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Natural Products as a Source for Rational Antichlamydial Lead-Discovery

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Natural Products as a Source for Rational Antichlamydial Lead-Discovery

Elina Karhu

ACADEMIC DISSERTATION

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ABSTRACT

Chlamydia pneumoniae is a very common intracellular bacterium in humans. It causes upper respiratory tract diseases, symptoms often occurring as normal seasonal flu. *C. pneumoniae* is shown to be the causative agent of approximately 10% of community-aquired pneumonia (CAP) cases. Pneumonia is one of the leading causes of death globally. The treatment and hospitalization costs to the society are remarkable. There are antibiotics to treat *C. pneumoniae* infections, but they are not without problems. One problem is the overall increasing resistance which is associated with the use of broad-spectrum antibiotics. As the diagnostic technology improves, and the pathogen can be specified in increasing number of cases, there is a need for rational drug treatment with more selective, narrow-spectrum antibiotics. With the targeted treatment, the development of resistant strains can be delayed and the patient's normal microbiota will be unharmed. Another problem with the antibiotics currently in use is that *C. pneumoniae* can turn into a persistent form which causes a chronic infection. This is associated with other chronic diseases, such as lung cancer, cardiovascular diseases and multiple sclerosis (MS). The persistent form, in turn, cannot be cured with current antibiotics.

In the present study, new antichlamydial compounds were discovered from plants and natural product libraries. This thesis presents a new antichlamydial group of compounds, dibenzocyclooctadiene lignans from the fruits of *Schisandra chinensis* (Turczaninov) Baillon, i.e. *Schisandra* lignans. Cough and pneumonia are among the ethnopharmacological uses of the extracts obtained from the fruits of *S. chinensis*. In this work, the extract completely inhibited the growth of *C. pneumoniae* cardiovascular strain CV6 and showed significant inhibition of the growth of a clinical isolate K7. The growth inhibition of two chlamydial species, *C. pneumoniae* and *Chlamydia trachomatis* was dose dependent and established with three different strains. These findings give raise to the potential of the extract from *S. chinensis* berries as a source of antichlamydial compounds. *Schisandra* lignans were shown to inhibit *C. pneumoniae* inclusion formation and production of infectious progeny. One of the lignans, schisandrin B, inhibited *C. pneumoniae* inclusion formation even when administered 8 hours post infection,

indicating a target that occurs in the mid-phase of the intracellular infection cycle of *C. pneumoniae*. Before infecting the host cells, the lignan-pretreated *C. pneumoniae* elementary bodies had impaired ability to form inclusions. The structure–activity relationship among the lignans was clear. Substitution and presence of methylenedioxy, methoxy and hydroxyl groups in the structure of the lignans had a substantial impact on the antichlamydial activity. In addition, the data suggest that the antichlamydial activity of the lignans is not caused only by their antioxidative properties. None of the lignans inhibited growth of seven other common metabolically active bacteria, suggesting a degree of selectivity of the antichlamydial effect. The lignans were shown to be non-toxic to the host cells, which is in line with the literature presenting these compounds.

Moreover, this work presents a novel strategy for lead discovery on *C. pneumoniae*. Ligand-based virtual screening (LBVS) of a natural product library with the ChemGPS-NP chemography tool, followed by validation of the activity with *in vitro* antichlamydial assays proved successful with a hit rate of 1.2%. Six non-cytotoxic lead compounds, ranging from active to highly active, belonging to new antichlamydial chemotypes were found in the process.

There is an urgent need of promising hit and lead molecules for antimicrobial drug discovery. The results presented herein suggest the described dibenzocyclooctadiene lignans from the fruits of *Schisandra chinensis* to be active against *C. pneumoniae*. Since these compounds proved to be active, non-cytotoxic to the host cells and selective in antichlamydial action, they present promising candidates for further lead development. Considering the lack of validated targets in antichlamydial drug discovery, ligand-based methods, such as the successful LBVS approach described in this work, are suitable for future projects in the field of natural product drug discovery.

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Elina Karhu

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications referred to in the text by the Roman numerals I–IV.

I Kapp K, Hakala E, Orav A, Pohjala L, Vuorela P, Püssad T, Vuorela H, Raal A. Commercial peppermint (*Mentha* × *piperita* L.) teas: Antichlamydial effect and polyphenolic composition. Food Research International 2013; 53(2): 758–66.

II Hakala E, Hanski L, Yrjönen T, Vuorela H, Vuorela P. The Lignan-containing Extract of *Schisandra chinensis* Berries Inhibits the Growth of *Chlamydia pneumoniae*. Natural Product Commununications 2015; 10(6): 1001–4.

III Hakala E, Hanski L, Uvell H, Yrjönen T, Vuorela H, Elofsson M, Vuorela PM. Dibenzocyclooctadiene lignans from *Schisandra* spp. selectively inhibit the growth of the intracellular bacteria *Chlamydia pneumoniae* and *Chlamydia trachomatis*. Journal of Antibiotics 2015; 68: 609–14.

IV Karhu* E, Isojärvi J, Hanski L and Fallarero A. Identification and characterization of priviledged antichlamydial structures using a novel ligand-based strategy. Manuscript.

*née Hakala

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CONTRIBUTION TO ORIGINAL PUBLICATIONS

I Designing and performing the cellular and antichlamydial experiments in collaboration, data analysis, statistics and constructing the graphs of the results gained in the antichlamydial experiments, writing the parts in the publication concerning the cellular and antichlamydial experiments and reviewing the article.

II Planning the experiments in collaboration, preparation and quality analysis of the extract, performing the cellular and antichlamydial experiments, data analysis, statistics, and constructing the graphs of the results gained from all of the experiments, writing the publication.

III Planning the experiments in collaboration, performing the cellular and antichlamydial experiments, data analysis, statistics, and constructing the graphs of the results gained from all of the experiments, writing the publication.

IV Planning the experiments in collaboration, performing the *in silico* chemical space analysis, performing the cellular and antichlamydial experiments, data analysis, statistics, and constructing the graphs of the results gained from all of the experiments, writing the publication.

ABBREVIATIONS

AB	aberrant body
ADME	absorption, distribution, metabolism, excretion
ATCC	American-type culture collection
CAP	community-acquired pneumonia
C. pneumoniae	*Chlamydia pneumoniae*
C. trachomatis	*Chlamydia trachomatis*
ChemGPS	Chemical Global Positioning System, Chemical Global Property Space, or Chemical Global Property Scores
cLPS	chlamydial lipopolysaccharide
CRP	cystein-rich protein
CV-6	*C. pneumoniae* coronary artery strain 6
DCFH-DA	dichloro-dihydro-fluorescein diacetate
DCF-DA	dichloro-fluorescein diacetate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EB	elementary body
ED	Euclidean distance
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HCS	high content screening
HL	human line (epithelial cells of lung origin)
HPLC	high performance liquid chromatography
HTS	high throughput screening
IC_{50}	Inhibitory concentration 50%
IFN-γ	interferon-γ
IFU	infection-forming units
IL	interleukin
K7	*C. pneumoniae* strain Kajaani-7
LBVS	ligand-based virtual screening
LPS	lipopolysaccharide
MCC	minimum chlamydiacidic concentration
MIC	minimum inhibitory concentration
MOI	multiplicity of infection
MOMP	major outer membrane protein
MPA	mycophenolic acid
NAC	*n*-acetylcystein
NP	natural product
PCA	principal component analysis
PBS	phosphate-buffered saline
p.i.	post infection
QSAR	Quantitative Structure–Activity Relationships
RB	reticulate body

RFU	relative fluorescent unit
ROS	reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute medium-1640
S. chinensis	*Schisandra chinensis*
SMILES	Simplified Molecular Input Line Entry Specification
TCM	traditional Chinese medicine
TLC	thin-layer chromatography
VS	virtual screening
WHO	World Health Organisation

“Natural products are privileged compounds in antibiotic discovery.
They are genetically encoded products of natural selection.
They have been molded by evolution to interact with biological targets;
as such they represent proven and outstanding leads for drug discovery.”

- *Gerard D. Wright, PhD, 2014*

1. INTRODUCTION

As a common pathogen in humans, *Chlamydia pneumoniae* (or *Chlamydophila pneumoniae*) has a remarkable role in the global disease burden (Wyrick, 2000; Roulis *et al*., 2013). The impact of the acute infection as respiratory diseases can be more clearly seen than that of the chronic latent forms of the infection.

Insufficient response to the first line antibiotics and the risk of persistence remain major challenges in treatment of chlamydial infections (Ekman *et al*., 1993; Hammerschlag & Kohlhoff, 2012; Kohlhoff & Hammerschlag, 2015). Despite the *in vitro* susceptibility of *C. pneumoniae* to several antibiotics, treatment failures are a matter of concern. The antibiotics currently in use against *C. pneumoniae* do not eradicate the chronic form of the disease (Gieffers *et al*., 2004). In addition, in clinical care rapid diagnostic methods for identification of pathogens causing an infection allow prescribing more targeted antibiotics for selective therapy (Caliendo et al., 2013). Pathogen-specific compounds can be used rationally against the infection causing pathogen, which will delay the development of resistant strains, and leave the body's natural microbiota unharmed.

Nature is a rich source of potential lead compounds. Many compounds obtained from the plant kingdom are in drug market as such, or serve as a basis for semi- or total synthesis of drugs. Plant derived compounds together with antibiotics and biological drugs, i.e. compounds of biogenic origin or natural products, constitute majority of the drug market and continue to provide lead compounds that have entered clinical trials (Newman & Gregg, 2007; Newman & Gregg 2012; Harvey *et al*., 2015). The original technical reasons why the pharmaceutical industry abandoned plant derived natural products are outdated. Development in the fields of mass spectrometry and nuclear magnetic resonance, compound purification and identification, genomics and metabolomics, as well as the diversity of screening libraries has brought natural products back as an even more fruitful source for lead compounds than ever before (Clardy & Walsh 2004; Lewis, 2012; Wright, 2014; Harvey *et al*., 2015). Natural products and natural product inspired compounds form a

diverse chemical space for computational and biological methods for screening of new hit and lead molecules.

Virtual screening (VS) can be used to facilitate high-throughput screening (HTS) of large compound libraries. The screening process generally begins with either an already known target protein, or a reference set of ligands which bind to the target and cause the wanted biological activity (Dobi *et al*., 2014). Ligand-based approaches are especially useful when the target is unknown.

In this thesis, the discovery of new antichlamydial compounds is based on phenotypic assays on chlamydial infections, with an aim towards selective antichlamydial agents. With the help of ligand-based virtual screening (LBVS) combined with *in vitro* experiments, a privileged antichlamydial chemical space can be defined. Focusing on the hit and lead compounds' properties, such as non-toxicity and lead-likeness, we aim to provide promising lead candidates for further drug development.

2. REVIEW OF THE LITERATURE

2.1 *Chlamydia pneumoniae*

Chlamydia pneumoniae is the most common *Chlamydia* in humans and virtually everyone is infected in a lifetime (Grayston, 2000). The seroepidemiological studies have shown an antibody prevalence of 50‒70%, the figures increasing by age of the studied population (Grayston, 1992; Benitez *et al*., 2012). The most commonly infected are the children at school age. Within the children the infection manifests as mild upper respiratory tract symptoms, whereas adults typically suffer from a more severe long-lasting pneumonia. As a common respiratory pathogen, the participation of *C. pneumoniae* in chronic pulmonary inflammations has strong evidence (Grayston, 1992; Hahn *et al*., 2002; Hahn *et al*., 2012). Acute complications of *C. pneumoniae* infection include some severe although rare diseases, such as reactive arthritis (Gérard *et al*., 2000). Yet, the chronic consequences are more significant considering the whole population.

There are many globally significant diseases that have been associated with chronic *C. pneumoniae* infections: chronic obstructive pulmonary disease (COPD), asthma, atherosclerosis, reactive arthritis and lung cancer (Gérard *et al*., 2000; Zhan *et al*., 2011; Hahn *et al*., 2012; Roulis *et al*., 2013). Four of five patients with chronic bronchitis, as also patients with adult or juvenile asthma, express signs of a persistent *Chlamydia* infection.

Acute *Chlamydia* infections are sensitive to quinolones, tetracyclines and macrolide antibiotics (Kohlhoff & Hammerschlag, 2015). Despite the good response, there is a remarkable risk of the infection relapsing and turning into chronic. Chronic *Chlamydia* infection in turn cannot be cured with antibiotics (Kern *et al*., 2009). Thus, the question is how we can affect chronic *Chlamydia* infection and the diseases with pathogenesis in which it plays a key role. Obviously, there is an urgent need for new biologically active compounds with antichlamydial activity.

2.1.1 History and epidemiology

Chlamydia pneumoniae was first thought to be a virus, most likely due to its intracellular life style and small size. Early reports from the 1930's and 1940's mention a pathogen causing atypical pneumonias. It was noted that the earlier described meningopneumonitis virus is a psittacosis-like virus (Eaton *et al*., 1941). This finding was one step closer to the reality, since psittacosis is an atypical pneumonia caused by *Chlamydia psittaci*, the third species of the genus *Chlamydophila*, which was known to consist of only two species, *Chlamydia trachomatis* and *Chlamydia psittaci*, by 1960's. The first clinical isolate of the pathogen was thought to be a new *Chlamydia psittaci* strain called TWAR, and it was obtained in 1965 from a conjunctivitis of a Taiwanese child, and named TW-183 (Grayston *et al*., 1986). From this finding it took over two decades until the strain "TWAR" was suggested to be an independent species, *Chlamydia pneumoniae* (Grayston *et al*., 1989). Consequently, the species *C. pneumoniae* is a relatively recently identified pathogen and the epidemiological studies made ever since indicate the remarkable role of this newcomer in the clinic.

C. pneumoniae is a common cause of community-acquired pneumonia (CAP) accounting for about 10% of all the cases (Hahn *et al*., 2002). The most (70%) of acute human *C. pneumoniae* respiratory tract infections are asymptomatic or only mildly symptomatic but a smaller part of them (30%) cause more severe respiratory illnesses including community-acquired pneumonia (CAP) which is the leading cause of hospitalizations and death among the patients over 65 years in developed countries (Vila-Corcoles *et al*., 2009). CAP cases in this population have increased as a consequence of an overall increase in the elderly and the patients at risk. Seroepidemiological surveys show, that in most populations, antibody prevalence is low in children below the age of five, rising during school years and then persisting throughout adulthood (Grayston, 1992; Hahn *et al*., 2002). Prevalence of a chlamydial antibody, measured with micro-immunofluorescence (MIF) assay, increases up to 40% to 50% between ages 5 and 20 rising only gradually thereafter (Grayston, 1992). This indicates that most primary infections occur in children and young adults. *C. pneumoniae* is distributed worldwide, with an estimate of up to 50% of adults

seropositive in all geographic locations examined (Hahn *et al*., 2002). *C. pneumoniae* causes outbreaks of pneumonia in all age groups in close-quarters living environments, such as military installations, prisons and universities (Miyashita *et al*., 2005; Coon *et al*., 2011; Fajardo *et al*., 2015).

2.1.2 Developmental cycle

Chlamydiales is a genus of small gram-negative bacteria that are classified as a *separate* taxonomic group due to their intracellular reproduction cycle (Figure 1). The characteristic developmental cycle is remarkably conserved throughout the whole Phylum Chlamydiae. *Chlamydia pneumoniae* infection is initiated by attachment of the infective extracellular forms, elementary bodies (EBs) to the host cell surface (Wolf *et al*., 2000). Several different surface proteins located in the outer membrane of EBs have been suggested as chlamydial adhesins. These serve as ligands in receptor-mediated endocytosis. *Chlamydia* bacteria use glycosaminoglycans (GAGs) as receptors for cell attachment (Wupperman *et al*., 2001). GAGs are the polysaccharide chains of proteoglycans, found ubiquitously on the surface of eukaryotic cells. Many pathogens use GAGs as receptors for cell attachment. For *Chlamydia pneumoniae* the cellular receptor is heparan sulfate-like GAG. An important virulence factor in certain intracellular gram-negative bacteria, type 3 secretion system (T3SS), is also essential for the infectivity of *Chlamydia* spp (Ouellette *et al*., 2005; Fields *et al*., 2003). T3SS is a protein appendage, also called injectisome, which allows direct effector protein injection into the host cell cytosol, creating an environment which favors bacterial growth and survival. During the entry-phase the chlamydial EBs internalize into the host cell within the first two hours of infection. The chlamydial phagosomal vesicle, called inclusion is not detected by the host cell, and it thereby escapes the lysosomal fusion. During the following 1−8 hours the EBs differentiate into the intracellular metabolically active forms, reticulate bodies (RBs). The RBs replicate inside the inclusion by binary fission. *Chlamydia* uses the host cellular nutrients for its own metabolism, for example cholesterol (Carabeo *et al*., 2003). Acute infection lasts for 48−72 hours and during that period of time the chlamydial RBs multiplicate and transform into EBs, which are then released from the cell to infect new cells. Occasionally, atypical growth of RBs is detected,

and these developmental forms, called aberrant bodies (ABs), are considered as a hallmark of the persistent *Chlamydia* infection (Kern *et al*., 2009). Persistence can be described as a stage of infection where viable but culture-negative, yet nucleic acid-positive organisms reside in the cells (Beatty *et al*., 1994; Bin *et al*., 2000).

2.1.3 Chlamydial envelope structure

Chlamydiae have a gram-negative special envelope structure which consists of the inner membrane and a lipopolysaccharide (LPS)-containing outer membrane (Tamura *et al*., 1971; Aistleitner *et al*., 2015). The known protein constituents include LPS, 60 kDa heat shock protein (HSP-60), 12 kDa protein and 40 kDa major outer membrane protein (MOMP). In the envelope structure of *Chlamydia* there are also some non-proteinaceous components such as LPS, other glycolipids, phospholipids and fatty acids.

