<https://helda.helsinki.fi>

Recent advances in technologies for developing drugs against *Chlamydia pneumoniae*

Hanski, Leena

2014-07

Hanski , L & Vuorela , P M 2014 , ' Recent advances in technologies for developing drugs against *Chlamydia pneumoniae* ' , Expert opinion on drug discovery , vol. 9 , no. 7 , pp. 791-802 . <https://doi.org/10.1517/17460441.2014.915309>

<http://hdl.handle.net/10138/169907>

<https://doi.org/10.1517/17460441.2014.915309>

unspecified

submittedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

Please download and read the instructions before proceeding to the peer review

Recent advances in technologies for developing drugs against Chlamydia (Chlamydophila) pneumoniae

Journal:	<i>Expert Opinion On Drug Discovery</i>
Manuscript ID:	Draft
Manuscript Type:	Review
Keywords:	Chlamydia pneumoniae, drug discovery, gram-negative bacterium, persistence, respiratory pathogen, atypical pneumonia

SCHOLARONE™
Manuscripts

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Recent advances in technologies for developing drugs against
Chlamydia (Chlamydophila) pneumoniae

Abstract

The unique morphological characteristics, capacity of manipulating host cell function, and association with chronic inflammatory diseases represent the features of *Chlamydophila pneumoniae* (or *Chlamydia pneumoniae*) that have fascinated scientists and medical professionals for several decades. In this paper, we review the current status of attempts to discover and develop drugs against *C. pneumoniae*, including discovery of nonconventional antichlamydial agents, targeting chlamydial type 3 secretion system (T3SS), approved drug repositioning and combination therapies. In addition, recent advances in *C. pneumoniae*-related research and technologies that are likely to have a significant impact on identifying efficacious treatments against this pathogen are discussed. While recent advances in understanding *C. pneumoniae* biology are likely to affect the potential for identifying and validating therapeutic targets with both the bacterium and its host cells, focusing on phenotypic assays, careful evaluation of the physicochemical properties of the lead candidates and attempts towards more narrow-spectrum antibacterial agents are considered critical elements for successful lead generation.

1. Introduction

Since the isolation of *Chlamydia pneumoniae* from atherosclerotic arteries in 1990s [1], the role of this obligate intracellular bacterium as a risk factor for atherosclerosis has been a matter of intense debate and extensive research. After the publication of negative results from clinical studies evaluating antibiotics as a secondary preventive medication for atherosclerosis in early 2000s [2], some skepticism has emerged towards the connection between *C. pneumoniae* and cardiovascular diseases. However, the value of the clinical trials for proving or disproving the hypothesis is highly questionable due to the pathophysiologically late endpoints selected for the trials and the lack of complete eradication of *C. pneumoniae* by the used antibiotics [3]. After a transient decrease of interest in *C. pneumoniae* and other microbes in this context, microbial burden hypothesis has re-emerged among cardiovascular disease risk factors [4], and the previous failures in the area will hopefully guide us to more sophisticated approaches and study designs in this respect in the future.

While the association with atherosclerosis has received immense attention and been the subject of various human, animal and molecular level studies [5], atherosclerosis is not the only chronic inflammatory disease that has been linked with *C. pneumoniae*. As a respiratory tract pathogen, *C. pneumoniae* is responsible for 5-10% of community acquired pneumonia (CAP) cases, and it is also the causative agent of various other respiratory tract illnesses with varying severity [6]. Numerous studies have evaluated the role of persistent respiratory infection by *C. pneumoniae* as predisposing factor in asthma and chronic obstructive pulmonary disease [7, 8], and the

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

well-known ability of *Chlamydia* spp. bacteria to disseminate into body sites distant from the primary infection via circulating white blood cells has formed the basis of the role of these pathogens also in reactive arthritis [9]. Isolation of *C. pneumoniae* from brain areas affected in Alzheimer's disease in post mortem samples initiated another research line focusing on the causal relationships between the bacterium and the disease also in this respect [10]. More research is needed for achieving detailed understanding on the role of *C. pneumoniae* in the etiology and pathological course of these diseases. Being able to reduce the risk of any of such prevalent illnesses could significantly alleviate the public health burden, and the idea of achieving this by eliminating *C. pneumoniae* has fascinated scientists in different areas within medicine. On the other hand, finding an effective chlamydiocidal agent suitable for human use would enable re-evaluation of the unanswered questions concerning causal relationships between *C. pneumoniae* and the above mentioned diseases and is thus of extreme importance also for evaluating the hypothesis of microbial burden in chronic disease etiology. Collectively, these aspects form the rationale for efforts on discovering drug candidates capable of eradicating *C. pneumoniae* infections.

