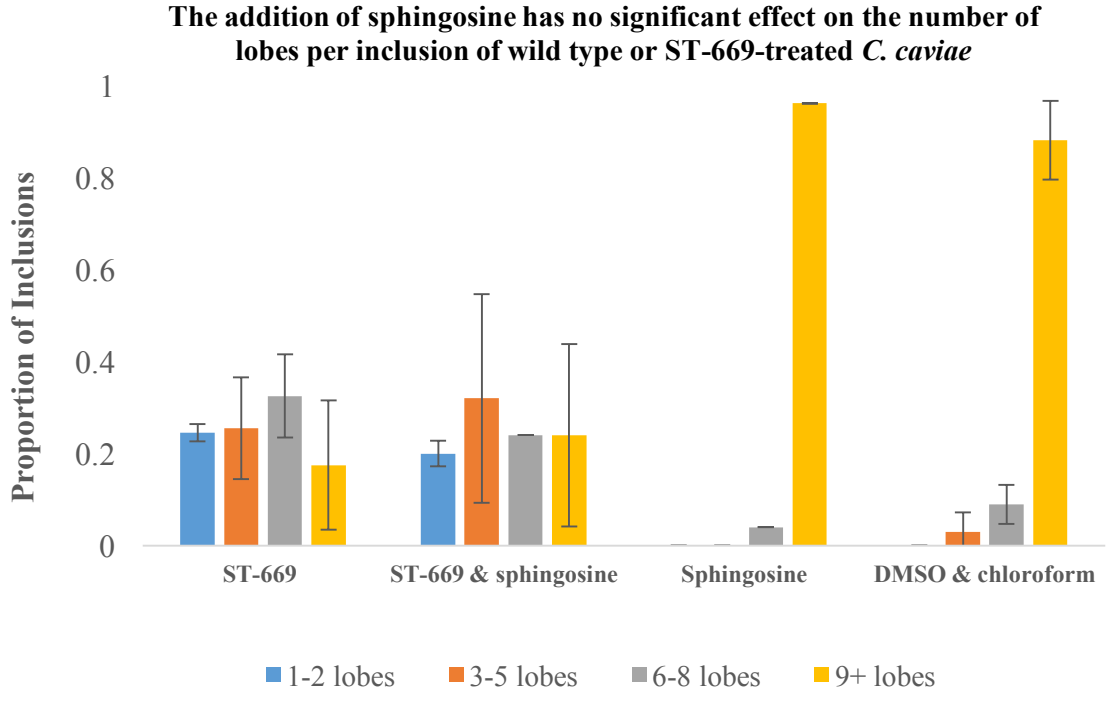

Figure 9: Sphingosine does not complement the effect of ST-669 treatment on the number of lobes/inclusion in *C. caviae*. Vero cells infected with *C. caviae* and cultured in the presence of: ST-669 and sphingosine, ST-669, sphingosine, and DMSO and chloroform (vehicles) were fixed with methanol 30 hpi. 50 randomly selected inclusions were observed per coverslip and the number of lobes per inclusions were counted and recorded categorically: 1-2 lobes, 3-5 lobes, 6-8 lobes, or 9+ lobes per inclusion.

Discussion

The molecular target of the broad-spectrum anti-infective compound, ST-669, is not known. Knowledge of the target is a critical aspect of understanding the utility of a candidate therapeutic. By understanding the mechanism of action of ST-669, both the toxicity of the compound and, perhaps, the identification of additional drugs to target the pathway can be pursued. We made the observation that treatment of cells with ST-669 led to similar inclusion structures as is seen with myriocin, a compound that inhibits sphingomyelin synthesis. Because of these observations, we hypothesized that the target of the drug may be discovered by measuring the effects of ST-669 in the presence of sphingomyelin precursors such as sphingosine.

Myriocin acts as a potent inhibitor of serine palmitoyltransferase (SPT), the first enzyme in *de novo* sphingomyelin synthesis. As we had expected, the addition of sphingosine, a sphingomyelin precursor late in the salvage pathway, reversed the inhibitory effects of myriocin (Figures 3, 6, 7). If ST-669 were acting on the sphingomyelin pathway using a mechanism similar to that of myriocin, we would expect the addition of sphingosine to complement the effects of ST-669, as seen in myriocin. However, treatment of infected cells with sphingosine did not reverse the effects of ST-669 on *C. caviae* inclusion morphology (Figures 8, 9).

Because the effects of ST-669 are not altered by the addition of sphingosine, we conclude that ST-669 acts on a different molecular target than that of myriocin. It remains possible, however, that ST-669 may act on the sphingomyelin pathway downstream of sphingosine as an inhibitor of ceramide synthase or sphingomyelinase (Figure 3). Alternatively, ST-669 may not be acting on the sphingomyelin pathway at all: ST-669 may target a host protein involved in a different pathway that impacts chlamydial inclusion membrane formation.

When Vero cells are treated with ST-669 following inoculation with a low or modest multiplicity of infection (MOI), inclusions with a single lobe are observed late in the infection, regardless of the initial MOI of bacteria. Nonetheless, *C. caviae* will initially grow in multiple vacuoles in the presence (as well as in the absence) of ST-669 when infected with a high MOI. Because of this, we hypothesize that fusion of the vacuoles takes place between the initial growth of *C. caviae* in the lobed inclusion leading to a mature inclusion with a single lobe. This fusion may be a result of the host protein that is the target of the drug: ST-669 may be targeting and up-regulating a host protein that is involved in membrane fusion, and the higher concentration of this protein may cause the chlamydial inclusions to fuse together. Alternatively, this fusion may be due to the biology of *C. caviae*: ST-669 could cause inclusion membranes to be loosely associated with the RBs early in infection, preventing rapid division of the inclusion membrane. The inclusions may then fuse through the processes used by other species of *Chlamydia*, which are functional in *C. caviae*, however the division of the inclusion membrane is normally faster than the fusion.

Uncovering the molecular target of ST-669 is a critical aspect of understanding the utility of this drug as a candidate therapeutic. Discovering the pathway upon which this novel, anti-infective acts may lead to the testing of additional drugs that target the same cellular process. The Rockey laboratory will continue to use the *C. caviae* model to provide insight into how ST-669 functions, both with a goal of understanding the mechanism of action of ST-669, as well as to study host-microbe interactions in the chlamydial system. Future studies in this line of research may help elucidate some of the differences between *C. caviae* and other species of chlamydia, and ST-669 may become a useful tool to study why *C. caviae* grows in a unique lobed inclusion within mammalian cells.

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