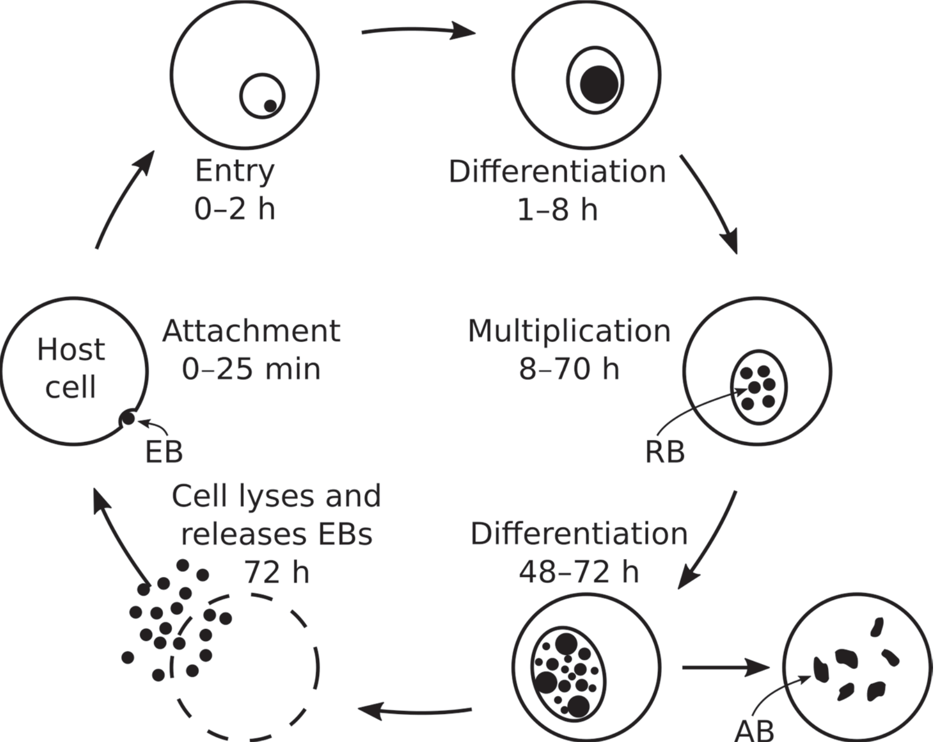


Figure 1. Life-cycle of *Chlamydia pneumoniae*

Lipopolysaccharide (LPS), a typical membrane molecule of gram-negative bacteria, is the main antigenic component of Chlamydiae (Nurminen *et al*., 1983). Chlamydial LPS (cLPS) has been shown to contain the characteristic constituents of bacterial LPSs (Nurminen *et al*., 1985). On the other hand cLPS is less immunogenic than other bacterial LPS (Kalayoglu, *et al*., 2000). One interesting feature of cLPS is that cLPS antigen has been shown to accumulate in the plasma membrane of infected cells (Karimi *et al*., 1989). This is followed by decreased membrane fluidities which the authors suggested to have possible consequences on endocytic processes, lysosome-endosome fusion and complement-mediated cytolysis. The described action of *Chlamydia* could be seen as the bacteria's way of hiding from the host cell's immunity system, as the most intriguing proposal.

It was noted a long time before the order Chlamydiales's taxonomic identification that the reticulate bodies of the meningo-pneumonitis are less rigid, highly labile forms that do not survive outside the host cell (Tamura & Manire, 1967). Since then it has been shown in several studies that the envelope structure varies between the different life cycle forms of Chlamydiae (Hatch, 1996). The overall assessment is that the envelope of metabolically inactive elementary bodies (EBs) consists of densely cross-linked cysteine-rich proteins (CRPs) which might respond to the osmotic stability of EBs. An opposing observation has been made of the reticulate bodies (RBs), which are metabolically active, but osmotically stable. RBs lack the disulfide cross-linked envelope proteins. To further indicate the difference, it is also seen in the formation of CRPs, such as 12 kDa CRP and a large 60 kDa CRP doublet that are present only in EBs but absent in dividing RBs (Hatch *et al*., 1984). It has been proposed, that this structural difference in CRPs might be the reason for the metabolic and osmotic divergences of EB's and RB's (Hackstadt *et al*., 1986). The earlier studies indicated that *Chlamydia* lacks the peptidoglycan (PG) in their cell wall, and it was suggested that major outer membrane protein (MOMP) may substitute to the PG as a structural component (Garrett *et al*., 1974; Newhall & Jones, 1983; Hatch *et al*., 1984). Later it has been shown that there is functional PG in the cell wall of *Chlamydia trachomatis* (Liechti *et al*., 2014).

In addition to the CRPs mentioned above, there are other disulfide cross-linked envelope proteins. Probably the most interesting structural protein, MOMP, consisting of disulfide

linked amino acid chains, is the main component of *Chlamydia*'s cell wall. It is similar in molecular weight and composition between the different chlamydial species although, there are some differences (Campbell *et al*., 1990; Perez Melgosa *et al*., 1991). It has been suggested, that *C. pneumoniae* MOMP would be less immunogenic and antigenically complex than those of the other chlamydial species. The main function of this protein is in maintaining cell wall rigidity. Similar to the other envelope proteins, the consistence of MOMP varies between the different life cycle forms of the bacteria. The MOMP of EBs is tightly cross-linked with disulfide bridges and it is insoluble in sodium dodecyl-sulfate (SDS) a detergent used to solubilize chlamydial inner membrane proteins in the absence of mercaptoethanol (Hatch *et al*., 1981). The solubility of MOMP in the presence of mercaptoethanol and insolubility in the absence of emphasizes the importance of disulfide bridges to the maintenance of the structural stability of the protein. Mercaptoethanol was used as a reducing agent, and reduction of the disulfide residues is assumed to cause envelope proteins tertiary conformation to denaturize.

2.1.4 Diagnostics and clinical challenges

In diagnosing pneumonia, specifying the causative pathogen organism is neither feasible nor cost-efficient in all patients, and resources for full diagnostic work-up are spared for moderate and high risk patients and nosocomial infections (Lim *et al*., 2009; Woodhead *et al*., 2011). The first line treatment in pneumonia are beta-lactam antibiotics which are not selective to *Chlamydia* or *Mycoplasma*. If the symptoms persist or there are *Chlamydia pneumoniae* or *Mycoplasma pneumoniae* epidemias, then an antibiotic selective to these will be added to the treatment. This procedure is preferred and used as a national healthcare guideline in Finland, because there is lack of unambiguous research evidence about benefits in taking the presence of *C. pneumoniae* or *M. pneumoniae* into consideration when choosing the antibiotic in the first line treatment (Käypä hoito -suositus, 2015: www.kaypahoito.fi).

Acute infection of *C. pneumoniae* can be diagnosed by serology measuring the level of *C. pneumoniae* antibodies. This can be done with microimmunofluorescence test (MIF) using elementary bodies (EBs) as antigens and measuring separately IgG and IgM levels

(Wang, 1999). Recently, a Food and Drug Administration (FDA)-approved molecular diagnostic test for respiratory pathogens, including *C. pneumoniae*, has become available (FDA, 2012: www.fda.gov). In the future this will enable standardized testing and targeted treatment of respiratory infections.

The first line treatment of chlamydial infection is azithromycin or doxycycline (Kohlhoff & Hammerschlag, 2015). One problem in treatment of pneumonia is that the penicillin antibiotics used to treat the most common causative agent of CAP, *Streptococcus pneumoniae*, are shown to trigger persistence in *C. pneumoniae* (Matsumoto & Manire, 1970; Schoborg, 2011). *C. pneumoniae* instead, is not very often diagnosed for the above mentioned reasons. In addition to that, the persistent form at the moment is very difficult to diagnose. A widely accepted criteria for serological diagnostics of the persistent form does not exist and diagnosing is based on the symptoms (Boman & Hammerschlag, 2002; Bunk *et al*., 2010). A major problem in diagnosing is that serological tests are not able to discriminate between past and persistent infections. There are no antibiotics to treat the persistent form, but there are some studies that show that in the treatment of acute infection the dose and the course of antibiotic treatment should be sufficient, because subinhibitory concentrations of antibiotics are shown to trigger persistence (Gieffers *et al*., 2004) and resistance *in vitro* by serial passage (Kutlin *et al*., 2005). However, the role of antibiotic resistance or persistent infections in treatment failures is not clear (Kohlhoff & Hammerschlag, 2015). Instead, the mass distribution of azithromycin has led to incidence of azithromycin-resistant fecal *Escherichia coli* (Seidman *et al*., 2014) and azithromycin-resistant *Streptococcus pneumoniae* carriage in young children (Coles *et al*., 2013).

C. pneumoniae and cardiovascular events are shown to have a connection (Saikku *et al*., 1988; Player *et al*., 2014). Strongest evidence is gained from the connection to atherosclerosis, and it suggests that it might be a risk factor in the onset and/or development of the disease. In respect to these findings of the relation of chronic *C. pneumoniae* with atherosclerosis, there are several papers that study the *Chlamydia* selective antibiotics in the treatment of coronary diseases and show no evidence of the connection (O'Connor *et al*., 2003; Andraws *et al*., 2005; Cannon *et al*., 2005). Due to the numerous inconsistent findings, a meta-analysis of randomized controlled trials suggested that there is no benefit

of antibiotic therapy in reducing mortality or cardiovascular events in patients with coronary artery diseases (Andraws *et al*., 2005). The lack of significance in these studies is stated to reverse the theory of connection of the chronic infection with atherosclerosis. The problem with this statement is the fact that these antibiotics do not have effect on the chronic infection, so the lack of their effect to atherosclerosis cannot disprove the connection.

2.1.5 Antichlamydial treatment

Antibiotics in the treatment of acute *Chlamydia pneumoniae* infection are macrolides and fluoroquinolones, of which more specific are ketolides and respiratory fluoroquinolones, respectively (Kohlhoff & Hammerschlag, 2015). The latter have a good intracellular/extracellular ratio and thus are suitable for treatment of intracellular pathogens (Tulkens, 1991; Rakita, 1998).

Macrolide antibiotics attach to the bacterial ribosome 50S subunit inhibiting their protein synthesis at translocation phase. In this class of compounds azithromycin is the most capable of transferring into tissues and also accumulating into white blood cells. Azithromycin is the gold standard in the treatment of *C. pneumoniae*. In addition to the protein synthesis inhibiting class of antibiotics tetracycline based compounds are also used for this purpose but they do not accumulate in cells and may be present at insufficient concentrations.

Fluoroquinolones inhibit bacterial DNA gyrase which in turn inhibits duplication of bacterial DNA. In comparison to the bacteriostatic macrolides, fluoroquinolones are bactericidal in their nature. Fluoroquinolones have also a good ability of penetrating into tissues.

The antibiotics whose mechanism of action is based on the weakening of bacterial cell wall, penicillins in general, do not have a remarkable effect on *Chlamydias* because of them lacking peptidoglycan (PG) in their cell walls (Garrett *et al*., 1974; Newhall & Jones, 1983). Inhibition of the cross binding of PG chains is the mechanism of action of penicillins. However, *Chlamydias* have some susceptibility to anti-PG antibiotics, which exhibits a phenomenon known as "chlamydial anomaly". Later this anomaly has been reversed, as it has been shown that there is functional PG in the cell wall of *Chlamydia trachomatis*

(Liechti *et al.*, 2014). It also was already shown earlier that *Chlamydias* have penicillin-binding proteins, and they are in that way somewhat sensitive to drugs that inhibit PG synthesis (Barbour *et al.*, 1982).

Chronic *Chlamydia* is resistant to antibiotics, and some antibiotics can be used to induce a persistent infection at subinhibitory concentrations. The first-choice antibiotics in the treatment, macrolides, tetracyclines, rifampin, and quinolones, are shown to induce persistence (Gieffers *et al.*, 2004). A number of other antibiotics which have no specific effect on *Chlamydia* are also used to induce persistence. Penicillin G is widely used in this meaning, although it's mechanism of induction remains unclear (Matsumoto & Manire, 1970; Schoborg, 2011). It has been suggested that it might be due to host cell gene silencing during a chlamydial infection (Peters *et al.*, 2005).

2.1.6 Antichlamydial drug research: therapies in clinical trials

There are several new antichlamydial agents in early clinical development. A novel DNA gyrase inhibitior AZD0914, developed by Astra Zeneca, has showed promising activity against *Chlamydia pneumoniae* and *Chlamydia trachomatis in vitro* (Kohlhoff *et al.*, 2014). The activities against both pathogens were comparable to levofloxacin and 16-fold less than the gold standard, azithromycin, based on MIC_{90} values. The authors declared that the results gained by the *in vitro* protocol used in the study has a validated correlation with clinical outcomes. Activities obtained measuring MBC values (minimal bactericidal value, vs. MCC, minimal chlamydiacidic value) were remarkably lower than that of azithromycin, indicating higher recovery rates of the isolates after the treatment. The role of AZD0914 in the treatment of chlamydial infections depends on the outcome of clinical studies assessing microbiological efficacy.

Two new quinolones, nemonoxacin and delafloxacin are introduced for the treatment of *C. pneumoniae* and *C. trachomatis*. Nemonoxacin (TG873870) is a novel broad spectrum non-fluorinated quinolone, which has shown antichlamydial activity against the both species (Chotikanatis *et al.*, 2014). It differs from fluoroquinolones only in that it lacks fluorine atom in R6 position. The *in vitro* activity of nemonoxacin against *C. trachomatis* was 2-fold lower than that of azithromycin, but against *C. pneumoniae* it was comparable

with that of levofloxacin, doxycycline, and azithromycin. However, as the authors noted, *in vitro* activity does not necessarily predict microbiologic efficacy *in vivo* against *C. pneumoniae*. Considering that the fluorine atom in the fluoroquinolones' position R6 has over ten-fold increase to the DNA gyrase inhibiting effect, and can decrease MIC-values 100-fold, the clinical relevance remains to be elucidated by *in vivo* experiments.

Delafloxacin, a novel fluoroquinolone lacking a basic substituent in position 7, has shown low MIC values against gram-positive and gram-negative bacteria, including atypical pathogens such as *C. pneumoniae* (van Bambeke, 2015). It is recently evaluated in Phase III trials and qualified for the treatment of community-acquired bacterial pneumonia (CAP), due to its high activity on pneumococci and atypical pathogens.

A new fluoroketolide antibiotic, solithromycin (CEM-101), has recently entered clinical trials (Golparian *et al.*, 2012). The MIC_{90} values against both *C. pneumoniae* and *C. trachomatis* were only two-fold less than that of azithromycin (Roblin *et al.*, 2010). Other new ketolides, cethromycin and telithromycin were considered attractive additions to antibacterial tool kit for mild-to-moderate CAP (Georgopapadakou, 2014). The first ketolide to be approved, Sanofi-Aventis' telithromycin (RU 66647, HMR 3647, Ketek®), was withdrawn from clinical development due to controversial FDA approval concerning rare, irreversible hepatotoxicity that included deaths. Cethromycin (ABT-773), originally developed by Abbott, completed phase III clinical trials and filed New Drug Application, but it was denied by the FDA in 2009. Enanta's modithromycin (EDP-420), is currently in Phase II in Japan. All of these above listed ketolides have activity against CAP causing atypical pathogens, including *C. pneumoniae*.

Despite the excellent activity in treatment of chlamydial infections, the use of rifamycins, rifampicin and a newer derivative rifalazil, in that indication is discouraged due to them developing resistance relatively fast (Kutlin *et al.*, 2005).

Sitafloxacin (DU-6859a), a new-generation oral fluoroquinolone with broad range *in vitro* activity against gram-positive and -negative bacteria, as well as against atypical bacterial pathogens, was approved in Japan in 2008 (Ghebremedhin, 2012). In Caucasian population its use is currently limited due to the potential for ultraviolet A phototoxicity.

In addition to investigational antimicrobials that have modifications to already existing antibiotics, there are studies concerning antichlamydial potential of drugs for other than antimicrobial indications. There is data about calcium channel blockers, such as verapamil in clinical use inhibiting *C. trachomatis* (Shainkin-Kestenbaum *et al.*, 1989), but opposite observations with L-type calcium channel blockers improving the growth of *C. pneumoniae* (Azenabor & Chaudhry, 2003).

2.1.7 Plant phenolic compounds' and extracts' antichlamydial effect

The health beneficial effects of polyphenolic compounds of plant origin are widely reported. There are several *in vitro* and *in vivo* studies that show the antichlamydial activity of multiple plant-derived phenols in acute infections (Vuorela *et al.*, 2001; Vuorela *et al.*, 2004; Törmäkangas *et al.*, 2005; Alvesalo *et al.*, 2006b; Salin *et al.*, 2010; Salin *et al.*, 2011a; 2011b). In a study, a susceptible cell line, HL (human line, lung epithelial cells) cells were infected with *Chlamydia pneumoniae* clinical isolate K7 (Kajaani 7) (Alvesalo, 2006b). Antichlamydial activity was seen in various compound groups of plant derived phenolic compounds. These substances belong to the chemical groups of flavones, flavonols, coumarins and gallates. Inhibition of the growth of *C. pneumoniae* clinical isolate K7 was 100% with some of the compounds. There were remarkable structure–activity variances between the same groups of compounds. With flavones and flavonols it was suggested that compounds with sugar moieties were generally less active against *C. pneumoniae* than those with only aglycone present. In contrast to most antibiotics that act only against metabolically active forms of bacteria, several phenolic compounds in the study, especially rhamnetin, were active also against the inactive chlamydial elementary bodies (EBs). It was also shown, that some of the natural phenolic compounds had the ability to accumulate inside the host cells or cell membranes and caused inhibition even when they were present only prior to infection. Coadministration of natural phenolic compounds, quercetin, luteolin, rhamnetin and octyl gallate, with calcium modulators, isradipine, verapamil and thapsigargin, resulted in potentiation of the phenolic compounds (Salin *et al.*, 2011a). The calcium modulators alone did not show any inhibitory effect on the growth of *C. pneumoniae*. The plant phenolic compounds were also assayed together

with doxycycline, and they did not potentiate the effect of this chlamydiacidic antibiotic *in vitro*. Another study showed that phenolic compounds resveratrol and quercetin improved the antichlamydial effect of clarithromycin and ofloxacin (Rizzo *et al*., 2014). Also in this study it was found that resveratrol and quercetin alone inhibit intracellular *C. pneumoniae* growth.

Highly active compounds against *C. pneumoniae* are also found from other chemical groups different from flavonoids. An *in vitro* study with *C. pneumoniae* clinical isolate CV-6 (CV, cardiovascular) and CWL-029 showed a natural lupane-class triterpene betulin and its derivatives to be highly active inhibitors of *C. pneumoniae* (Salin *et al*., 2010). One synthetic derivative, betulin dioxime, had a minimal inhibitory concentration (MIC) of 1 μM against CWL-029 and was active with nanomolar concentrations.