In this paper, we review the current status of attempt to discover and develop drugs against *C. pneumoniae* and describe recent advances in *C. pneumoniae* research and technologies that are likely to have a significant impact on identifying efficacious treatments against this pathogen. In the end of the article, we highlight the key technologies and approaches we consider critical for successful generation of viable drug candidates against *C. pneumoniae*.

2. Challenges related to *C. pneumoniae* as a drug discovery target

Chlamydia pneumoniae (alternatively called *Chlamydophila pneumoniae*, according to the current yet not widely accepted taxonomical classification [11]) is a small spherical bacterium, which is an obligate intracellular parasite. The multiplication of the bacterium can only occur inside eukaryotic cells, in a membranous organelle detached from the host cell's endocytic vesicle system upon the bacterium's entry into the cell. In Figure 1 the life cycle of *C. pneumoniae* is presented as a schematic illustration. As other gram-negative bacteria, the outermost structure of *C. pneumoniae* itself is a lipopolysaccharide (LPS) -rich outer membrane, which poses significant challenges for drug penetration. In general the outer membrane is the primary reason for inherent resistance of gram-negative bacteria towards various antibacterial agents, and in the case of *C. pneumoniae*, additional permeability barriers are formed by host cell plasma membrane and the inclusion membrane surrounding the bacterium in its replicating (RB) form. Any compound targeting proteins or other components within RBs must thus be able to penetrate all these membranes in order to reach its target site. One strategy to overcome such challenges posed to the physicochemical properties of antichlamydial compounds is to focus on targeting components not residing inside the inclusion membrane, such as host cell factors or chlamydial effector proteins attached to the inclusion membrane or secreted to the host cell cytoplasm.

While the extracellular, non-dividing form of the bacterium, referred as elementary body (EB), is rigid and osmotically stable, the intracellular

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

replicating form (reticulate body, RB) lacks the structural components required for such integrity. The dramatic changes in rigidity and osmostability between EB and RB reflect the unique structure of the bacterium's cell wall, which contains only very marginal amounts of peptidoglycan [12]. Instead, the EB form maintains its structure by disulfide bridged protein complexes, which are released as separate protein molecules upon EB differentiation into RB after entry to a host cell [13-15]. Within RB to EB maturation and exit of the newly formed bacteria from the host cell, disulfide bonds are re-formed. These features explain the resistance of *C. pneumoniae* and other *Chlamydia* spp. bacteria to antibiotics targeting bacterial cell wall biosynthesis, and in fact, penicillins are known to trigger the development of persistent infection rather than leading to effective eradication [16,17].

Propensity to persistence is the hallmark of chlamydial infections that forms the most obvious challenges in antichlamydial therapy. This viable but non-replicating state, known to be induced by a spectrum of environmental factors, is characterized by a specific intracellular form known as aberrant body (AB) (Figure 1). Chlamydial persistence, along with the factors triggering it and the consequences of this infection state have been extensively reviewed in the literature over the past decades [18-20]., and it is generally accepted that despite their low or non-existing multiplication rate, the bacteria residing in ABs maintain active metabolism and continue to manipulate their host cell by secreting effector proteins interfering with host structures and signaling pathways [21]. Considering antibacterial therapy, the persistent infection is not sensitive to antibiotics affecting bacterial replication machinery,

and treatment failures have been reported even after prolonged treatment with first choice antibiotics such as azithromycin and erythromycin [22].

Detailed characterization of the persistent infection, as well as other aspects of *C. pneumoniae* infection have been significantly limited by the fact that *C. pneumoniae* remains genetically intractable. Lack of success with standard bacterial genetics techniques has been attributed to the metabolically inert state of EBs and fragile nature of RBs, and to date, not a single laboratory generating mutant strains of *C. pneumoniae* has been reported. The absence of suitable tools for genetic modification has also confronted the studies on individual chlamydial proteins, making it very difficult to identify or validate potential drug targets within the bacterium. However, as discussed below, the recent advances in inserting *C. trachomatis* derived plasmid into *C. pneumoniae* [23] as well as producing mutant strains of related bacterial species shed some hope on overcoming these major limitations also with *C. pneumoniae* in the future.

C. pneumoniae has an established role as a causative agent in atypical pneumonia and other respiratory infections, but according to recent findings it may not be the only chlamydia-related pathogen associated with such a disease. Since the discovery of novel families *Parachlamydiaceae*, *Simkaniaceae* and *Waddliaceae* within the order *Chlamydiales* more than 10 years ago [24, 25], accumulating evidence has indicated that these chlamydia related bacteria possess a broad host range and may be associated with respiratory tract infections also in humans. PCR studies have reported the prevalences between 1 to 10% of these species in human respiratory samples [26, 27], and preliminary susceptibility studies have indicated that some of the

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

newly discovered species are not sensitive to quinolones which are often used for treat atypical pneumonia [28]. As soon as our understanding increases concerning the relevance of these species as causative agents of human infections, it may change also the requirements of an effective therapeutic agent aimed for the treatment of atypical respiratory pneumonia.

3. Existing antibiotic therapies

Current treatment strategies for chlamydial infections have recently been reviewed by Hammerschlag and Kohloff [29]. *C. pneumoniae* is generally considered susceptible to antibiotics interfering with prokaryotic DNA, RNA or protein synthesis, such as quinolones, tetracyclines and macrolides, but in contrast to *C. trachomatis*, not sensitive to trimethoprim or sulfonamides [29, 30]. Furthermore, aminoglycosides are not useful in treating *Chlamydia* infections since they fail to penetrate eukaryotic cell membranes. In most countries, the recommended antibiotic therapy involves the use of azithromycin. However, even though azithromycin and several other antibiotics effectively eradicate *C. pneumoniae* infection in most cell types, including epithelial cells which are most often used for in vitro susceptibility testing, the infection in peripheral blood mononuclear cells (PBMNC) is refractory to antibiotics and treatment failures have been reported even after prolonged azithromycin therapy [23, 31]. In clinical settings, another therapy-related challenge is the widespread use of penicillins for treating respiratory tract infections. Emergence of beta-lactam resistant strains of *Streptococcus pneumoniae* and other common respiratory pathogens has shifted the CAP treatment guidelines to more common use of quinolones [32], but beta-lactam

antibiotics are still widely used for CAP and other respiratory tract infections [33]. Furthermore, besides the challenges in non-optimal antibiotic selection, even the first-choice antichlamydial antibiotics may trigger persistent infection if used at suboptimal concentrations [34].

4. Investigational approaches for novel anti-chlamydial compounds

Given the challenges in treating *C. pneumoniae* with conventional antibiotics, new approaches are needed for treating the acute infections and providing means for eradication of the infection in all affected cell types. Several classes of organic small molecules have been identified that are able to inhibit *C. pneumoniae* replication in vitro or in vivo. Table 1 lists the nonconventional *C. pneumoniae* inhibitors published during the past 10 years, and selected examples of these compounds are discussed in the following chapters as examples on different discovery strategies. As illustrated by the physicochemical parameters presented in the table, most but not all of these compounds follow Lipinsky rules describing the classical drug-like properties of small molecules. Despite the widespread use of these rules in medicinal chemistry, it is generally known that antibiotics commonly violate these rules and strict following may even limit the changes in identifying novel antibacterial agents [35]. In fact, restricting the chemical collections by such rules has even been suggested as one major factor underlying the recent failures in antibiotic drug discovery [35, 36]. As regards to antichlamydial compounds, no general rules on physicochemical requirements for active compounds can be drawn based on either classical antibiotics or the nonconventional antichlamydial agents, and considering the compounds

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

presented in Table 1 it is not even clear which of these compounds act by directly targeting the replicating bacteria and which act on targets outside the inclusion.

An essential factor in screening for new inhibitors is the access to a robust and reproducible bioassay capable of identifying antichlamydial compounds based on a biologically meaningful endpoint. A time-resolved fluorescence (TRF)-based assay reported by Tammela et al. [37] was described to meet these basic requirements, and the 96-well format has allowed small to moderate scale screens [38-40]. As regards to image-based screening platforms, application of a DNA chip imaging system coupled with a customized software tool was recently reported for quantification of *C. pneumoniae* inclusions [41]. Yet no bioactivity screening data has been presented by using this technique, the platform may potentially be useful in screening environment given that the reagent cost issues related to sample staining can be managed upon high sample numbers. Alternative approaches have relied on using the related more widely studied species *C. trachomatis* as the primary target and assayed inhibition of *C. pneumoniae* in follow-up studies. A significant step forward with screening chemical libraries against *C. trachomatis* is the high-content screening (HCS) assay capable of determining chlamydial inclusion number and size in infected HeLa cells [42]. The shorter life cycle and higher in vitro infectivity of *C. trachomatis* compared to *C. pneumoniae* contribute to its popularity as primary screening target, but it is, however, good to remember that despite the relatively close phylogenetic relationship between the two species some major differences in their susceptibility as antibacterial agents are known to exist [29, 43].