In addition to the above mentioned *in vitro* studies there is *in vivo* data showing the antichlamydial activity of plant polyphenolic compounds. An *in vivo* study with a mouse model presented the effects of two flavonoid compounds, quercetin and luteolin, and an alkyl gallate, octyl gallate, on acute *Chlamydia pneumoniae* infection (Törmäkangas *et al*., 2005). Luteolin and quercetin were found to be effective in both suppressing the lung inflammatory response and decreasing the chlamydial load in lung tissue. The luteolin treatment also lowered the levels of *C. pneumoniae*-specific antibodies in serum. Octyl gallate did not display any significant effect on the course of infection. The authors speculated the best inhibitory activity of luteolin to be due to its better bioavailability of the free aglucon form compared with the other two compounds. Another mouse model of *C. pneumoniae* infection with isolates K7 and CWL-029 assayed the antichlamydial effect of corn mint (*Mentha arvensis,* L.) extract. The extract was able to diminish the inflammatory parameters relate to *C. pneumoniae* infection and genome equivalents (Salin *et al*., 2011b). The main phenolic components in the extract linarin and rosmarinic acid inhibited the growth of strain K7 by over 60% at a concentration of 100 μM. Also tea polyphenol product was tested *in vitro* against both *C. pneumoniae* strains AR-39 and AC-43, as well as *C. trachomatis* strains D/UW-3/Cx and L2/434/Bu (Yamazaki *et al*., 2003). The product showed complete inhibition of both pathogens, and authors believed this to encourage the topical use of tea polyphenols in treatment of chlamydial infections.

2.1.8 Other experimental compounds

Cathelicidin peptides which are natural defense compounds in mammalian leukocytes could be considered as lead compounds for antichlamydial use (Donati *et al*., 2005). This theory was challenged by a finding of a chlamydial plasmid-encoded virulence factor Pgp3, which was shown to neutralize the antichlamydial activity of human cathelicidin LL-37 (Hou *et al*., 2015). Cationic antimicrobial peptides (AMPs) are shown to interact with negatively charged microbial membranes, thus permeabilizing the membrane phospholipid bilayer, resulting in lysis and the death of microbes (Hancock *et al*., 2002). Several plant peptides showed activity against *Chlamydia trachomatis* (Balogh *et al*., 2014). However, the exact mechanisms of action of AMPs are poorly understood.

A ligand binding to mannose receptor, M6P-PAA, was active against both *C. trachomatis* and *Chlamydia pneumoniae* with 72% decrease in infectivity compared to infected control to the latter pathogen (Kuo *et al*., 2007). Inhibition was shown *in vivo* in a mouse model. Since chlamydial mannose oligosaccharide is shown to mediate attachment and infectivity of *Chlamydia trachomatis* and *Chlamydia pneumoniae in vitro*, the mannose receptor inhibiting ligands affect the attachment and entry to the host cell. The authors suggested M6P-PAA for topical use, for example in mouthwashes for preventing pneumonia.

Other non-antibiotic compounds with antichlamydial activity are heparin, (Wupperman *et al*., 2001), some COX-2 inhibitors (Yan *et al*., 2008), statins slightly reduced *C. pneumonia* growth (Kothe *et al*., 2000) and rapamycin (immunosuppressant compound) (Yan *et al*., 2010). Contradictory data has been obtained with corticosteroids. One study shows that corticosteroids increase *C. pneumoniae* infection (Malinverni *et al*., 1995), but the other study demonstrates that a steroid receptor antagonist mifepristone inhibits the growth of *C. pneumoniae* (Yamaguchi *et al*., 2008).

2.2 Antimicrobial drug discovery

2.2.1 Current status

Discovery of penicillin in 1928 started the golden era of antimicrobial drug discovery, and during that time nearly all of the antimicrobials used nowadays were invented. Since then the discovery of new antimicrobials has ceased and resistance to the existing antibiotics increases all the time. According to many sources including researchers and health care authorities we have entered a post-antibiotic era meaning that the era of antibiotics may have come to an end (WHO, 2011: www.who.int; Viens, 2015). According to the World Health Organization (WHO) (WHO, 2014: www.who.int), 'the problem is so serious that it threatens the achievements of modern medicine. A postantibiotic era — in which common infections and minor injuries can kill — is a very real possibility for the 21st century'.

According to some estimations bringing a new drug to market takes approximately 10–15 years (antibiotics, reviewed in Fowler, 2014) and costs 1.8 billion dollars (Paul *et al.*, 2010) (See Figure 2. for schematic presentation of drug discovery process). These estimations vary intensively depending on the therapeutic area. Also what comes to costs the figures vary depending on the time frame of discovery process, company in question and whether cash or capitalized costs are compared (Chit *et al.*, 2015). Since the drug discovery process gets more expensive as it continues, it is reasonable to eliminate the possible risks in as early phase as possible. There is always a high risk for failure in what comes to absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) properties of the drug candidate. In addition to that antimicrobials, when they finally reach the market, are normally used only for a small period of time, in comparison to regularly used drugs, such as for example antihypertensive drugs or drugs to treat diabetes (Wright, 2014). Thus antimicrobials do not generate as much revenue for the company as the regularly used medicines (Fowler *et al.*, 2014; Wright, 2014). Taken into account the relatively poor risk/benefit-ratio, it is obvious that from the pharmaceutical industry point of view discovery of antimicrobials does not always seem like the most promising investment.

However, antimicrobial resistance is a well acknowledged global threat, which has been compared to catastrophes such as major flooding, terrorist attacks and pandemic outbreaks by health authorities (Viens, 2015; Cabinet Office UK, 2015: www.gov.uk). WHO has announced that urgent actions are necessary if the effectiveness of antibiotics is to be ensured in the future (WHO, 2011: www.who.int). It is said that the disaster of antimicrobial resistance is not inevitable so now is at last time to take action (Fowler, 2014; Viens, 2015).

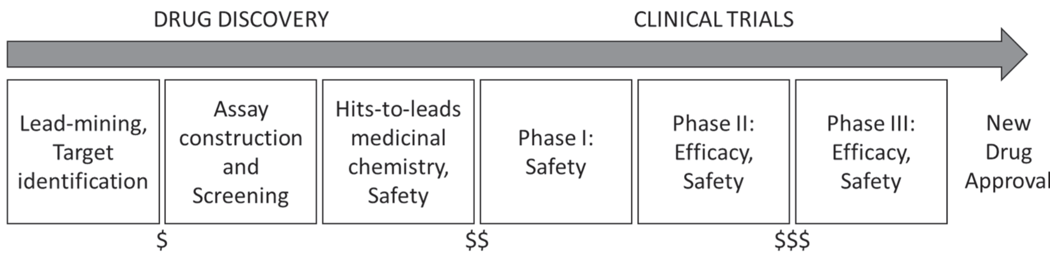


Figure 2. Drug discovery pipeline

2.2.2 Strategies

In early drug discovery the lead finding strategies can be roughly divided into two categories: target based methods and cell based or phenotypic assays (Macarrón & Hertzberg, 2011). In the first approach an isolated target molecule is screened against a compound library for finding a molecule that interacts with the target. By the early 2000s the disappointments with the success rates of molecular-based hit discovery campaigns made the industry to change the course back towards cell-based high throughput screening (HTS). The whole cell based *in vitro* environment proved naturally better in mimicking the *in vivo* physiology than the isolated target molecules. In some cases experiments with isolated targets give the best answers, but this depends on the hypothesis. To discover a new antimicrobial drug there are as well several strategies and their variations (Lipinski & Hopkins, 2004). These attempts begin with either a known target or a promising positive result from a phenotypic experiment. In the latter approach the screening protocol begins similarly as with the target screening, but in these experiments also drug candidate compounds are included. In an antimicrobial screen the experimental set up consists of

infected control, uninfected control and drug treated control. Usually HTS methods are applied in order to find lead molecules that interact with the target (Keserü & Makara, 2009). The libraries are either commercial or in-house libraries consisting of hundreds to thousands or even tens of thousands of compounds. These are then screened in multiwell-plate system. Despite the huge number of candidate compounds, compared to the virtually infinite number of molecules in the chemical space, even a few million molecules in a HTS library make little difference in sampling. Also the possible drug–protein interaction varieties are immense (Lipinsky & Hopkins, 2004). The drug can bind to a receptor, a different subpopulation of a receptor, ion channel or an enzyme, the protein can be located on the cell membrane, or be intracellular and the binding can have different affinities and selectivities. Another strategy, a developed version of HTS is high content screening (HCS), which has proven successful in screening of antichlamydial compounds (Hanski & Vuorela, 2014; Marwaha *et al.*, 2014).

2.2.2.1 Ligand-based virtual screening

Despite the huge financial investments and expectations in HTS in yielding new promising lead molecules, the overall result has been somewhat disappointing (Keserü & Makara, 2009). Taken into account the infinite possibilities of the chemical space and biological space and their interactions, without validated screening protocols and tools finding new drug candidates may seem more hopeless than finding a needle in a haystack. Virtual screening (VS) offers a very ecological and time saving approach to facilitate HTS. VS tools are proposed for avoiding the synthesis of trivial analogues and instead search through chemical space for topologically similar entities using known active compounds or pharmacophores as references (Bleicher *et al.*, 2003). Combining computational methods to HTS has enabled a move from purely random-based testing to high content screening (HCS) and focused libraries. VS also diminishes costs and time when the vast compound library can be tested virtually, which allows reducing costly and laborious *in vitro* experiments. VS methods can be either target-driven or ligand-based. Especially when the target is unknown ligand-based virtual screening (LBVS) approaches offer a solution to hit and ligand screening. LBVS are typically based on a collection of molecules known to bind

to a set of related targets (Dobi *et al.*, 2014). The reference set of ligands is used to perform similarity searches against one or more databases of a selected library. The amount of reference ligands can vary from a single molecule to multiple molecules, but the careful selection of the reference set is highly important, since it defines the chemical space for similarity search (Walters *et al.*, 2003). One key element in performing these types of searches is novelty. Using a similarity metric that is too uniform tends to identify molecules that are close analogues of the known ligands used to perform the search. In an ideal case the similarity metric identifies molecules that are functionally equivalent to the reference set, but chemically distinct enough to bring additional novelty to the search.

2.2.2.2 (Q)SAR studies based on similarity searches

Designing the optimal drug requires the best physicochemical and pharmacodynamics features from a candidate molecule. Because the number of all the possible analogues of a molecule and their combinations is infinite, structure–activity relationship (SAR) or quantitative SAR (QSAR) studies can help in guiding the synthesis. Another application is in defining a chemical space where to find active lead candidates. These studies aim to identify the physicochemical properties of a compound that are responsible for the biological activity and/or other desired features. One way of determining structure–activity correlations is to use data matrices derived from molecule similarity calculations (Good *et al.*, 1993). The computational similarity searches are based on the idea that called similarity principle (Martin *et al.*, 2002), which states that similar molecules are likely to have similar physicochemical properties and therefore might have similar biological activity. The similarity metrics can use 2D or 3D structure representation of molecules, structural fingerprints and molecular descriptors in the calculations, and the data is compared using a similarity index. This depends on the method in use, and there are several different similarity measures. Most methods use simple distance measures such as Euclidean distance and association coefficients such as Tanimoto coefficient or Tanimoto index.

2.2.2.3 ChemGPS-NP as a tool for screening

In a review in Nature 2004 Lipinski & Hopkins compared chemical space with the cosmological universe: "Chemical space can be viewed as being analogous to the cosmological universe in its vastness, with chemical compounds populating space instead of stars" (Lipinski & Hopkins, 2004). This analogy was well accepted by the scientific community, as it describes very well the challenge for drug discovery to find the biologically active compounds in the chemical space. That challenge is to identify those regions that are likely to contain biologically active compounds, the biologically relevant chemical space. For example the compounds that bind to same target classes, such as G protein-coupled receptors (GPCRs), are clustered together in discrete regions of chemical space. The regions can be defined by particular chemical descriptors. For chemical compounds one can calculate a range of properties, such as charge, number of atoms, number of rotatable bonds etc. (Stockwell, 2004). These properties, called descriptors, can be calculated using commercially available software. A chemography tool, ChemGPS (chemical global positioning system), is created of data set which consists of 423 structures selected for a balanced chemical space, largely based on the Lipinski's rule of five (Oprea & Gottfries, 2001). It combines predefined rules and objects as dimensions to provide a global drugspace map. These rules include general properties of a chemical compound, such as size, lipophilicity and hydrogen bond capacity. The coordinates of the drugspace map are extracted by principal component analysis (PCA) from a list of molecular descriptors that evaluate the rules on a selected set of molecules. PCA is a mathematical method, widely used in drug discovery to transform a multidimensional descriptor space into a more manageable low dimensional space (Larsson *et al.*, 2007). The possibly correlated variables of a data set are compressed into a smaller number linearly uncorrelated variables called principal components (PCs). Using the chemography tool PCA-score prediction is used to project new molecules on the map, to explore the chemical space which these particular compounds occupy. The comparison to the reference set is described numerically as Euclidean distances. It has been noted, that natural products (NPs) tend more often to fall outside the ChemGPS defined chemical space (Larsson *et al.*, 2005). The reason is thought

to be that in comparison to the druglike synthetic molecules for which the system was initially designed, NPs are very different in terms of structure and chemical properties. Larsson and coworkers proposed that ChemGPS as such is not suitable for complex and atypical NPs, and the expansion of ChemGPS was made into ChemGPS-NP, where NP stands for natural products (Larsson *et al*., 2005; Larsson *et al*., 2007; Rosén *et al*., 2009).

2.2.3 Hit identification

Since HTS hasn't produced as much new drugs as anticipated, researchers have elaborated on the methodology to high content screening (HCS) and more targeted smaller libraries. Also quality of the screen has been addressed and the factors in a particular screen that affect finding a hit, such assay quality parameters like Z'-factor, signal/noise or signal/background ratio, IC_{50} and statistical significance (Walters *et al*., 2003). There are also several other issues in the screen that should be taken into consideration in hit or lead discovery. Careful compound selection which take into account physical properties, ADME properties and drug-likeness, or lead-likeness, are things to pay attention in designing the screen. It is said that in compound selection no factor has a larger role than the compounds used for the screen. In addressing the issues of the compounds selection according to their properties computational approaches can be of great value. These include methods such as protein–ligand docking, similarity searching, pharmacophore searches and property profiling. There has been debate about including the requirements for ADME properties in the early drug discovery, and some researchers suggests that, it might be premature to use predictive ADME at the hit screening stage, because the compounds will usually undergo significant changes during the lead-optimization process. Some analyses of drug-discovery programs have pointed out that drug candidates are typically larger and more lipophilic than the initial lead (Hann *et al*., 2001; Oprea, 2002). This is speculated to be a result of a tendency of medicinal synthetic chemists to more often add functional groups to the lead candidates, rather than eliminating them. On the basis of these observations, it has been concluded that it might be beneficial to select such compounds in the early screens that are predicted to be soluble and orally bioavailable. Not only optimizing the screening protocol, it is essential to choose the correct strategy for finding appropriate leads (Bleicher *et al*., 2003). Among

other things this includes the questions whether a target or ligand-based approach would be suitable (See chapter 2.2.2: Strategies).

2.2.4 Hit-to-lead validation

When the natural source, for example an extract, has proven biological activity, it is essential to define the compound or compounds responsible for that activity. This kind of molecule can act as a hit or lead compound. In the early drug discovery process it is important to define lead molecules from hit molecules, and it is not enough for a successful lead molecule to show biological activity. As the cost of drug discovery process increases the further it gets, possible failures must be identified and eliminated as early as possible (Bleicher *et al*., 2003). To distinguish the drug-like and nondrug-like molecules from each other there are some requirements for a hit molecule that fit the description lead-likeness or drug-likeness (Ajay *et al*., 1998). The most famous rule of a thumb to look for drug-likeness is the Lipinski's rule of five. It states that "historically 90% of orally absorbed drugs had fewer than five hydrogen bond donors, less than ten hydrogen bond acceptors, molecular masses of less than 500 daltons and log$P$ values (a measure of lipophilicity) of less than five" (Lipinski *et al*., 1997; Lipinski *et al*., 2012). This rule is created to estimate solubility and permeability of a drug in discovery and development. The aqueous solubility, in terms of hydrophilicity, is crucial for a compound to get in contact with the absorption site, for example gut wall. On the other hand, if the drug is too hydrophilic, it is not able of penetrating biological barriers, like the gut wall or cell membranes. The hydrophilicity–hydrophobicity balance is often described as log$P$ value. In addition to solubility and absorption, a successful lead candidate needs to possess adequate bioactivity, appropriate physicochemical properties to enable formulation development, reasonable metabolic stability and appropriate safety and efficacy in humans (Pritchard *et al*., 2003). Nowadays in the drug discovery process information about the toxicity of a compound is required in a very early phase. The overall cell toxicity is tested *in vitro*, and gives a very good exclusion criterion for a compound. This will save effort and expenses in the further process. For predicting the optimal properties of a lead compound and finding it from vast chemical

libraries the computational methods offer time and resources saving processes to the discovery.

2.2.5 Natural products

Nature is a rich and mainly unexplored source of biologically active compounds. Many active substances in drugs sold in the pharmacy nowadays are originally isolated from a natural source, such as morphine, cocaine, digitalis, quinine, nicotine, muscarine, paclitaxel (taxol) and tubocurarine. Compounds that are used as such or serve as a basis for semi- or total synthesis from plant kingdom, together with antibiotics produced by microbes and biological drugs, comprise majority of the drug market (Newman & Gregg, 2007; Newman & Gregg, 2012). Thus, it can be said that nature has served, and will serve, as the most abundant source of drugs, and most of it still remains unexplored (Wright, 2014). Natural products (NPs) are developed by evolution and they have very often been selected for properties that allow them to cross biological barriers, such as bacterial cell wall. Nature has taught us invaluable lessons in the logic of biosynthesis for developing new drugs, as well as properties of different functional groups, which helps in synthesizing new molecules (Clardy & Walsh, 2004). During the recent 20 years there has been a trend within the pharmaceutical industry to downscale efforts in NP research, with the present knowledge and technology this could be reconsidered. The problems with NPs that made the companies withdraw from the field are outdated (Clardy & Walsh, 2004; Lewis 2012; Wright, 2014). These problems included challenges with identifying the active components from natural product extracts and difficulty in synthesis. With current technological advances especially in the field of mass spectrometry and nuclear magnetic resonance, compound purification and identification are brought to a wholly new level. Going back to explore new chemical entities and therapeutically relevant chemical diversity in the nature seems to offer great potential for drug discovery industry.