4.1. Drug repositioning and combination therapies

One approach on suppressing *C. pneumoniae* infection and its inflammatory consequences has involved the evaluation of clinically approved non-antibiotic drugs against the bacterium, and particularly the drugs used for treating cardiovascular diseases have been of interest. Among the first reports in this respect was the study on the effects of calcium channel blockers on *C. pneumoniae* infected macrophages, showing that L-type calcium channel blockers such as nifedipin can reactivate persistent infection to a phenotype closer to acute infection [44]. While the calcium channel blockers alone did not have any significant impact on *C. pneumoniae* titers in macrophages, combining them with erythromycin, doxycycline or rifampin improved the susceptibility of *C. pneumoniae* to these antibiotics. In epithelial cells L-type specific (isradipin) as well as unspecific (verapamil) calcium channel blockers rather improved than suppressed *C. pneumoniae* growth [45], which reflects the indigenously different infection state in the two cell types and the properties of calcium channel blockers as infection modulators rather than chlamydial growth inhibitors. Effects of calcium channel blockers on *C. pneumoniae* have also been studied in combination with the phenolic compounds quercetin, rhamnetin and luteolin [45]. These three naturally occurring compounds have been shown to suppress *C. pneumoniae* growth in vitro and in vivo [38, 46, 47], and the connection of high dietary phenolic compound intake to lowered atherosclerosis risk has opened the speculations on the relevance of also antichlamydial effects in this respect. Combining quercetin, rhamnetin or luteolin with isradipin or verapamil did not

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

result in improved *C. pneumoniae* growth inhibition in epithelial cells, but combining the same phenolic compounds with thapsigargin, a modulator of endoplasmic reticulum transport protein responsible for intracellular calcium homeostasis, was reported to result in a significant synergistic effect on *C. pneumoniae* growth [45]. Impact of such combinations on *C. pneumoniae*-infected macrophages or other models of the persistent infection is not known, and in vivo studies with the phenolic compounds should take into account the well-known anti-inflammatory effects besides the direct antichlamydial activities, but further studies on combining the phenolic compounds would be of importance not least due to the abundance of these compounds in our daily diets.

The significance of immunomodulatory effects on the overall outcome of antichlamydial therapy is presented also by the effects of statins, widely used lipid lowering drugs, on *C. pneumoniae* infections. Simvastatin has been reported to decrease *C. pneumoniae* load in the lungs of infected mice [48], while a more hydrophobic statin analogue pravastatin did not have a similar effect, potentially due to its weaker penetration to alveolar tissue [49]. Cerivastatin, a statin analogue no longer in clinical use, has been reported to moderately inhibit *C. pneumoniae* infectivity in macrophages and suppress the transmission of *C. pneumoniae* from infected macrophages to vascular endothelial cells in vitro [50, 51]. However, no systematic evaluation involving different statin analogues and cell types has been presented. The hypotheses presented on the mechanisms of action of statins in this respect have pointed to the *Chlamydia* spp. bacteria's dependence on host cell cholesterol, but the relevance of this phenomenon in the observed effects is not clear.

1
2
3 Considering inflammatory responses, simvastatin has been shown to
4 suppress *C. pneumoniae*-induced cytokine expression in endothelial cells and
5 prevent *C. pneumoniae*-induced overexpression of lectin-like oxidized low-
6 density lipoprotein receptor 1 (LOX-1) in cell cultures [52, 53]. However, the
7 net immunomodulatory effect of statins in the lungs of *C. pneumoniae*-infected
8 mice seems to vary between different analogues, as illustrated by the
9 proinflammatory rather than anti-inflammatory effect of pravastatin [49].
10 Studies combining statins with conventional or nonconventional antibiotics
11 have not been presented thus far yet they could help to elucidate potential
12 synergistic effects on acute or persistent infections.
13

14 Also rapamycin (sirolimus), which is an anti-inflammatory and
15 antiproliferative agent used for prevention of neointima formation, has been
16 shown to prevent *C. pneumoniae* replication in epithelial cells [54]. This drug
17 is used for restenosis prevention in patients with coronary artery disease in
18 the form of rapamycin eluting stents, providing a targeted release of the drug
19 at the affected vascular area, which enables local concentrations high enough
20 for the chlamydiocidal effects.
21

22 Besides the findings described above, the antichlamydial activities of
23 heparins and other drugs and drug-like molecules binding to heparan sulfate
24 receptors on host cell surface [55, 56] support drug repositioning as one
25 approach offering means towards more effective treatment against *C.*
26 *pneumonia*, and assaying the currently used drugs either alone or as
27 combinations should be further encouraged.
28

29 **4.2. Targeting type 3 secretion system**

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Targeting microbial virulence factors has been suggested as one major strategy to combat antimicrobial resistance and affect slowly growing or non-dividing infectious agents. Among gram-negative bacteria, type 3 secretion system (T3SS) represents a major virulence mechanism that is well conserved among several bacterial species. Several details on chlamydial T3SS machinery structure and function have been elucidated within the past 5 years, demonstrating its significance for invasion of the bacterium to host cells and in its intracellular survival [57]. It is currently generally accepted that the system is a central mediator of chlamydia-induced changes in host cell function, which further increases the attractiveness of chemotherapy against the system [21].

Screening strategies on T3SS have exploited the well-conserved nature of the system among gram-negative bacteria and relied on the use of surrogate bacterial species that allow the construction of recombinant systems with screening-friendly reporters [58]. More recently, ectopic expression of *C. pneumoniae* T3SS components in *E. coli* is emerging as a new tool for functional studies on the putative *Chlamydia* T3SS inhibitors originally identified against other species [59]. A series of *Yersinia* T3SS inhibitors identified by a luminescent screening assay has been demonstrated to suppress also *C. trachomatis* and *C. pneumoniae* growth [60, 61]. The most potent of the derivatives have proceeded to preclinical efficacy trials and pharmacokinetic evaluation [62].

Another line of research within inhibition of T3SS has been the use of peptidomimetics for blocking protein-protein interactions between components of the secretion apparatus. Using epitope mapping, Stone et al. [63] were able

to identify regulatory protein binding site in an T3SS associated CdsN ATPase, and by exploiting a homologue structure from *E. coli* they could design a peptide capable of blocking the invasion of *C. pneumoniae* EBs into epithelial cells. The 28 amino acid peptide used in the study is not a viable candidate for developing an orally bioavailable antichlamydial drug, but it could potentially serve as a starting point for designing small molecule weight peptidomimetics with similar activity. In addition, the authors suggest the application of similar targeted peptides as chemical probes to compensate the genetic intractability of *C. pneumoniae* in studying the functions of bacterial proteins. Considering the impact on chlamydial life cycle, the described peptide seems to suppress T3SS in a manner different from the known small molecule inhibitors, which do not affect the invasion of *Chlamydia* into host cells but rather impair the replication phase [61].

4.3. Drug design based on in silico data

To date, only a few crystal structures of *C. pneumoniae* proteins have been published, and the efforts on structural analysis on the chlamydial proteins have focused on understanding the immunogenic properties of proteins exposed to EB surface [64, 65]. Some of the surface proteins have been indicated with essential roles in attachment of the bacterium onto host cells, and the existing structural data could potentially be useful for designing ligands binding to these proteins, but no attempt to this end have been reported.

Besides the immunogenic surface proteins, only two 3D structures of *C. pneumoniae* proteins have appeared in Protein Data bank: a NMR-

resolved structure of chlamydia-specific oxidoreductase located in periplasmic space [66] and the crystal structure of a *C. pneumoniae* homolog of chlamydial type 3 secretion system (T3SS) associated protein Cpn0803 [67].

Given the lack of structural data on actual *C. pneumoniae* proteins, sequence data has been successfully used for homologue-based approaches. Full genomes of several *C. pneumoniae* strains have been sequenced and comparative bioinformatics studies can thus be applied to improve our understanding of chlamydial proteins having homologues in other species. This approach has been successfully followed for the identification of *C. pneumoniae* growth inhibitors by conducting a virtual screen with a RNA methyltransferase structure from *Bacillus subtilis*, which has highly similar amino acid sequence to that of the *C. pneumoniae* dimethyladenosine transferase [68]. Based on the in silico screen of 300 000 compounds against this surrogate target, 33 compounds were selected to perform a growth inhibition screen on *C. pneumoniae*, resulting in eight active hits. Among these eight compounds, two molecules were identified harboring a benzimidazole structure. Further on, design and synthesis of 33 new derivatives bearing this skeleton resulted in the identification of several new derivatives with improved antichlamydial activity and allowed structure activity relationship (SAR) studies in this respect [39, 69]. Several derivatives with MIC values in low micromolar range determined for a cardiovascular *C. pneumoniae* isolate CV-6 were identified as the most potent antichlamydial compounds containing 2-arylbenzimidazole scaffold. The main finding from conformation studies of 2-arylbenzimidazoles was that compounds which can more easily adopt a non-planar conformation show higher antichlamydial

activity. Collectively these results form basis for construction of a pharmacophore model to identify more potent inhibitors.