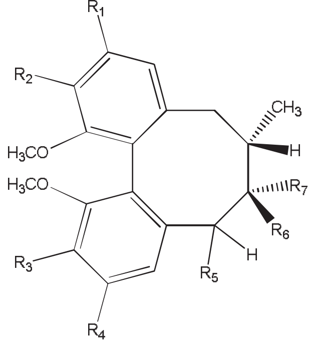
2.3 *Schisandra* lignans

Schisandra chinensis (Turczaninow) Baillon, “Wu Wei Zi” in Mandarin, is a liana native to the forests of Eastern Asia (Hancke *et al*., 1999). It produces bright red fruits spherical in shape and growing in clusters. *S. chinensis* is known for its use in Traditional Chinese Medicine (TCM) — and overall in Far East, ethnopharmacologically — and is used to treat several disorders of the reproductive, respiratory, nervous, and digestive systems (Hancke *et al*., 1999; Panossian & Wikman, 2008). Traditionally in Russia, the fruits of *S. chinensis* are considered a stimulant and adaptogen. Nowadays the herb is used in dietary supplements mainly as an adaptogen.

Schisandra lignans have various different synonyms in the literature (Opletal *et al*., 2004). The constituents of *S. chinensis* berries can be divided into four classes: dibenzocyclooctadiene lignans, which represent the dominant secondary metabolite component in the berries, monoterpenes, sesquiterpenes and other compounds of various structures (Opletal *et al*., 2004). The lignans are predominantly dibenzocyclooctadiene in structure, which also are responsible for the majority of the declared biological activities of the berries (Lu & Chen, 2009). In the group of dibenzocyclooctadiene lignans the dominant compounds are schisandrin and schisandrin B, which are also the most studied ones. In this thesis some of the major dibenzocyclooctadiene lignans are presented (Table 1.).

Dibenzocyclooctadiene lignans from plants in the genus *Schisandra*, i.e. *Schisandra* lignans, are shown to have various pharmacological activities, such as neuro-, cyto- and hepatoprotective, as well as anti-cancer and anti-inflammatory effects (Guo *et al*., 2008; Stacchiotti *et al*., 2009; Lee *et al*., 2012; Liu *et al*., 2012; Lv *et al*., 2015). These activities were obtained with either schisandrin or schisandrin B at 5 and 6.25 µM concentrations respectively in cell models, and with schisandrin B at 1–30 mg/kg in animal models, as the lowest concentrations to give statistically significant results. *Schisandra* lignans are also shown to have anti-viral activities, including anti-HIV activity (Chen *et al*., 1997; Yang *et al*., 2010), inhibition of HSV-2 and adenovirus (Song *et al*., 2013), and inhibition of Epstein–Barr virus early antigen activation (Chen *et al*., 2002). The lowest EC_{50} values obtained in these studies against HIV-1 were 0.005 µg/ml for gomisin G (Chen *et al*., 1997)

Table 1. *Schisandra* lignans presented in this work; core structure and functional groups



***Schisandra* lignan**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**
Schisandrin	OCH_3	OCH_3	OCH_3	OCH_3	H	OH	CH_3
Schisandrin A	OCH_3	OCH_3	OCH_3	OCH_3	H	OH	CH_3
Schisandrin B	-O-CH_2-O-		OCH_3	OCH_3	H	OH	CH_3
Schisandrin C	-O-CH_2-O-		-O-CH_2-O-		H	H	CH_3
Schisandrol B	-O-CH_2-O-		OCH_3	OCH_3	H	OH	CH_3
Schisantherin A	-O-CH_2-O-		OCH_3	OCH_3	O-C-O-Bz	CH_3	OH

and 1.4 µg/ml for new lignans identified in the same study (Yang *et al*., 2010). The lowest EC_{50} values for HSV-2 and adenovirus were 5.85 µg/ml and 22.10 µg/ml respectively, gained with new lignans identified in the same study (Song *et al*., 2013). The effect of *Schisandra* lignans on respiratory pathogens is not known, but *Schisandra chinensis* (Turcz.) Baill. is used in TCM for several indications, and one of them is treatment of chronic cough (Chinese pharmacopoeia, 2010). In a clinical study results have suggested that a tincture of *Schisandra* fruit has alleviating effect on the symptoms of pneumonia (Pavluschenko *et al*., 1981, reviewed in Panossian & Wikman, 2008). In the study the causative agent of pneumonia was not determined. Also other clinical studies made in the former USSR with *Schisandra* tinctures show a reduced prevalence of influenza in study subjects treated with *Schisandra* tincture (Panossian & Wikman, 2008). In addition there are modern *in vitro* and *in vivo* studies that show *Schisandra chinensis*' protecting effect in alveolar inflammation. *Schisandra chinensis* water extract prevented airway inflammation both *in vitro* and *in vivo* (Bae *et al*., 2012). In human alveolar epithelial cells *Schisandra*

extracts inhibited cytokine mixture induced NO production and reduced IL-8 and MCP-1 secretions. The extracts also suppressed neutrophil and macrophage infiltrations of lung tissues and increased IL-6 and TNF-α levels in bronchoalveolar lavage fluid in LPS-treated BALB/c mice. A pure compound, schisantherin A, was shown to alleviate acute respiratory distress syndrome through downregulating NF-κB and MAPKs signaling pathways *in vivo* in a mouse model at 10 mg/kg concentration (Zhou *et al*., 2014).

2.3.1 *Schisandra* lignans' antioxidative activity

Schisandra lignans are widely studied for their antioxidant properties, which is considered one major mechanism responsible for the cytoprotective effects (Liu *et al*., 1982; Opletal *et al*., 2004; Guo *et al*., 2008; Zeng *et al*., 2012) (Table 2.). The antioxidant or reducing properties of the lignans are also shown to mediate the anti-inflammatory responses. For example, in a study schisandrin B displayed anti-inflammatory activities which were due to alteration of cellular redox status leading to inhibition of nuclear factor (erythroid-derived 2) 2 -like 2 (Nrf2) and NF-κβ (Checker *et al*., 2012). Schisandrin B suppressed Iκβα degradation and nuclear translocation of NF-κβ in activated lymphocytes. In the same study schisandrin B was shown to inhibit mitogen-induced phosphorylation of c-Raf, MEK, ERK, JNK and p38. Schisandrin B-mediated activation of Nrf2 was proposed to suppress NF-κβ, which is a redox sensitive pathway. Nrf2 induces production of antioxidative enzymes, as NAD(P)H:quinone oxidoreductase, glutathione S-transferase, HO-1, glutathione peroxidase, glutamate cysteine ligase, and peroxiredoxin 1. These factors are known to diminish reactive oxygen species (ROS). Thus it can be concluded that Nrf2 activation of schisandrin B leads to anti-inflammatory responses that are mediated by ROS. It was also noted that schisandrin B inhibited anti-CD3/CD28 mAb-induced secretion of IL-2, IL-4, IL-6 and IFN-γ by T-cells. In two other studies schisandrin B enhanced apoptosis through activation of a redox-sensitive Nrf2 pathway (Chiu *et al*., 2009; Chiu *et al*., 2011). The antioxidative activity of schisandrin B was further confirmed in a study which showed schisandrin B to inhibit the production of ROS and NADPH oxidase activity in microglia (Zeng *et al*., 2012). NADPH oxidase-dependent superoxide O_2 and ROS formation were LPS induced.

Table 2. *Schisandra* lignans' and *Chlamydia pneumoniae*'s effects on selected host cellular targets

	Host cell target	***Schisandra* lignan / *Chlamydia*: Effect on host cells***	***Schisandra* lignan**	**Reference: *Schisandra* / *C. pneumoniae***
Intracellular				
APOPTOSIS	Bcl-2 protein family	- / ±	schisandrin B	Lv *et al*., 2015 / Fischer *et al*., 2004; Schöier *et al*., 2006
APOPTOSIS	Caspase-3 pathway	+ / -	schisandrin B	Wu *et al*., 2004 / Airenne *et al*., 2002
APOPTOSIS	Cytochrome c	- / -	schisandrin B	Chiu *et al*., 2009 / Airenne *et al*., 2002
OXIDATIVE STATUS	GSH	+ / -	schisandrin B	Ip & Ko, 1996 / Azenabor *et al*., 2006
OXIDATIVE STATUS	NADPH oxidase	- / +	schisandrin B	Checker *et al*., 2012 / Mouithys-Mickalad *et al*., 2004
OXIDATIVE STATUS	ROS	± / +	schisandrin B	Checker *et al*., 2012; Zeng *et al*., 2012 / Mouithys-Mickalad *et al*., 2004
OTHER SIGNALLING AGENTS	Ca_2^+	- / +	schisandrin A, schisantherin A	Fu *et al*., 2008 / Wang *et al*., 2010
OTHER SIGNALLING AGENTS	ERK1/2	- / +	schisandrin B & C, schisantherin A	Oh *et al*., 2010 / Jiang *et al*., 2008
OTHER SIGNALLING AGENTS	Heat shock proteins	Hsp25 + ; Hsp70 ± ; Hsp72 + / Hsp60 +	schisandrin B	Chiu & Ko, 2004; Wu *et al*., 2004 / Hirono *et al*., 2003
OTHER SIGNALLING AGENTS	JNK	- / +	schisantherin A, schisandrin B & C	Oh *et al*., 2010 / Jiang *et al*., 2008
OTHER SIGNALLING AGENTS	MEK	- / +	schisandrin B	Guo *et al*., 2008 / Jiang *et al*., 2008
OTHER SIGNALLING AGENTS	NF-κB	- / +	schisandrin A & B, schisantherin A	Guo *et al*., 2008 / Wahl *et al*., 2001
OTHER SIGNALLING AGENTS	p38 MAPK	- / +	schisandrin A & B, schisantherin A	Guo *et al*., 2008 / Jiang *et al*., 2008
OTHER SIGNALLING AGENTS	TRAF-6	- / +	schisandrin B	Zeng *et al*., 2012 / Jiang *et al*., 2008

Membrane proteins			
EGFR	- / +	schisandrin B	Waiwut *et al*., 2011 / Mölleken *et al*., 2013
Toll-like receptor 2 & 4	- / +	schisandrin B	Zeng *et al*., 2012 / Jiang *et al*., 2008
Secreted factors			
COX_2	- / +	schisandrin, schisandrin B	Guo *et al*., 2008 /Mouithys-Mickalad *et al*., 2004
iNOS	- / -	schisandrin B	Zeng *et al*., 2012 / Shimada *et al*., 2009
NO	- / -	schisandrin, schisandrin B, schisantherin A	Guo *et al*., 2008 / Shimada *et al*., 2009
IFN-γ	- / +	schisandrin B	Checker *et al*., 2012 / Buss *et al*., 2010
IL-1	- / +	schisandrin B, schisantherin A	Zeng *et al*., 2012 / Kaukoranta-Tolvanen *et al*., 1996
IL-6	- / +	schisantherin A	Zeng *et al*., 2012 / Heinemann *et al*., 1996
IL-8 (CXCL8)	+ / +	schisandrin, schisandrol B	Lin *et al*., 2011 / Al-Bannawi *et al*., 2011
Matrix metalloproteinases 2 & 9	- / +	schisandrin B	Lee *et al*., 2012 / Kol *et al*., 1998
PGE_2	- / +	schisandrin, schisandrin B	Guo *et al*., 2008 / Rödel *et al*., 2004
TNF-α	- / +	schisandrin B, schisantherin A,	Zeng *et al*., 2012 / Jiang *et al*., 2008
Vascular endothelial growth factor (VEGF)	- / +	schisandrin B	Lv *et al*., 2015 / Wang *et al*., 2010

*inductor effect indicated with + and inhibitory effect indicated with -. Opposing effects are indicated with grey background.

2.3.2 Immunity, inflammation, LPS-induced mechanisms

Schisandra lignans are shown to be able of suppressing the inflammatory responses induced by bacterial lipopolysaccharide, LPS (Guo *et al*., 2008; Oh *et al*., 2010) (Table 2.). Schisandrin was found to inhibit *in vitro* LPS-stimulated production of nitric oxide (NO), prostaglandin E2 (PGE2), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which was shown to result from the inhibition of nuclear factor-kappaB (NF-κβ) reporter gene expression and activation of c-Jun N-terminal kinase JNK and p38 mitogen-activated protein kinases (MAPKs) (Guo *et al*., 2008). In another study a group of different *Schisandra* lignans, Gomisin J, N and schisandrin C, were shown to reduce LPS-induced NO production and expression of pro-inflammatory cytokines by blockage of p38 activated MAPK, ERK1/2 and JNK phosphorylation (Oh *et al*., 2010). They also decreased mRNA levels of IL-1 and IL-6 and TNF-α in LPS-activated cells. Schisandrin C reduced phosphorylated ERK1/2 to the level of untreated control. Gomisin N did not affect p38 MAPK or ERK1/2, but LPS-induced phosphorylation of JNK was reduced by gomisin N pretreatment, and also by gomisin J and schisandrin C pretreatment. One report that studied the anti-neuroinflammatory effects of schisandrin B showed that this schisandrin inhibited LPS-induced neuronal cell death and LPS-induced production of NO, PGE2 and TNF-α, and downregulated expression of IL-6 and IL1beta mRNA in LPS treated microglia (Zeng *et al*. 2012). Schisandrin B significantly reversed the LPS-induced phosphorylation of IKKα/β and Iκβ and reduced the total Iκβ expression level in a concentration dependent manner. Pretreatment with schisandrin B significantly decreased NO and PGE2 production by inhibiting iNOS and COX-2 protein expression. Schisandrin B inhibited the interaction of toll-like receptor 4 (TLR4) and TLR2 with Toll adapter proteins MyD88, IRAK-1 and TRAF-6, which was proposed to indicate that schisandrin B may inhibit the IKKα/β-Iκβ-NF-κβ inflammatory signaling pathway via selective antagonism of TLR4.

3. AIMS OF THE STUDY

The study of this thesis is focused on whole-cell assays on chlamydial infections, careful evaluation of the physicochemical properties of the lead candidates and attempts towards more narrow-spectrum antichlamydial agents as elements for successful lead generation.

The specific aims of this thesis were:

1. To study the antichlamydial activity of peppermint tea water extracts. To optimize further the acute cell culture model for detection of *Chlamydia pneumoniae* infection *in vitro*, which can be used for screening new drug candidates in drug discovery process. (I)

2. To investigate the antichlamydial effect of *Schisandra chinensis* water–methanol extract and compare the effect with isolated *Schisandra* lignans. (II)

3. To study the *Schisandra* lignans' effect on the host cell–*Chlamydia* interaction and on the life-cycle of *Chlamydia pneumoniae*. (III)

4. To evaluate the promising antichlamydial compounds' suitability as successful lead compounds. (III–IV)

5. To study the structure–activity relationships of a variety of antichlamydial compounds. Build a training set for ChemGPS-NP for screening of antichlamydial hit compounds from a natural product library. To validate the *in silico* results with *in vitro* antichlamydial assays. (IV)

4. MATERIALS AND METHODS

4.1 Plant material and preparation of the extracts (I–II)

Commercially available peppermint herbal teas (n = 27) in the form of crude herb were purchased from food markets, health shops and retail pharmacies, originating from Egypt, Estonia, EU, Finland, Germany, Latvia, Poland, The Netherlands, UK and USA (I). The infusions from the herbal teas were made according to manufacturer's instructions with distilled water, filtered and centrifuged at 4000 rpm for 15 min. at 20 °C. The total polyphenol content and quantification of individual polyphenols was done by HPLC-UV-MS/MS analyses.

The powdered berries of *Schisandra chinensis* (Turcz.) Baill. were purchased from Loitsukeller, Estonia (II). Their identity and schisandrin content were investigated by macroscopic and microscopic identification, as well as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) tests according to European Pharmacopoeia guidelines (European Pharmacopoeia, 2010).

The 80% aqueous methanol extract was prepared from 5.0 g of finely powdered *S. chinensis* berries by sonicating the mixture for 10 min at room temperature following that the extract was vacuum-filtered and evaporated to dryness using a rotary evaporator. The residue was dried under N_2-flow and frozen at -20 °C overnight. The frozen extract was lyophilized and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml for the *in vitro* experiments. This stock solution was used for making dilutions of 10, 25, 50, 100 and 250 µg/ml final concentrations in growth medium the concentration of DMSO not exceeding 0.25%.

4.2 Pure compounds (II–IV)

Dibenzocyclooctadiene lignans ≥ 98% (HPLC) (Fine Tech Industries, London, UK) were dissolved to dimethyl sulphoxide (DMSO) as 20 mM stock solutions. *L*-ascorbic acid at 250 µM concentration, *l*-dithiothreitol and *n*-acetyl-l-cysteine at 10 mM concentration (solid pure compounds obtained from Sigma–Aldrich, Germany) were dissolved in deionized sterile water prior to experiments (III). Antichlamydial virtual hits that were tested *in vitro* were obtained from Screen-Well® natural product library version 7.1 (Enzo Life Sciences), as 2 mg/ml stock solutions in DMSO, and diluted in cell culture medium for in vitro antichlamydial assays (IV). More detailed information about the test compounds is listed in Table 3 and Table 4. As control antibiotics for chlamydial growth inhibition was used 12 nM rifampicin (II) and 20 nM azithromycin (III, IV).