5. Omics approaches

Besides comparative genomics studies, *C. pneumoniae* genome sequencing has provided the basis for transcriptional analysis studies on whole genome level. While various earlier studies have investigated expression profiles of individual sets of genes in the course of infection, Mäurer et al. described in 2007 the first microarray study on transcriptional regulation of *C. pneumoniae*, reporting 12 differentially expressed gene clusters in the course of acute infection [70]. Comparison of the gene expression profiles of acute infection and iron depletion-mediated persistent infection suggested that the transcriptional changes associated with the persistent infection represent an arrest in mRNA production occurring at the midway of a typical acute infection cycle, rather than a distinct transcriptional profile as such. The state of transcriptional arrest instead of reprogramming implies that reactivation of the infection might be possible if suitable triggers could be identified to overcome the arrest. The concept of reactivating the persistent infection as a means for improving antibiotic efficacy against *C. pneumoniae* is actually not new but has been suggested already ten years ago [44]. In vivo evidence on the potential of eradicating persistent infections by this approach is not available, but the microarray data encourage more detailed studies in this respect. Similar comprehensive data are not available for persistent infections induced by other factors, such as penicillin or interferon gamma, but gene expression studies conducted by more limited sets of target genes indicate that

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

expression profiles are dependent on the factor used to induce the persistence, and increasing evidence indicates that the phenomenon known as persistent infection actually reflects a heterogenic group of states with differential transcriptional profiles [71]. The complexity of the in vivo persistent infection is likely to affect the outcome of potential reactivation-based therapeutic approaches and should be carefully considered when evaluating this hypothesis.

Despite the obligate intracellular nature of *C. pneumoniae*, analysis of the specific proteins expressed in the course of the infection can be conducted by applying cycloheximide, an inhibitor of eukaryotic cell protein synthesis, in combination with ³⁵S methionine to label newly synthesized bacterial proteins [72]. By this means differential expression of *C. pneumoniae* proteins at different stages of the infection cycle, as well as between acute and persistent infection, have been confirmed [73, 74]. In addition, the proteome of the extracellular EB form of *C. pneumoniae* has been characterized [75] and is known to correlate well with the late transcriptional clusters described by Mäurer et al. [70].

Besides the protein expression levels, it is generally accepted that also translocation of the *C. pneumoniae* proteins are tightly regulated in each stage of its life cycle, but analysis on these processes in whole proteome level has been limited by inability to isolate host cell cytoplasm without rupturing the chlamydial inclusions. However, comparative analysis on host cell cytoplasm samples and isolated bacteria has been successfully used for identification of *C. pneumoniae* effector proteins, identifying for example the relatively well-

studied chlamydial protease-like activity factor [76] and an autotransporter protein Cpn0796 [77].

In addition to the attempts on establishing more comprehensive picture on *C. pneumoniae* gene and protein expression profiles in different infection states, several studies have addressed the changes in host cell transcriptome and proteome upon infection. Microarrays on host cell responses to *C. pneumoniae* have identified several human genes the expression of which is altered at certain points upon *C. pneumoniae* infection [78-80]. When searching for host cell factors suitable for potential antichlamydial targets, interpretation of the observed changes should distinguish between the active host manipulation by *C. pneumoniae* and innate immunity responses resulting mostly from the recognition of bacterial LPS or other surface structures. While both aspects are probable important in promoting the inflammatory state and could be applied as targets for suppressing the pathological changes induced by the infection, the latter may not represent a target for suppressing the bacterial growth or survival as such. Therefore, essentiality of the observed changes in gene expression for the bacterial survival and replication has been confirmed with RNAi studies or other similar techniques.

While the *C. pneumoniae*-induced changes in host cell gene expression have been characterized in different cell lines, relatively little is known about the *C. pneumoniae*-induced changes in host cell proteome. One study has addressed this question upon infection in epithelial cells, describing a significant proteolytic changes in host cell cytoskeleton proteins, and identifying a cleavage site of the relatively well studied chlamydial secretory protease-like activity factor [81].

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

6. Towards genetic modification of *Chlamydia* spp. bacteria

Until very recently, *Chlamydia* spp. bacteria have resisted all efforts on generating mutant strains, which has significantly limited the molecular biology studies on the infection. To date, not a single *C. pneumoniae* mutant has been intentionally generated, but limited success has been achieved with a closely related species *C. psittaci*. Binet et al. [82] described the replacement of an endogenous *C. psittaci* sequence by an *E. coli*-amplified mutant counterpart, using electroporation of the plasmid to *C. psittaci* elementary bodies. Yet being limited to only one target allele, this report represents the first achievements on site-directed mutagenesis of *Chlamydia* spp. bacteria.