Table 3. Test compounds used in publications II and III

Compound	**Purpose**	**Publication**	**CAS nr.**
Schisandrin	*Schisandra* lignan	II, III	7432-28-2
Schisandrin A	*Schisandra* lignan	III	61281-38-7
Schisandrin B	*Schisandra* lignan	II, III	61281-37-6
Schisandrin C	*Schisandra* lignan	III	61301-33-5
Schisandrol B	*Schisandra* lignan	III	58546-54-6
Schisantherin A	*Schisandra* lignan	III	58546-56-8
L-ascorbic acid	reducing agent	III	50-81-7
L-DTT	reducing agent	III	16096-97-2
N-acetyl-l-cysteine	reducing agent	III	616-91-1

Table 4. Virtual hits used in publication IV

Compound	CAS nr.	Compound	CAS nr.
6-Acetamido-6-deoxy-castanospermine	134100-29-1	Graveoline	485-61-0
		Harmaline HCl	304-21-2
Aphidicolin	38966-21-1	Hesperidine	520-26-3
Apigenin-7-O-glucoside	520-36-5	Honokiol	35354-74-6
(−)-Asarinin	133-05-1	7-Hydroxyflavone	6665-86-7
Baccatin III	27548-93-2	Hypocrellin A	77029-83-5
Betulin	473-98-3	Indirubin	479-41-4
Caffeic acid phenethyl ester	104594-70-9	Isoquercitrine	21637-25-2
Caryophylleneoxide	1139-30-6	Jervine	469-59-0
Catharanthine base	2468-21-5	Laudanosoline HBr	485-33-6
Cevadine	62-59-9	Leucomisine	17946-87-1
Chartreusin	6377-18-0	Lupinine	545-47-1
Chrysine	480-40-0	Manool	596-85-0
Cinobufagin	470-37-1	Mycophenolic acid	24280-93-1
Convolvamine HCl	500-56-1	Nalidixic acid	389-08-2
Coumesterol	479-13-0	Nonactin	6833-84-7
Cycloheximide	66-81-9	Norleaginine	16502-01-5
Deguelin	522-17-8	Oleanolic acid	508-02-1
Dehydrokawain	15345-89-8	Oridonin	16964-56-0
Digitoxin	71-63-6	Osthole	484-12-8
Dihydroergocristine mesylate	24730-10-7	Peganole	36101-54-9
		Pimaricin	7681-93-8
Dipterocarpol	471-69-2	Podocarpic acid	5947-49-9
Epicathecin	490-46-0	Prostaglandin B1	13345-51-2
Eriocitrin	13463-28-0	Radicicol	12772-57-5
Flavanomarein	577-38-8	Remerine HCl	17669-16-8
Gossypol	303-45-7	Syrosingopine	84-36-6

4.3 Host cell lines and chlamydial strains

Human epithelial HL cells (Human line, respiratory origin,) are a sensitive cell line for propagation of *Chlamydia pneumoniae* (Kuo & Grayston, 1990) and were used as host cells for *C. pneumoniae* strains K7 (Kajaani 7) clinical isolate (Ekman *et al.*, 1993) and CV6 cardiovascular strain recovered from atherosclerotic lesions (Gieffers *et al.*, 1998). HL cells were grown in Roswell Park Memorial Institute medium-1640 (RPMI-1640, Lonza,

Verviers, Belgium) supplemented with 2 mM *l*-glutamine (Lonza), 7.5% fetal bovine serum (FBS) (Gibco, NY, US) and 20 µg/ml gentamicin (Sigma, MO, USA) at 37 °C, 5% CO_2 and 95% humidity to confluence in cell culture flasks. *C. pneumoniae* stocks were stored in 0.2 m sucrose (Sigma), 0.02 m sodium phosphate (Sigma–Aldrich, Germany) (pH 7.4) and 5 mM glutamic acid (Sigma) sucrose-phosphate-glutamic acid (SPG) buffer at -80 °C until use.

HeLa-229 cells (CCL-2.1; ATCC, Manassas, VA, USA) for the infections with *Chlamydia trachomatis* serovar L2 (VR-902B; ATCC), were grown in RPMI-1640 supplemented with 10% FBS, 20 mM HEPES (Sigma) (pH 8.0), 8 µg/ml gentamicin, 1 µg/ml amphotericin B (Sigma) and 2 mM *l*-glutamine at 37 °C, 5% CO_2 and 95% humidity. *C. trachomatis* stocks were stored as above (as *C. pneumoniae*).

4.4 Infections

HL cells were grown to confluence in cell culture flasks, washed with phosphate-buffered saline (PBS) (Lonza, Verviers, Belgium) and detached with trypsin (Lonza) 0.25% in PBS. The cell suspension was seeded into 24-well plates (Greiner, Germany) with coverslips (VWR international) at 4×10^5 cells per well and incubating for 24 h at 37 °C, 5% CO_2 and 95% humidity. The growth medium was removed and cell monolayers were inoculated with a suspension of *Chlamydia pneumoniae* EBs suspended in cell culture medium supplemented with 0.5 µg/ml cycloheximide (Sigma, China), a mammalian cell 80 S ribosome inhibitor, with the multiplicity of infection (MOI) 0.2. The plates with inoculated cells were centrifuged at 550 *g* for 1 h and incubated at 37 °C for 1 h to allow the EBs to internalize into the host cells. Medium supplemented with 1 µg/ml cycloheximide containing the samples or controls was added. Non-treated infected cells, infected cells treated with 0.01 µg/ml rifampicin and non-infected cells served as controls. In each well the concentration of DMSO was adjusted to 0.25%. Plates were incubated for 70 h at 37 °C, 5% CO_2 and 95% humidity. Seventy-two hours post infection the infection was terminated by washing the wells with PBS fixing the cells with methanol. The coverslips were removed from the wells and dried in room temperature. The *Chlamydia* inclusions were labeled with

Pathfinder® Chlamydia Culture Confirmation System (Bio-Rad Laboratories, Berkeley, CA, USA) according to manufacturer's protocol. The inclusion counts were determined with fluorescent microscope at × 200 magnification.

The infections done with *Chlamydia trachomatis* were carried out in a similar manner as the *C. pneumoniae* infections with the following exceptions. Confluent HeLa-229 cells were infected with *C. trachomatis* serovar L2 diluted in Hank's balanced salt solution (HBSS) at multiplicity of infection 0.3. After 1 h incubation at 37 °C, HBSS was replaced with growth medium including test compounds or solvent alone (1% DMSO). Cell cultures were fixed at 24 h, immunostained with Pathfinder® and examined under fluorescent microscope.

4.5 Evaluation of the target: phase of infection

4.5.1 Infectious progeny production

HL cells were seeded into 24-well plates with and without coverslips, infected and treated with the samples as described above. After 72 h infection (first passage) the number of infectious progeny was determined by washing the wells with PBS and replacing the sample containing medium with fresh medium without the samples. The wells with coverslips were fixed and stained to calculate the inclusions (first passage). The infected cell layers were then disrupted and cell lysates from the wells without coverslips were used to infect fresh HL cell monolayers. After another 72 h infection (second passage) the cells were fixed and stained to determine the amount of infectious progeny.

4.5.2 Elementary body infectivity

To evaluate direct effects on chlamydial elementary bodies, *Chlamydia pneumoniae* stocks were diluted in the RPMI medium supplemented with 0.5 μg/ml cycloheximide to yield a 400 000 IFU/ml suspension and the studied compounds were added in aliquots of the EB suspension. The suspension was incubated at +4 °C for 1 h and was then used to infect HL cells as described above.

4.6 Intracellular ROS detection

The level of intracellular reactive oxygen species (ROS) were measured with modifications to an earlier presented protocol (Wang & Joseph, 1999). HL cells were seeded into 96-well plates to 60 000 cells per well, incubated overnight to confluence and loaded with 20 µM 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma, MO, USA) for 30 min at 37 °C. The excess label was removed from the wells and washed once with Hank's balanced salt solution (HBSS) prior to the exposure of the cells to the samples for 1 h at 37 °C. RFUs (relative fluorescent units) were measured at 503/523 (ex/em) with VarioskanFlash plate reader (Thermo Fischer Scientific, Vantaa, Finland). 1 mM hydrogen peroxide (Aldrich, Germany) and *N*-acetylcysteine served as positive and negative controls. To determine a possible interference between the samples and the dye, a similar experiment was performed with only samples and DCFH-DA (Sigma, MO, USA) (no cells). The potential effects of dye de-esterification or efflux were excluded with a 2'7'-dichlorofluorescein diacetate (DCF-DA) assay.

4.7 Host cell viability assays

Extract- and compound-treated HL and HeLa cell viabilities were confirmed by resazurin assay. Seeding of the HL-cells into a 96-wellplate to a density of 5×10^3 (growing) and 6×10^4 cells (confluent) per well followed by overnight incubation at 37 °C. One column was left without cells and served as a background. Triton X (Sigma–Aldrich) (II) and 50 µM usnic acid (Aldrich, Switzerland) (I, IV) were used as positive controls. The following day the samples and controls were added onto cell monolayers. DMSO concentration was adjusted to 0.25% in every well. After that the plate was incubated at 37 °C in 5% CO_2, the exposure corresponding the acute infection period. To measure the amount of metabolically active cells, a profluorescent dye, resazurin in PBS, was added into the wells. The plate was incubated for 2 h at 37 °C and fluorescence was measured with VarioskanFlash plate reader at 570/590 nm (ex/em).

4.8 Selectivity assays: turbidity and fluorescence measurements

The growth inhibition of metabolically active bacteria, other than *Chlamydia*, by the *Schisandra* extract and the lignans were assayed with turbidity and fluorescence measurements of the bacterial suspension (protocol adapted from Sandberg *et al*., 2009). Gram-negative bacteria; *Enterobacter aerogenes* (ATCC; 13048), *Escherichia coli* (ATCC; 25922), *Proteus mirabilis* (ATCC; 43071), *Pseudomonas aeruginosa* (ATCC; 27853) and gram-positive bacteria; *Bacillus subtilis* (ATCC; 6633), *Staphylococcus aureus* (ATCC; 25923) and *Staphylococcus epidermis* (FOMK; in-house strain) were grown on Bacteriological agar Type A (VWR international) (III). Gram-positive bacteria *Streptococcus pneumoniae* (DSM 11867, DSMX Collections) and *Streptococcus pyogenes* (ATCC; 12351) were grown in Todd–Hewitt broth (BD, France) (II). Gram-negative *Klebsiella pneumoniae* (HAMBI culture collection; 1332) was grown in nutrient broth (Sigma–Aldrich, Spain) (II). The bacterial culture was inoculated to 10 ml of nutrient broth and grown overnight at 37 °C in a shaker at 100 rpm. A 500 × dilution of the inoculum in the broth was seeded into a 96-well plate. The samples were added at 50 µM concentration and non-treated cultures, cultures treated with either 12 nM (0.01 µg/ml) rifampicin or 20 µg/ml ampicillin were used as control samples. Wells with nutrient broth alone served as background. The amount of DMSO was adjusted to 0.25% in each well. Starting point turbidity was measured as light absorbance at 590 nm with VarioSkan Flash plate reader. The plates were incubated for 24 h on a shaker at 37 °C, 100 rpm. The change in turbidity was measured at 590 nm. Resazurin at 20 µM in PBS was added and incubated for 5 min at 37 °C, on a shaker in darkness and the fluorescence was measured with VarioSkan flash plate reader at 560/590 nm (ex/em).

4.9 Structure–activity relationship studies with ChemGPS-NP

4.9.1 Constructing of the reference set of compounds

Reference antichlamydial compounds were selected according to literature by the following criteria: nature derived structurally diverse compounds shown to completely inhibit the

growth of a same strain and isolate of *Chlamydia pneumoniae* at 50 µM concentration. The following compounds fulfilled the criteria with 100% growth inhibition of *C. pneumoniae* clinical isolate K7 (Kajaani 7): acacetin, apigenin, coumarin 106, 7-diethylamino-3-thenoylcoumarin, dodecyl gallate, luteolin, methoxy sporalen, methyl gallate, morin, myricetin, octyl gallate, propyl gallate, rhamnetin, rotenone (Alvesalo *et al.*, 2006b); biochanin A (Hanski *et al.*, 2014), schisandrin A, schisandrin B, schisandrin C, schisantherin A (original publication III). In the paper IV linear notations of the molecules were used, canonical SMILES (Simplified molecular-input line-entry system) codes. SMILES annotations for these 19 reference compounds were obtained from web based databases ChemSpider (www.chemspider.com) and PubChem (www.pubchem.net).

4.9.2 Chemical space analysis

The SMILES codes were exported to ChemGPS-NP (Larsson *et al.*, 2007; Rosén *et al.*, 2009) which is freely available online: http://www.chemgps.bmc.uu.se/. This platform takes into account size, shape, lipophilicity, polarity, polarizability, flexibility, rigidity and hydrogen bonding capacity in forming an eight-dimensional chemical space. ChemGPS-NP space map is constructed of t-scores from principal component analysis (PCA) (Larsson *et al.*, 2007). In paper IV the four first principal components (PC1−PC4) were used because they are the most significant PCs and explain 77% of the variance. PC1 consist of descriptors for size, shape and polarizability, PC2 for aromatic- and conjugation-related properties, PC3 for lipophilicity, polarity and H-bond capacity and PC4 describes flexibility and rigidity.

The target library, Screen-Well® natural product libarary version 7.1 Cat. No. BML-2865-0500 (Enzo Life Sciences, Inc., Enzo Biochem, Inc., Farmingdale, New York), that was screened against the 19 antichlamydial reference compounds consisted of 502 compounds covering alkaloids, coumarins, flavones, isoflavones, macrolides, peptolides, terpenoids and synthetic derivatives of previous compound groups. The chemical space analysis was made from 2D descriptors (total of 35) that describe physical-chemical properties of the compounds and are calculated from canonical SMILES. All salts, hydration information, and counter-ions were excluded from the SMILES annotation, and differences in

stereochemistry ignored, since ChemGPS-NP only uses 2D descriptors. Mapping of the chemical space was done in a two-dimensional or a tri-dimensional space, using Grapher for Mac (version 10.4). For the *in vitro* assays the pure compounds that were in the library as 2 mg/ml in dimethylsulfoxide (DMSO) stock solutions were diluted in cell culture medium and used at 50 μM final concentration in the well.

4.9.3 Ligand-based virtual screening

Euclidean distances (EDs) were computed between the reference compounds (19) and the natural products library compounds (502). EDs were calculated between points P = (p1, p2,...,p4) and Q = (q1, q2,...,q4) in Euclidean 4-dimensional space provided by the ChemGPS-NP coordinates:

$$ED = \sqrt{(p_1 - q_1)^2 + (p_2 - q_2)^2 + (p_3 - q_3)^2 + (p_4 - q_4)^2}$$

For graphical visualization of EDs a heat map was constructed using Gnuplot (version 4.5). The virtual hits were selected by choosing the three closest compounds to the reference set of compounds. Compounds that appeared repeatedly were chosen only once. One already known antichlamydial compound, betulin (Salin *et al.*, 2010), was excluded.

4.10 Statistical analyses

Data is expressed as the ± SEM, and analyses are performed by either the Student's t-test or ANOVA using SPSS Statistics 21.0 software. *P*-values below 0.05 are regarded as statistically significant and indicated in the figures with a sign *. Accordingly, *P*-values below 0.01 are indicated with ** and *P*-values below 0.001 with *** (I–IV). SPSS Statistics 21.0 was used to obtain the best curve fit for constructing the dose–response curves for the estimations of IC_{50} values.

5. RESULTS

5.1 Inhibition of the growth of *Chlamydia* by the studied extracts and compounds (I–III)

Seven commercial peppermint tea water extracts inhibited the growth of *Chlamydia pneumoniae* clinical respiratory isolate K7 at 250.0 µg/ml (n = 3) (I). The tea samples inhibited the chlamydial growth in the range of 20.7% to 69.5%.

The aqueous methanol extract of *Schisandra chinensis* berries was administered to the *Chlamydia* infected host cells and the respiratory pathogen cultures (II). The final concentrations of the extract were 10, 25, 50, 100 and 250 µg/ml in each experiment. The extract inhibited all the chlamydial species and strains in a dose-dependent manner. The MIC values against *C. pneumoniae* strains CV6 and K7 were < 100 µg/ml, whereas *C. trachomatis* was less susceptible with the MIC value exceeding 250 µg/ml.

There were remarkable differences in the antichlamydial activities of *Schisandra* lignans (Table 5.). However, there is no consistent pattern in functionalities that could contribute to the increased activity. In all of the experiments schisandrin B inhibited chlamydial growth remarkably more than schisandrin with an over two-fold lower IC_{50} value against *C. pneumoniae*. Schisandrin had virtually no activity on the growth of *C. trachomatis*. All of the six *Schisandra* lignans inhibited the growth of *C. pneumoniae* and *C. trachomatis* in a dose-dependent manner with three of them having a MIC value of 50 µM against *C. pneumoniae*, and one of them, schisandrin C, a MIC value of 25 µM.

Table 5. Minimum inhibitory concentration (MIC) µM, Inhibitory concentration 50% (IC_{50}) µM and host cell viabilities (%) of studied extract and compounds (II–IV).

Studied extract/compound	**MIC µg/ml (the extract), µM (pure compounds)**		**IC_{50}), µg/ml (the extract), µM (pure compounds)**						**Host cell viability %**
	C.pne.K7	C.trac.L2	C.pne.CV6	C.pne.K7	C.trac.L2	K.pne.	S.pne.	S.pyo.	
Schisandrin	>50.0	-	>50.0	52.7 ± 10.3	>50.0	>50.0	>50.0	>50.0	119.0 ± 4.9
Schisandrin A	50 .0	100.0	nd	6.1 ±3.8*	nd	nd	nd	nd	115.3 ± 3.6
Schisandrin B	50.0	100.0	12.2 ± 4.9	5.4 ± 1.3	25.8	>50.0	>50.0	>50.0	102.5 ± 5.8
Schisandrin C	25.0	50.0	nd	5.0 ± 12.3*	nd	nd	nd	nd	98.0 ± 7.3
Schisandrol B	>50.0	-	nd	6.0 ± 8.0*	nd	nd	nd	nd	122.0 ± 3.0
Schisantherin A	50.0	100.0	nd	25.2 ± 2.7*	nd	nd	nd	nd	97.0 ± 8.6
Extract from *Schisandra chinensis*	>250.0*	nd	54.2 ±12.5	36.4 ± 12.4	>250.0	>250.0	>250.0	>250.0	9.3 ± 7.1
Catharanthine	>100.0*	nd	nd	52.6 ± 16.1	nd	nd	nd	nd	88.9 ± 2.8
Jervine	100.0*	nd	nd	25.6 ± 15.1	nd	nd	nd	nd	74.8 ± 5.4
Manool	100.0*	nd	nd	35.5 ± 12.9	nd	nd	nd	nd	88.6 ± 3.3
Mycophenolic acid	1.0*	nd	nd	0.31 ± 0.1	nd	nd	nd	nd	86.6 ± 2.1
Peganole	50.0*	nd	nd	35.1 ± 20.3	nd	nd	nd	nd	95.2 ± 4.1
6-Acetoamido-6-deoxycastano-spermine	>100.0*	nd	nd	67.2 ± 10.4	nd	nd	nd	nd	86.6 ± 2.0

* = unpublished data; nd = not determined

MIC, IC_{50} and host cell viability values are given with ± standard error of the mean (SEM). None of the extracts or the compounds had statistically significant reduction of the host cell viability in comparison to non-treated cells. C.pne.CV6 = *Chlamydia pneumoniae* strain CV6, C.pne.K7 = *Chlamydia pneumoniae* strain K7, C.trac.L2 = *Chlamydia trachomatis* strain L2, K.pne. = *Klebsiella pneumoniae*, S.pne. = *Streptococcus pneumoniae*, S.pyo. = *Streptococcus pyogenes*.

5.2 Target phases of infection

5.2.1 Effect on formation of infectious progeny (II–III)

The impact on production of infectious progeny was studied with the aqueous methanol extract of *Schisandra chinensis* berries and the lignan pure compounds. Assaying the extract at 10–250 µg/ml concentrations, there was no statistically significant difference between the dose responses of *Chlamydia pneumoniae* strains K7 and CV6 either at the first or second passage, indicating that the extract inhibited the formation of the second progeny at the same intensity as the first passage at 250 µg/ml concentration (Figure 4.). Inhibition of the second passage was statistically significant at the assayed concentrations of ≥ 50 µg/ml, $P < 0.01$–0.001, with both *C. pneumoniae* strains. At the first passage the inhibition ranged from 0.54% to 98.7% for K7 and from 18.4% to 100.0% for CV6. The corresponding figures at the second passage were 26.9% to 100.0% and -12.7% to 100.0%.

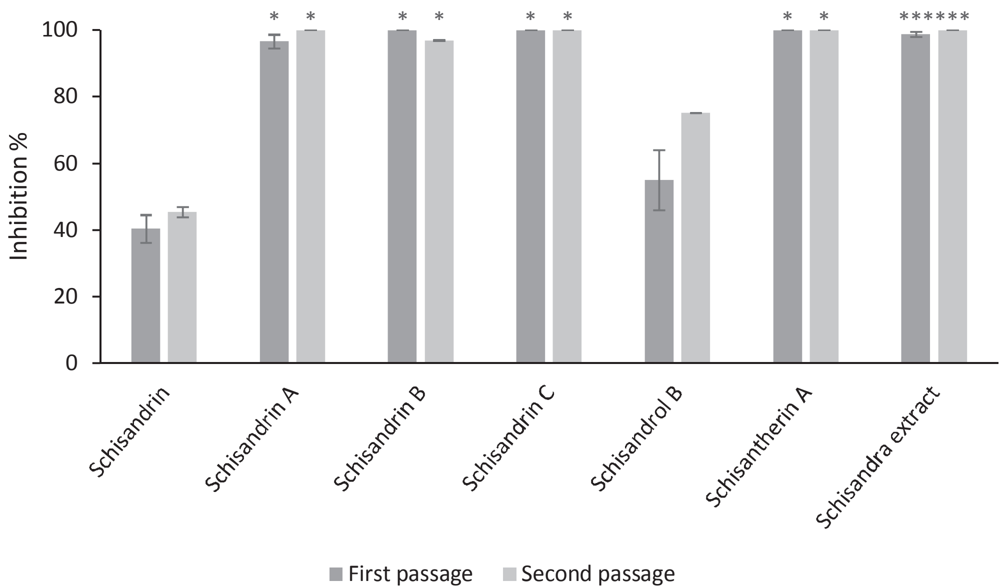


Figure 4. *Chlamydia pneumoniae* clinical isolate K7 inhibition with the extract at 250 µg/ml concentration and the *Schisandra* lignans at 50 µM concentration. Inhibition percentages are calculated comparing to the infected non-treated controls. Inhibition of the first passage was measured after 72 h p.i. (acute infection) and the second passage after two acute infection cycles (72 + 72 h). All data are means ±SEM (n=4), *(* $P < 0.05$, *** $P < 0.001$).*

The six lignan pure compounds were assayed on *C. pneumoniae* infectious progeny production (Figure 4). Consistent with previous growth inhibition experiments, the inhibition at the first passage at 25 and 50 µM were lowest with schisandrin (26.3% at highest) and highest with schisandrin C (99.5%, 25 µM), schisandrin B, schisandrin C and schisantherin A (100.0%, 50 µM), each. The same tendency was seen at the infectious progeny production as the inhibition percentages were again lowest with schisandrin (45.6% at highest) and highest with schisandrin C (98.3%, 25 µM) and schisandrin A, schisandrin C and schisantherin A (100.0%, 50 µM), respectively. Schisandrin C showed the highest inhibition percentages at both concentrations and both passages.

5.2.2 Delayed administration of compounds (III)

Schisandrin and schisandrin B at a concentration of 50 µM were administered to *Chlamydia pneumoniae* infected cell cultures 2, 8 and 24 h after the initiation of the infection (Figures 5. and 6.). Delaying the administration of schisandrin B and schisandrin to 8 h did not affect the lignans' ability to inhibit *C. pneumoniae* inclusion formation, and inhibitory activity of schisandrin B remained over 20% also upon administration of the lignans at 24 h post infection compared to the uninfected control.

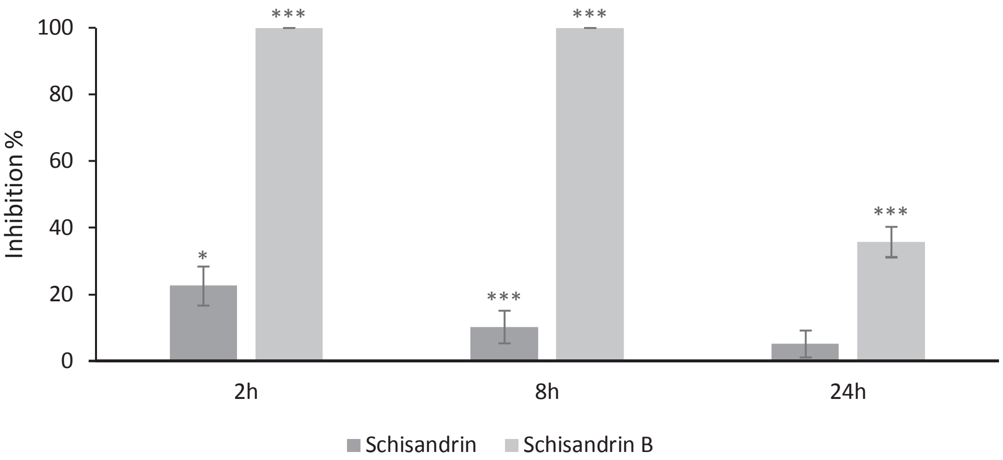


Figure 5. The impact of delayed administration of schisandrin and schisandrin B on *C. pneumoniae* K7 inclusion counts. The lignans were administered at 50 µM concentration at either 2 h, 8 h or 24 h post infection. Data represents mean ± SEM, n = 12 (* $P < 0.05$, *** $P < 0.001$).

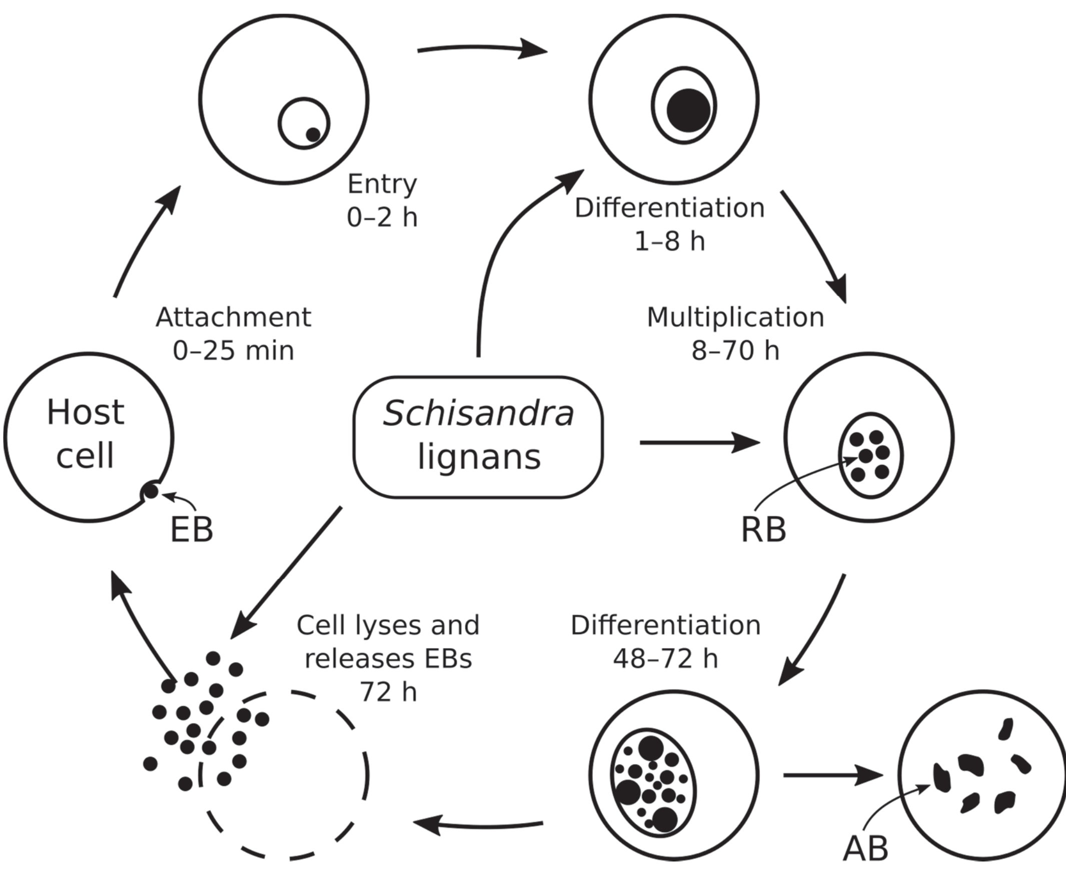


Figure 6. *Schisandra* lignans inhibit the growth of *Chlamydia pneumoniae* at different phases of the chlamydial life-cycle.

5.2.3 Pretreatment of elementary bodies (III)

To study the effect of the extract and the lignans on *Chlamydia pneumoniae* extracellular form (elementary body, EB) and its ability to infect host cells, a pretreatment of EBs was carried out. *Schisandra* extract at 250 µg/ml concentration inhibited infectivity by 94.6% (significant, data not shown). The impact of the extract was greater than that of the positive controls', 10 µM dithiothreitol (DTT), which inhibited infectivity by 84.0%. When the chlamydial EB particles were pretreated with the six lignans at 50 µM the chlamydial infectivity inhibition ranged between 11.2% (schisandrin) and 55.2% (schisandrin B) (Figures 6. and 7.).

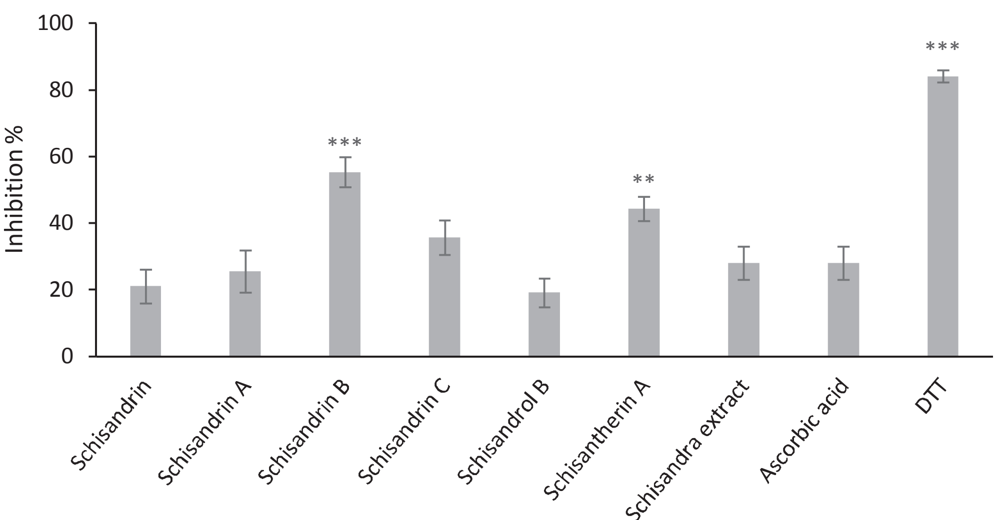


Figure 7. To evaluate direct effects of the 250 µg/ml extract, 50 µM lignans, 250 µM ascorbic acid and 10 mM dithiothreitol (DTT) on chlamydial elementary bodies, *C. pneumoniae* K7 stocks were diluted in the infection medium to yield a 400 000 IFU/ml suspension. The IFUs as elementary bodies in the medium were pretreated at +4 °C with the samples for 1 h prior to the inoculation on HL cell monolayers. Data represents mean ± SEM, n=12. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

5.3 Role of cellular redox status modulation in the antichlamydial effects (III)

The impact of the lignans on changes in cellular basal reactive oxygen species (ROS) levels was determined. HL cells were loaded with a profluorescent ROS probe DCFH-DA and were exposed to each of the lignans at 50 and 25 µM concentrations. 25 µM schisandrin A and schisandrin B reduced the amount of intracellular ROS with 30.5% and 32.3% respectively ($P < 0.01$), when at 50 µM the change was not statistically significant. In the presence of 1 mM H_2O_2, an inductor of intracellular ROS levels, none of the lignans at 25 or 50 µM had an effect on the amount of intracellular ROS. Only 50 µM schisandrin B reduced the amount of ROS by 6.5% *(P* < 0.05, Figure 8.) in *Chlamydia pneumoniae* infected cells (MOI 1.0). The baseline ROS producing activity was not significantly increased upon *C. pneumoniae* infection compared to uninfected controls.

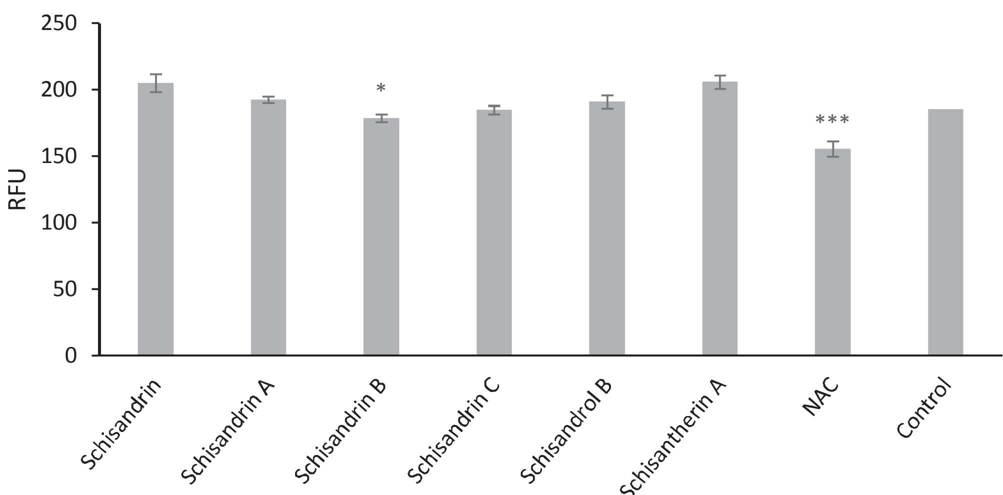


Figure 8. The impact of the *Schisandra* lignans on *C. pneumoniae* induced intracellular ROS levels. HL cells were loaded with the ROS probe DCFH-DA for 1 h, after which the cells were infected with C. pneumoniae K7 MOI 1 simultaneously with administration of 50 µM lignans and incubated for an additional hour before the fluorescent readout. Intracellular ROS levels were measured after 2 h post infection. 10 mM *N*-acetylcysteine (NAC) was used as a positive control. Data represent mean ± SEM, n = 4 (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

To detect the possible antioxidant effect of *Schisandra* lignans on *C. pneumoniae*, common reducing agents ascorbic acid and *N*-acetylcysteine (NAC) were assayed against *Chlamydia*. The reducing compounds were assayed alone and in combination with schisandrin and schisandrin B at concentrations generally known effective in diminishing oxidative stress (Figure 9.). Ascorbic acid, a mild and non-toxic reducing substance, as well as NAC, showed a remarkably weaker inhibitory action than the lignans alone or in combination with the lignans. Ascorbic acid at 250 µM concentration produced only 11.4% inhibition (not significant). NAC had a similar effect with 20.2% inhibition when administered alone at 10 mM concentration. Administering simultaneously with schisandrin and schisandrin B, neither ascorbic acid nor NAC affected the inhibitory activity the lignans. Inhibition of the growth of *Chlamydia* was highest with 50 µM schisandrin B.

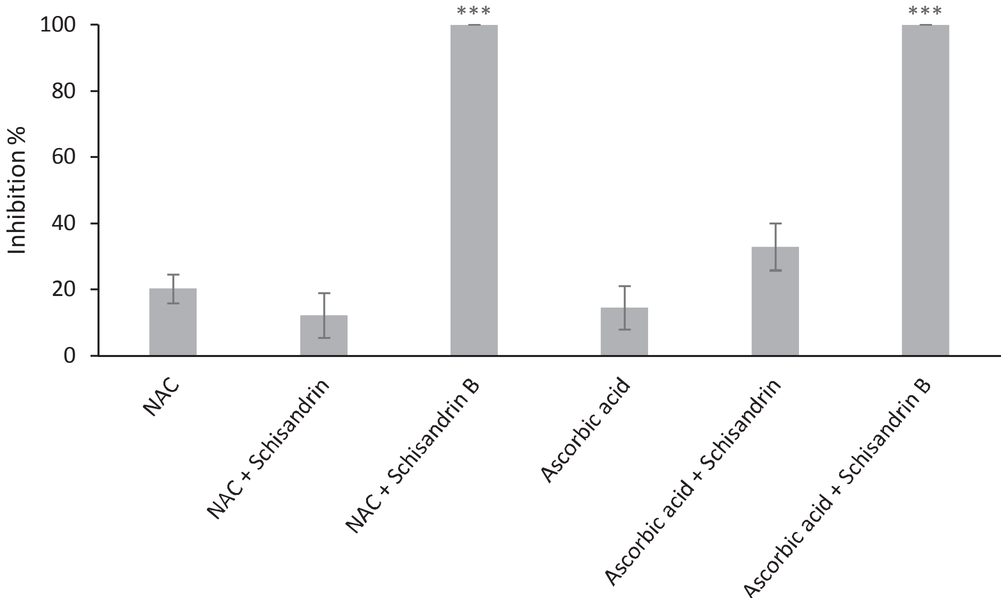


Figure 9. The growth inhibition of *C. pneumoniae* K7 as % to the infected control in the presence of 250 µM ascorbic acid, 10 mM *N*-acetylcysteine (NAC), schisandrin and schisandrin B. All the experiments are performed in three parallel replicates with 12 samples (N = 3, n = 12; mean ± SEM), (*** $P < 0.001$).