More recently, the breakthroughs in forward genetics on another related species, *C. trachomatis*, have emerged. In an influential work by Nguyen and Valdifie it was demonstrated that treatment of *C. trachomatis* with a chemical mutagen resulted in generation of bacterial strains with altered phenotypes, and the mutations could be tracked by whole genome sequencing of the isolated bacteria [83, 84]. Furthermore, co-infection of epithelial cells with the mutant and wild-type strains of *C. trachomatis* resulted in transfer of the mutated gene between the strains, allowing the connection between the mutation and the altered phenotype in question. These results may represent the beginning of a new era in *Chlamydia* research, yet, neither of the two approaches are directly applicable to *C. pneumoniae*. The isolation of mutants relies on the cytopathic properties of the applied *C. trachomatis*

1
2
3 strain via the plaque purification method, which cannot be applied to non-
4
5 cytotoxic *C. pneumoniae* strains.
6

7 Another recent advancement in genetic modification of *C. trachomatis*
8
9 was the delivery of a plasmid shuttle vector into a plasmid-free strain of the
10
11 bacterium, allowing the generation of a green fluorescent protein (GFP)
12
13 containing strain [85]. One major genetic difference between *C. trachomatis*
14
15 and *C. pneumoniae* is the absence of this 7.5 kb plasmid [endogenous to
16
17 most *C. trachomatis* strains) from *C. pneumoniae*. However, the first report on
18
19 delivering the plasmid artificially into *C. pneumoniae* has newly been
20
21 described [23].
22
23

24
25 Even though most of the newly described techniques may not directly
26
27 enable targeted *C. pneumoniae* mutant generation, they are expected to have
28
29 a deep impact on the molecular level studies of related chlamydial species
30
31 and will therefore improve our understanding on the biology of also *C.*
32
33 *pneumoniae* via comparative genetic analyses.
34
35
36
37
38
39
40

41 **7. Expert Opinion**

42
43 During the past decade, our understanding on the biology of *C. pneumoniae*
44
45 infections has dramatically increased, and potential molecular targets for
46
47 therapeutic intervention have been suggested based on molecular biology, in
48
49 silico and omics studies. Inspired by both bacteria host cell interaction studies
50
51 and the general trends in intracellular pathogen therapy approaches, such
52
53 targets could include both *C. pneumoniae* and host cell proteins. These
54
55 proteins have not, however, been subjected to comprehensive target
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

validation process, and it is thus too early to declare any of them as suitable starting points for large scale bioactivity screening campaigns. On the other hand, the general focus of high-throughput screening community has shifted from the reductionistic target-based approach back to the more holistic view offered by phenotypic screening. A recent study carried out by Swinney and Anthony [86] investigated the discovery strategies behind recently approved new chemical entities, revealing the striking fact that vast majority of drugs entering the market as “first-in-class” drugs (acting through a new molecular target) were originally identified in phenotypic screens. This finding demonstrates the value of phenotypic screening for the interplay between drug discovery and basic research, as it is the most likely means for identifying potent antichlamydial compounds and provides an inherent possibility for identifying bacterial and host cell targets essential for the infection.

Generally speaking success rate in antibiotic drug discovery has been disappointing and development of most lead compounds identified during the past decade has been discontinued [36, 87]. It has been stated that one major reason for these failures has been the unsuitable nature of the screened chemical libraries for antibiotic discovery, and several authors have emphasized that moving back to natural products would provide the chemical collections with physicochemical properties more likely to yield developable antibiotics. In fact, many compound groups presented in Table 1 as well as several approved drugs mentioned in this review are of natural product origin supporting the relevance of this view also in the case of antichlamydial drug discovery.

The major therapeutic challenge of *C. pneumoniae* infections is the persistent infection. Prolonged treatment with broad-spectrum antibiotics is needed for complete eradication of the bacterium, and treatment failures have been reported even after extensive antibiotic treatment [22]. Yet it might be theoretically possible to find a chemical agent capable of eradicating the persistent infection after single dose or short-time treatment, the more realistic scenario is that patients would be treated for weeks if not months with the therapeutic agent. Two major obstacles prevent the use of currently available antibiotics for this purpose: adverse effects on host normal microbiota and the risk of antimicrobial resistance. To minimize both risks, a *Chlamydia*-specific or selective agent would be highly desirable. None of the antibiotics in current use bear such a feature, as *Chlamydia* has never been the primary target of industrial antibiotic discovery campaigns but has rather been assayed as one minor group among others within antibiotic spectrum determination. While most efforts in antibiotic discovery in past decades have been put on screening for broad-spectrum agents, focusing on more narrow-spectrum compounds has been suggested as one success factor in tomorrow's antibacterial drug discovery [88]. As regards to *C. pneumoniae*, finding a specific or selective inhibitor can be considered a realistic goal given the phylogenetically distinct nature of these bacteria and the unique feature related to it. The second half of the issue is choosing the indication of the potential new drug to be developed. Targeting *C. pneumoniae* alone may be considered as too narrow indication by regulatory authorities. Atypical pneumonia, on the other hand, could be considered as an indication, thus