5.4 Effect on host cell viability (I–IV)

The host cell viability after treatment with the seven commercial peppermint herbal teas ranged from 82.4% to 99.4% after 72 h exposure (I). The effect of the *Schisandra* extract and the *Schisandra* lignans on logarithmically dividing cells and confluent cell cultures were assayed at a density of 5×10^4 and 6×10^5 cells per well, respectively (II). The viability of the 250 µg/ml *Schisandra* extract treated HL host cells was 90.3% for the confluent cells and 97.3% for the growing cells, with no statistically significant difference from the non-treated controls (Table 5.). Host cell viabilities of confluent cells (60 000 cells/well) ranged from 90.3% to 122.0% and from 72.7% to 97.4% with growing cells (5000 cells/well) when assayed with *Schisandra* lignans. The viability of either confluent or growing cells was not reduced by lignans, except schisandrin, which reduced the viability of the growing cells by 18.9% ($P < 0.01$).

5.5 Chlamydia-selectivity of studied extract and compounds (II–III)

To evaluate the sensitivity of the bacteria to the 250 µg/ml extract, the inhibition was measured on gram-positive *Streptococcus pneumoniae* and *S. pyogenes*, as well as gram-negative *Klebsiella pneumoniae*. These pathogens typically cause respiratory tract diseases, including pneumonia. None of the lignans or the extract inhibited the growth of bacteria other than Chlamydiae (Table 5.). Growth inhibition of seven other metabolically active bacterial species was investigated by turbidity measurements and resazurin staining. To examine the *Chlamydia*-selectivity of lignans the bacteria were assayed at 50 µM of the lignan against both common gram-negative (*Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*) and gram-positive (*Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermis*) bacteria. *Schisandra* lignans did not show growth inhibition of these bacteria (Table 5.).

5.6 Ligand-based *in silico* screen (IV)

The Enzo natural compound library of 502 compounds was screened against 19 antichlamydial reference compounds. The reference compounds were our research group's previous work and found from the literature (Alvesalo *et al*., 2006b; Salin *et al*., 2010; Hanski *et al*., 2014; original publication III). The reference compounds belonged to the chemical classes of lignans, flavones, isoflavones, flavonols, gallates, synthetic coumarins and synthetic flavonoids. Considering that phenotypic antichlamydial testing of a small fraction of the library takes at least 72 hours, and thus is not ideal of rational high throughput screening, the initial screening process was done *in silico* using ChemGPS-NP chemography tool (Figure 10., flow chart). The virtual hits were chosen according to their proximity to the reference set of compounds calculated as Euclidean distances (EDs) (Figure 11.). These virtual hits were selected from the natural product library using ChemGPS-NP, including three closest compounds to the reference antichlamydial compounds excluding overlapping compounds that already belonged to the references, as the same compounds that appeared repeatedly as closest compounds and one already known

antichlamydial compound (betulin) (Salin *et al*., 2010). *In silico* screening of the whole library resulted in 54 virtual hits that were chosen to be tested further *in vitro*. The selection process of virtual hits for antichlamydial testing included also *in vitro* cytotoxicity testing to exclude toxic compounds (Figure 10.). Assayed at 50 µM concentration, 27 compounds were excluded as cytotoxic, and for 27 compounds the host cell viability was ≥ 75%. The selected non-cytotoxic virtual hits were assayed against *C. pneumoniae* clinical isolate K7 at 50 µM concentration. This yielded six identified lead compounds showing ≥ 50% *C. pneumoniae* growth inhibition: jervine, mycophenolic acid (MPA), manool, peganole, 6-acetamido-6-deoxy-castanospermine and catharanthine base (Figure 11.). These compounds belong to the following chemical groups: steroidal alkaloid, phenolic acid, diterpene, quinazole, indolizine alkaloid and terpene indole alkaloid, respectively.

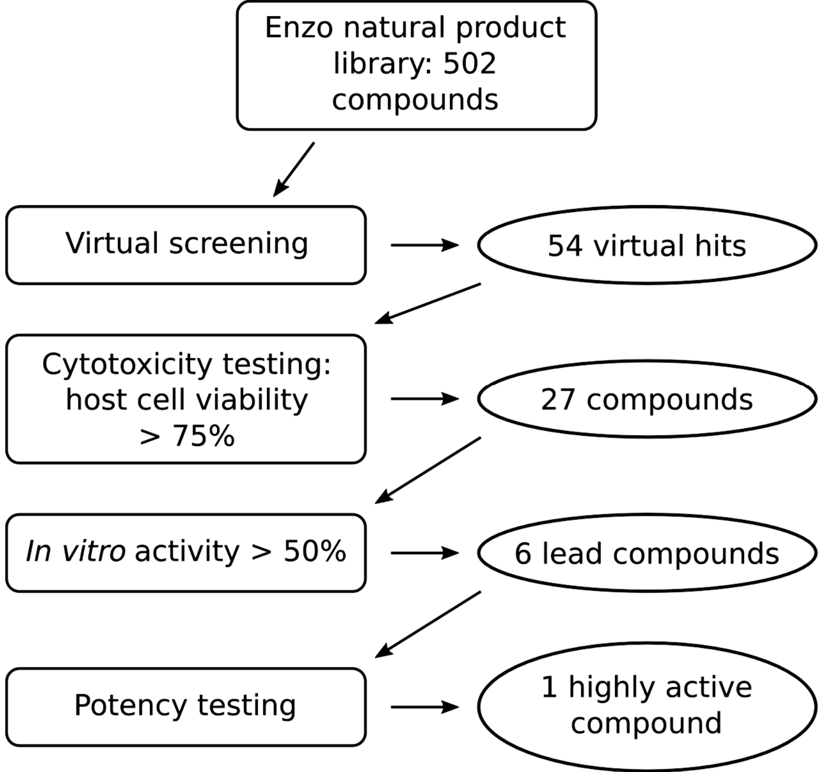


Figure 10. Screening process flowchart.

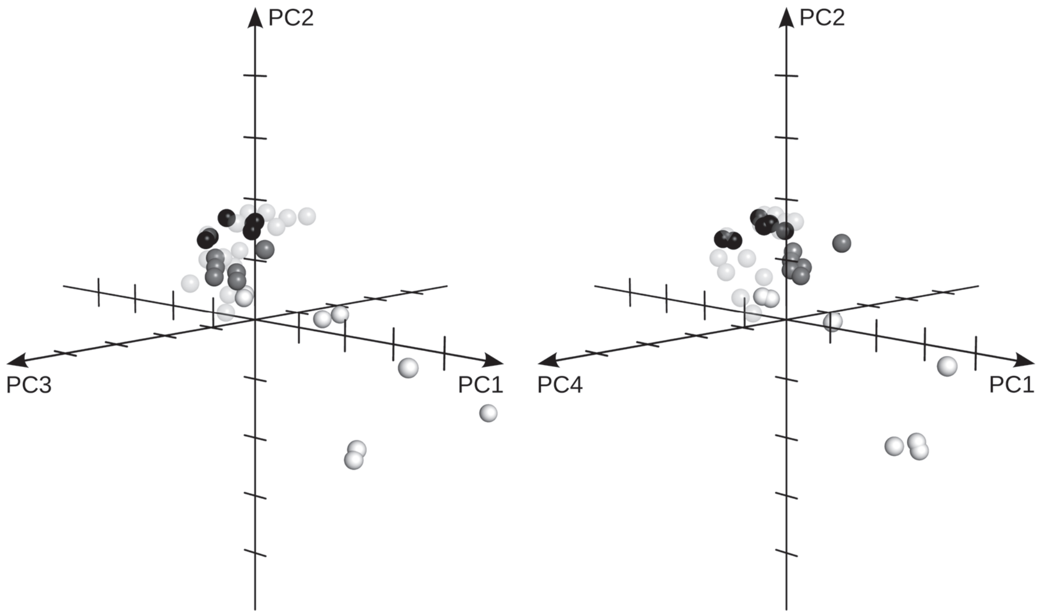


Figure 11. The chemical space analysis shows the regions populated by the six lead compounds (IV) (black dots), *Schisandra* lignans (grey dots) (III), reference compounds other than *Schisandra* lignans (faded dots) (IV) and eight commercial antibiotics which have activity against *Chlamydia pneumoniae*: azithromycin, ciprofloxacin, doxycycline, erythromycin, minocycline, ofloxacin, rifampicin and streptomycin (white dots). **A** PC1 (principal component 1) presents descriptors in size, shape and polarizability, PC2 describes aromatic- and conjugation-related properties and PC3 lipophilicity, polarity and H-bond capacity. **B** PC4 presents flexibility (and rigidity). In general, *Schisandra* lignans and the six lead compounds are smaller and slightly more aromatic than commercial antichlamydial drugs.

6. DISCUSSION

In this work, the principal source for finding antichlamydial hit and lead compounds was plant extracts. The commercial peppermint herbal teas were selected as representatives to the crude drug *Mentha* × *piperita* (L.) consisting of samples having both relatively high and low total polyphenol contents (I). The tea samples which had the highest antichlamydial activity were high in contents of luteolin and apigenin glycosides. These findings are in accordance with studies reported earlier in the literature, since these two flavonoids, luteolin and apigenin as aglycones have been shown to have high antichlamydial activity (Alvesalo *et al*., 2006b). Also another species of *Menthae*, *Mentha arvensis* (L.) has been shown to have antichlamydial activity (Salin *et al*., 2011b). The aqueous methanol extract from *Schisandra chinensis* inhibited the growth of *Chlamydia pneumoniae* and *Chlamydia trachomatis* in a dose-dependent manner (II). These results are in line with results obtained with the isolated pure compounds schisandrin and schisandrin B, which showed a notable inhibition of the two chlamydial species, with less inhibition activity on *C. trachomatis* than *C. pneumoniae*. These findings, however, do not exclude the possibility of other compounds in the extracts having antichlamydial potency.

In traditional Chinese medicine (TCM) — and overall in Far East ethnopharmacologically — the plant *Schisandra chinensis* (Turcz.) Baill. has commonly been used for hepato-, neuro- and cardioprotection (Panossian & Wikman, 2008). Although *Schisandra* lignans are widely studied there are no previous reports considering their antibacterial effects. However, some authors have reported on antiviral activities against HIV and some DNA viruses by the lignans (Chen *et al*., 1997; Chen *et al*., 2002; Chen *et al*., 2006; Yang *et al*., 2010; Song *et al*., 2013). A previously made high content screening (HCS) campaign has identified schisandrin B as an antichlamydial compound with activity against *C. trachomatis* (Hanski *et al*., unpublished). In the paper III it is shown that the investigated six lignans exhibit inhibition on chlamydial growth as decreased inclusion counts. This effect is dose-dependent and demonstrated with two distant, clinically important species of *Chlamydia*, *C. pneumoniae* and *C. trachomatis*.

The acute infection protocol (I–IV) mimics the natural acute *C. pneumoniae* infection, as the chlamydial EBs are brought to close proximity with the host cells and allowed to internalize for 2 hours. Duration of the acute infection is 72 hours, including the 2 hours infection period after which the cells are fixed and stained. Quantification of *Chlamydia* inclusions is done by a direct fluorescent procedure with a commercial fluorescein-conjugated monoclonal antibody. Methods based on immunofluorescence and monoclonal antibodies to detect *Chlamydia* in cell cultures have high specificity and reproducibility (Stamm *et al*., 1983). The infectivity of *Chlamydia* can be increased with a concentration of cycloheximide which depresses, but not completely inhibits, the metabolism of the eucaryotic host cells without affecting prokaryotic cells, like *Chlamydia* (Ripa & Mårdh, 1977). By this protocol the key antimicrobial parameters effect of a compound, such as IC_{50}, IC_{90} and MIC values, as well as the growth curves, can be determined. The protocol gives a basis for the antichlamydial experiments presented in this thesis.

The six *Schisandra* lignans inhibited the growth of *C. pneumoniae* and *C. trachomatis* in a dose-dependent manner with MIC values of 50 µM against *C. pneumoniae*, and one of them, schisandrin C, a MIC value of 25 µM (III).

Altough the *Schisandra* lignans' antichlamydial activity is not outstanding compared to the current antibiotics in use, they present a new chemical group of compounds for antichlamydial treatment. All the new antichlamydial agents in early clinical development are only slight modifications to the existing antibiotics (Golparian *et al*., 2012; Gebremedhin, 2012; Georgopapadakou, 2014; Chotikhanatis *et al*., 2014; Kohlhoff *et al*., 2014; van Bambeke, 2015), and therefore susceptible to cross-resistance.

The acute infection cycle of *C. pneumoniae* is 72 hours, in *in vitro* experiments termed as first passage, following the infectious elementary bodies release from the host cells to infect new host cells allowing the cycle to begin again (second passage). *In vitro* this method is used in order to gain a value referred to as the MCC value (minimum chlamydiacidic value), and has been defined as the concentration that completely inhibited further development of inclusions (Hammerschlag *et al*., 1983; Kuo & Grayston, 1988). In other words, it is used to express the amount of infective progeny after 72 hours infection period (duration of the acute infection) following another 72 hours infection period.

The impact on production of infectious progeny was studied with the aqueous methanol extract of *Schisandra chinensis* berries and the lignan pure compounds (II, III). As there was no statistically significant difference between the dose responses of *C. pneumoniae* strains K7 and CV6 either at the first or second passage, it can be concluded that the extract inhibited the formation of the second progeny at the same intensity as the first passage.
With pure compounds, the inhibition against *C. pneumoniae* at the first passage at 25 µM ranged from 26.3% (schisandrin) to 99.5%. At the infectious progeny production the same tendency was seen with inhibition percentages ranging from 45.6% (schisandrin) and 98.3% (schisandrin C).
It is possible that a compound inhibits the growth of *Chlamydia* at the first passage, but the *Chlamydia* recovers in the second passage of the infection. In an earlier study broad-spectrum cephalosporins and other lactam antibiotics were assayed against *C. trachomatis*, and it was seen that a higher concentration of antibiotics was needed for preventing the formation of a second passage (Hammerschlag *et al*., 1983). In the results presented here this is not seen. Rather the opposite findings describing this phenomenon can be observed, as the overall inhibition rates are slightly higher at the second passage.
Administration of the compounds at different time points (for example 2, 6, 12 and 24 hours p.i., post infection) gives information on the strength of the antichlamydial effect at different phases of the infection.
The results presented herein demonstrate that *Schisandra* lignans' antichlamydial activity is targeted to the chlamydial growth early/mid-cycle, 2−18 hours p.i. This phase initiates the rapid logarithmic binary division (Wolf *et al*., 2000). During the first two hours the EBs internalize into the host cell. After internalization they remain within individual, tightly membrane-bound vesicles. By 8 h p.i., differentiation into RBs is evidenced, although some EBs containing a condensed nucleoid may still be present. At 12 h p.i., the morphologically typical RBs are observed, and by 19 h p.i., multiplication is in full speed. At 24 to 36 h p.i. the RBs continue to multiply within the inclusion and no EBs are yet detected. The inhibitory effect of schisandrin and schisandrin B dramatically drops when they are administered at 24 h p.i. The next notable change in the developmental cycle is by 48 h p.i., as the RBs start to differentiate back into EBs. The division at this phase is asynchronous,

since although EBs and intermediate developmental forms are detected, some typical RBs are still in the process of binary fission. By 60 and 72 h p.i. there is an increasing percentage of EBs ready for exocytosis to release for subsequent rounds of infection. The *Schisandra* lignans' effect is targeted to the early/mid-phase of the infection, which marks the conversion of EBs into RBs. However, the inhibitory effect is maintained when the lignans are administered 8 h p.i., a time point in which majority of the EBs have differentiated into RBs (Wolf *et al*., 2000).

Besides the inhibitory activity of the studied extracts and compounds on the intracellular forms of *Chlamydia*, it is essential to know their effect on the outer cellular EBs. Since these results demonstrate that the inhibitory effect of the *Schisandra* lignans on *C. pneumoniae* EB pretreatment is significant, it can be concluded that these lignans have inhibiting effect on the EBs which is not dependent on the host cell. However, as the inhibition is remarkably higher during the infection, the effect is not specifically targeted to the EBs residing outside the host cell. The *Chlamydia* inhibiting effect of quercetin has been shown earlier (Törmäkangas *et al*., 2005; Alvesalo *et al*., 2006b), but the effect on the extracellular infectious elementary bodies (EBs) has not been studied before. In the publication III it was shown that quercetin reduces the infectivity by 79%. During the infection 50 µM quercetin was shown to inhibit the growth of *C. pneumoniae* by 90% (Alvesalo *et al*., 2006b).

It has been shown that reducing compounds, such as DTT, are able to disturb the assembly of MOMP and other disulfide-bridged protein in *Chlamydia* (Jones 1983; Hackstadt *et al*., 1985). As the studied lignans are known for their antioxidant activity (See chapter 2.3.1), this might explain why some of these lignans show partial inhibition when the EBs are pretreated with them. In addition, a common reducing agent, ascorbic acid decreased the infectivity by 27.9% (not statistically significant). Inhibitory effect upon the acute infection model, on the other hand, might depend on intracellular mechanisms.

Schisandra lignans have been widely studied for their antioxidant and pro-oxidant properties (Ip & Ko, 1996; Chiu *et al*., 2006; Chiu *et al*., 2009; Chiu *et al*., 2011; Checker *et al*., 2012; Zeng *et al*., 2012). Since *C. pneumoniae* infection is known to induce an increase in host cell ROS levels and also the extracellular EB form of the bacterium is

known to be sensitive to reducing agents (Kim *et al.*, 2009; Di Pietro *et al.*, 2013), the relevance of these aspects in the antichlamydial effect of the lignans was studied.

In this study it was observed that 25 µM schisandrin A and schisandrin B reduced the amount of basal intracellular ROS levels by 30.5% and 32.3% respectively. At 50 µM concentration schisandrin B did not have this effect, which was also smaller with schisandrin A at this concentration. In a study the cellular basal levels of ROS were elevated by schisandrin B in lymphocytes (Checker *et al.*, 2012). The differing results might be due to a different cell lines used in these studies. During the *Chlamydia pneumoniae* infection (MOI 1.0) only 50 µM schisandrin B modestly reduced the amount of ROS (6.5%, $P < 0.05$). However, the ROS reducing activity of schisandrin B was higher when the basal ROS levels were measured. These results suggest that the antichlamydial effect of *Schisandra* lignans is not due to interference with the *C. pneumoniae*-induced ROS production, nor solely the antioxidant effect. Also the time course of the infection-induced increase in ROS levels speaks against the ROS-related mechanistic effect of *Schisandra* lignans. The ROS levels are normally elevated 1 hour after the first contact between *Chlamydia* and the host cell, whereas administration of schisandrin B after 8 hours p.i. still shows efficient inhibition of the infection. Schisandrin B has been shown to inhibit the production of ROS in microglia (Zeng *et al.*, 2012). In this study by Zeng *et al.* NADPH oxidase-dependent superoxide O_2 and ROS formation were LPS induced. This result, however, is not directly comparable to the results gained in this study, because in addition to different cell lines used in these studies, Zeng *et al.* used a smaller concentration of schisandrin B (5−20 µM). Moreover, the ROS production in microglia was induced with LPS produced by *Escherichia coli.* Chlamydial LPS is shown to be immunogenically different from other bacterial LPS, in terms of evoking a weaker immune response (Kalayoglu, *et al.*, 2000).

The common reducing agents ascorbic acid (vitamin C) and N-acetylcysteine (NAC) were assayed against *C. pneumoniae* alone and in combination with schisandrin and schisandrin B. The reducing agents showed a remarkably weaker inhibitory action than the *Schisandra* lignans and did not have any synergistic effect when administered with the lignans.

Chlamydia has intracellular redox dependent mechanisms that are specific for its own metabolism. *C. pneumoniae* is shown to induce monocyte oxidation of LDL in a dose-

dependent manner (Kalayoglu, 1999). LDL oxidation was enhanced with longer incubation periods and with increasing concentrations of LDL, whereas it was inhibited by the antioxidant α-tocopherol (vitamin E). Since vitamin E inhibits *C. pneumoniae*-induced macrophage LDL oxidation, one might assume that also the modest inhibitory action of vitamin C presented herein stems from the general antioxidative activity of these vitamins.
As *Schisandra* lignans have various effects on human cells, and some of them are manipulated by *C. pneumoniae*, there are several possible mechanisms of action, which might contribute to the antichlamydial action of *Schisandra* lignans. According to the literature reviewed in this thesis it seems that the common things between *Schisandra* lignans' and *Chlamydia*'s effects are, to a large extent, in the host cell's immunity system (Table 2.).
Apoptosis is yet another interesting potential target of the Schisandra lignans' mode of action. The mammalian cell apoptosis is regulated by pro-apoptotic and anti-apoptotic signals. Several members of the species *Chlamydia*, including *Chlamydia pneumoniae* have been shown to inhibit the pro-apoptotic signaling molecules, such as Bcl-2 family of proteins, cytochrome c and caspase-3 pathway (Airenne et al., 2002; Fischer et al., 2004). The *C. pneumoniae* infected host cells were human laryngeal carcinoma cell line Hep2 (Fischer et al., 2004). In contrast, schisandrin B has been shown to induce apoptosis in human lung adenocarcinoma A549 cells, by inducing pro-apoptotic components Bax, cytochrome C and caspase-3 and -9, but decreasing Bcl-2 and proliferating cell nuclear antigen (PCNA) expression (Lv et al., 2015). However, the net effect of schisandrin B was an increase of apoptosis as the number of cells died by apoptosis. In this study the apoptosis mediated by schisandrin B was through mitochondrial pathway. By coincidence, *Chlamydia*-inhibited apoptosis was blocked upstream of the mitochondrial activation of Bax/Bak (Fischer et al., 2004). These results, however, depend largely on the cell line in question. For example *C. pneumoniae* has also been shown to induce apoptosis in human coronary artery endothelial cells (Schöier et al., 2006).
Chlamydia pneumoniae is found to upregulate and downregulate host cellular genes and proteins in HL cells (Alvesalo *et al*., 2008; Savijoki *et al*., 2008). Some of these effects are contrary to the effects of *Schisandra* lignan's. Some of the heat shock proteins (HSPs)

function as molecular chaperones. *C. pneumoniae* is shown to downregulate genes that are responsible for chaperone regulator activity in HL cells (Alvesalo *et al*., 2008). *Schisandra* lignans in turn are shown to upregulate heat shock proteins Hsp25, Hsp70 and Hsp72 (Chiu & Ko, 2004; Wu *et al*., 2004 (Table 2.). *C. pneumoniae* downregulates these genes 12 hours post infection, which is a time point where *Schisandra* lignans display antichlamydial activity according to the results presented herein.

Another important transmitters which are affected by *Schisandra* lignans and also important for *Chlamydia*, are calcium ions. In cultured hippocampal neurons schisandrin A (deoxyschisandrin) inhibited oscillations of intracellular Ca^{2}+ by decreasing the influx of extracellular calcium and the initiation of action potential (Fu *et al*., 2008). *C. pneumoniae* downregulated genes that code calcium channel regulatory activity in HL cells (Alvesalo *et al*., 2008). This effect was measured at 24 hours post infection, which is a time point where schisandrin B still showed over 20% statistically significant inhibitory activity on the growth of *C. pneumoniae.* A study determined the effects of modules associated with various cellular functions which were activated during *C. pneumoniae* entry phase, including calcium transport (Wang *et al*., 2010). The authors concluded the calcium transport to be one of the key components for successful *C. pneumoniae* entry into the host cell. Concidering the relevance of calcium as a target for antichlamydial action of a compound, it should be taken into account that calcium is a very common signaling transmitter present nearly everywhere in the human body. It is present in intracellular and extracellular space and its action is regulated by different types of calcium channels, as well as by the genes that code the activity of these receptors.

It must be kept in mind that the antichlamydial results presented in this thesis are gained in the presence of cycloheximide, which is a compound that inhibits mammalian protein synthesis. Therefore it is unlikely that the mechanism of action of the presented antichlamydial compounds is in mammalian protein synthesis. The results suggest rather that the principal mechanism of action of *Schisandra* lignans is in the host cell activated reticulate bodies. However, the possibility of a host cellular target can not be excluded, since many cellular events do not require protein synthesis. For example phosphorylation of certain kinases which are upregulated or downregulated by *Schisandra* lignans or

C. pneumoniae, often with opposing effects (Table 2., pages 40–41). These kinases, among other factors, could be the targets of the antichlamydial mechanism of action of *Schisandra* lignans.

The studied *Schisandra* extract and the lignans did not have an effect on the viability of logarithmically dividing cells and confluent cells. The only exception was schisandrin, which reduced the viability of the growing cells by 18.9% ($P < 0.01$).

These results are in line with the literature, as most of the lignans did not affect the host cell viabilities at the studied concentrations. Toxicology of these lignans has been studied in *in vitro* cell models and *in vivo* animal studies and considered non-toxic with rats (schisandrin B 30 mg/kg/day × 2, intraperitoneally), mice (schisandrin B 3 mmol/kg/day × 3, orally, schisandrin 100 mg/kg) and dogs (10 mg/kg daily for 4 weeks) (Chang *et al*., 1986; Ip & Ko., 1996; Lee *et al*., 2012). Several clinical studies made in the former USSR suggest the herbal products of *Schisandra chinensis* to be well tolerated by healthy volunteers (reviewed in Hancke *et al*., 1999 and Panossian & Wikman, 2008). In these studies, schisandrin was not found to induce adverse effects in healthy humans. Schisandrin was administered to a total of 153 healthy human subjects in doses of 3.6 mg (n = 17), 5 mg (n = 44), 10 mg (n = 47) or 20 mg (n = 45), and only four subjects experienced excitation and three complained of depression. No drug dependency or significant adverse effects were reported in a study of 1200 patients with 1162 individuals who had been taking infusions of *Schisandra chinensis* leaves regularly for 8 months. Only 38 patients (3.3%) had ceased taking the infusions because of excitation of the central nervous systems. In another study, no adverse effects were detected in 415 patients taking *Schisandra chinensis* seed extract for 1 month during influenza epidemic. The problem of validating the adverse events or toxicity within the clinical studies made with *Schisandra chinensis* herbal products is that they are not standardized for their content of active lignans and that many of the assays do not include determination of the lignans present.

The *Schisandra* extract did not affect the growth of gram-positive *Streptococcus pneumoniae* and *S. pyogenes*, nor the growth of gram-negative *Klebsiella pneumoniae*. The three latter pathogens typically cause respiratory tract diseases, including pneumonia. *Schisandra* lignans didn’t show growth inhibition of seven other metabolically active gram-

negative bacterial species *Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa*, *Proteus mirabilis*, nor of the gram-positive bacteria, *Bacillus subtilis, Staphylococcus aureus* and *S. epidermidis* at 50 µM concentrations. Although very useful, broad-spectrum antibiotics are definitely not completely without problems. The overuse of them has led to a global problem of antibiotic resistance (Gootz, 2010). Also the use of them has a significant damaging impact on the human gut microbiota (Rea *et al*., 2011). It is suggested that the therapeutic use in the treatment of *Clostridium difficile* infection in the gastrointestinal tract may even be compromised by their broad spectrum of activity. The use of more narrow-spectrum antibiotics could be favored to minimize the collateral damage to the normal microbiota. In addition, narrow-spectrum antibiotics could be one key solution to the resistance problem, since they only target a specific pathogen (Lewis, 2013). As *C. pneumoniae* alone would most likely be too narrow as a sole indication for an antibiotic, the indication could possibly be broadened to atypical pneumonia (Hanski & Vuorela, 2014). This would, however, require the efficacy to be shown also against other atypical respiratory pathogens, such as *Legionella pneumophila* and *Mycoplasma pneumoniae*.

The *Schisandra* lignans identified in this study as highly active antichlamydial compounds were further utilized for building a reference set of antichlamydial compounds for virtual screening (VS). The screening process made with ChemGPS-NP resulted in 6 lead compounds with ≥ 50% *Chlamydia pneumoniae* growth inhibition. The compounds are new antichlamydial compounds belonging to different chemical groups than the reference compounds.

In 2004 Lipinski and Hopkins described the challenge of drug discovery in the chemical space to be in identifying those regions that are likely to contain biologically active compounds, the biologically relevant chemical space (Lipinski & Hopkins, 2004). In the model presented in paper IV, the close proximity of compounds to the references predicted very well the biological activity as chlamydial growth inhibition, as the lead compounds showed good antichlamydial activity. Four of the six lead molecules, jervine, mycophenolic acid (MPA), manool, and peganole gave close to full inhibition at 100 µM concentration and three of them at 50 µM concentration, namely jervine, MPA and peganole. The most active compound was MPA with an IC_{50} value of 0.31 ± 0.1 µM. In Table 6. there are listed

different lead mining strategies for *C. pneumoniae* antichlamydial drug discovery. A synthesized betulin derivative, betulin dioxime, had an IC_{50} value of 0.29 µM against strain CWL-029, defined by the same *in vitro* method that was used in the work presented in this thesis (Salin *et al*., 2011a). The effect is thus comparable with that of MPA, which also had an IC_{50} value in nanomolar range. MPA was tested against a clinical isolate K7, which brings the result slightly closer to the clinical situation. Betulin dioxime had a MIC value of 1 µM against strain CWL-029, which is exactly the same as that of MPA's MIC value against the strain K7. Betulin dioxime was also assayed against a clinical isolate CV6 and had a MIC value of 2.2 µM. Comparing the lead properties, other than activity, betulin dioxime has a log*P* value of 4.9, which is high enough for the compound to have potential problems in solubility (Salin *et al*., 2010). As a rule of thumb, a compound with log*P* value higher than 2.5 is likely to cross blood brain barrier. This may result in central nervous system-related adverse effects. The log*P* value of MPA is 2.92 and it doesn't have violations of Lipinsky's rule of five. Five other antichlamydial compounds that have been found with different screening methods, 2-arylbenzimidazole 42 (Keurulainen *et al*., 2010), benzimidazole MB4 (Alvesalo *et al*., 2006a), biochanin A (Hanski *et al*., 2014), linarin (Salin *et al*., 2011b) and luteolin (Alvesalo *et al*., 2006b) were not as potent inhibitors of the growth of *C. pneumoniae* as MPA. The result of the ligand-based virtual screening (LBVS) combined with *in vitro* validation has proved this method to be an effective strategy for antichlamydial lead mining.

It is said that one key element in performing LBVS is novelty (Walters *et al*., 2003). This is because a similarity metric that is too uniform is prone to identify molecules that are close analogues of the known ligands used to perform the search. The reference set of compounds that was used to perform the search was relatively heterogeneous, as it consisted of compounds from different chemical groups, such as flavonoids and lignans, for example. This resulted in finding hit molecules that are functionally equivalent to the reference set, but chemically distinct, which brings novelty to the search. A target based virtual screening approach has been used before in *Chlamydia* research. In a study of finding antichlamydial compounds, a similarity based virtual screening approach has been used as a tool for targeted library design (Alvesalo *et al*., 2006a). In the ligand-based approach presented in

this study, the identified ligands occupy the same location in the Euclidean 4-dimensional chemical space, (Results, Figure 11., page 63). This privileged space can be further used in medicinal chemistry projects in order of finding new compounds with antichlamydial activity.

Table 6. Antichlamydial lead-mining strategies

Lead mining method	Compound	Chemical structure	IC_{50}/MIC/ inhibition%, strain	Publication
SAR-guided synthesis of ligands	2-arylbenzimidazole 42		6.3 μM (MIC), CV6	Keurulainen *et al.*, 2010
Target-based virtual screen with a small targeted library	Benzimidazole MB4		39.5% (inhibition) at 1 μM, K7	Alvesalo *et al.*, 2006a
Literature based screen	Betulin dioxime		1 μM (MIC), 0.29 μM (IC_{50}), CWL-029, 2.2 μM (MIC), CV6	Salin *et al.*, 2010
High-content screening	Biochanin A		12 μM (IC_{50}), K7	Hanski *et al.*, 2014
Analysis of natural phenols containing extract for active pure compounds	Linarin		100 μM (MIC), CWL-029	Salin *et al.*, 2011a
Screening of dietary phenolic compounds	Luteolin		8.8 (MIC) μM, K7	Alvesalo *et al.*, 2006b
Ligand-based virtual screening confirmed with *in vitro* -assays	Mycophenolic acid		1 μM (MIC), 310 nM (IC_{50}), K7	IV

7. Conclusions

In this work, with the *in vitro* assays on *Chlamydia pneumoniae* infection, new lead compounds were screened for drug discovery process. New biologically active extract and molecules were found to inhibit the growth of *C. pneumoniae*. These molecules showed varying activity on two different strains of *C. pneumoniae*, CV-6 and a clinical isolate K7. Especially promising were some of the dibenzocyclooctadiene lignans, *Schisandra* lignans, from the berries of *Schisandra chinensis* (Turcz.) Baill. These compounds were shown to act at the mid-cycle of the infection, 2−18 hours post infection, the phase which initiates the rapid binary division and exponential growth. The inhibitory effect of the *Schisandra* lignans on pretreated *C. pneumoniae* elementary bodies (EBs) was significant, indicating an effect on the EBs independent of the host cell. On the other hand, since the inhibition is remarkably higher during the infection than in the pretreatment, it can be concluded that the effect is more targeted to the intracellular forms of the bacteria rather than to the extra cellular EBs.

Since the *C. pneumoniae*-induced ROS levels were not affected by *Schisandra* lignans, the antichlamydial effect of these lignans is not likely to be due to the direct interference with the *C. pneumoniae*-induced ROS production. Moreover, the common reducing agents ascorbic acid or *N*-acetylcysteine (NAC) did not show remarkable inhibition of chlamydial growth, nor did they have any synergistic effect when administered together with the lignans. These results speak for the antichlamydial mechanism of the lignans to be specific to the host cell–*Chlamydia* interaction, rather than dependent on their antioxidative properties.

The *Schisandra* lignans showed also inhibitory activity on the sexually transmitted disease-causing pathogen, *Chlmaydia trachomatis*. However, the antichlamydial activity against *C. pneumoniae* was higher with all of the six lignans. In contrast, the *Schisandra* extract did not affect the growth of three common respiratory tract pathogens, and *Schisandra* lignans did not inhibit the growth of seven common metabolically active bacterial species. These results suggest a *Chlamydia*-selective action of the *Schisandra* lignans.

Most of the lignans did not decrease host cell viability at the studied concentrations. This is in line with literature, since these lignans are showed to be non-toxic in several *in vitro* and *in vivo* animal studies. Also several clinical studies suggest the herbal products of *Schisandra chinensis* to be well tolerated.

As the *Schisandra* lignans presented in this study are effective and selective growth inhibitors of *Chlamydia pneumoniae*, non-toxic to the human lung epithelial HL cells at 50 µM concentration, and most of them not violating the Lipinski's rule of five, these lignans present excellent lead candidates for further optimization.

The ligand-based virtual screening process with ChemGPS-NP yielded six non-toxic lead compounds, with a hit rate of 1.2%. These molecules belong to new antichlamydial chemotypes. The most active compound, mycophenolic acid, had an IC_{50} value in the nanomolar range. In the model presented here, the *in silico–in vitro*-correspondence was good, since the *in silico*-calculated proximity of compounds to the reference set predicted very well the chlamydial growth inhibition. With this screening process, a new chemical space for antichlamydial compounds was defined, which can be further utilized in future antichlamydial drug discovery projects.

8. References

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