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

demanding data on efficacy against also other respiratory pathogens such as *Mycoplasma*, *Moraxella*, *Legionella* and *Haemophilus* species.

Besides finding effective treatment alternatives against the acute respiratory infections, preventing, suppressing or ultimately eradicating persistent *C. pneumoniae* infection remains a challenging but attractive approach as an attempt to affect public health burden by chronic inflammatory diseases. As illustrated by the previous experience on clinical trials on the secondary prevention of atherosclerosis by antibiotics, evaluating such hypotheses requires not only the careful selection of therapeutic agents used and endpoints to be measured but also on the profound understanding on which disease progression state to target [3]. Considering rational optimization of lead molecules and formulation development, one major challenge lies in defining the target tissues of the intended chlamydiocidal effect. For example, a need to deliver the drug molecule into central nervous system in the case of intended Alzheimer's disease related application sets specific needs for blood-brain barrier penetration, while the properties of a molecule to be delivered into vascular wall would be somewhat different. It does not seem likely that a single antichlamydial compound, even a highly potent one, would be suitable for all suggested applications, but differential lead optimization and formulation development is rather necessary to take the specific requirements of each target tissue into account. In conclusion, to reach such a stage within drug discovery process, the *C. pneumoniae* research community must be able to merge the increasing understanding on the biology of this pathogen, careful analysis of the previous failures in clinical

1
2
3 trials, as well as the knowledge on the specific requirements on lead
4
5 molecules set by an intracellular gram-negative bacterium.
6
7
8
9
10

11 Acknowledgements

12
13 The authors thank researchers related to the topics presented in this paper
14
15 for informative correspondence.
16
17
18
19

20 Declaration of interest

21
22 The authors state no conflict of interest and have received no payment in
23
24 preparation of this manuscript.
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

8. Bibliography

1. Maass M, Bartels C, Engel PM, et al. Endovascular presence of viable Chlamydia pneumoniae is a common phenomenon in coronary artery disease. J Am Coll Cardiol 1998;31:827-32.

2. Andraws R, Berger JS, Brown DL. Effects of antibiotic therapy on outcomes of patients with coronary artery disease: a meta-analysis of randomized controlled trials. JAMA 2005;293(21):2641-7.

3. Anderson JL. Infection, antibiotics, and atherothrombosis-end of the road or new beginnings? N Engl J Med 2005;352:1706-9.

4. Rosenfeld ME, Campbell LA. Pathogens and atherosclerosis: update on the potential contribution of multiple infectious organisms to the pathogenesis of atherosclerosis. Thromb Haemost 2011;106:858-67.

****Reviews the significance of C. pneumoniae and other microbes in cardiovascular diseases according to current knowledge.**

5. Vainas T, Sayed S, Bruggeman CA, Stassen FR. Exploring the role of Chlamydia pneumoniae in cardiovascular disease: a narrative review. Drugs Today (Barc) 2009;45Suppl B:165-72.

6. Hahn DL, Azenabor AA, Beatty WL, Byrne GI. Chlamydia pneumoniae as a respiratory pathogen. Front Biosci 2002;7:e66-76.

7. Atkinson TP. Is asthma an infectious disease? New evidence. *Curr Allergy Asthma Rep* 2013;13:702-9.
8. Papaetis GS, Anastasakou E, Orphanidou D. *Chlamydia pneumoniae* infection and COPD: more evidence for lack of evidence? *Eur J Intern Med* 2009;20:579-85.
9. Carter JD, Inman RD, Whittum-Hudson J, Hudson AP. *Chlamydia* and chronic arthritis. *Ann Med* 2012;44:784-92.
10. Shima K, Kuhlenbäumer G, Rupp J. *Chlamydia pneumoniae* infection and Alzheimer's disease: a connection to remember? *Med Microbiol Immunol* 2010;199:283-9.
11. Stephens RS, Myers G., Eppinger M., Bavoil PM. Divergence without difference: phylogenetics and taxonomy of *Chlamydia* resolved. *FEMS Immunol Med Microbiol* 2009;55:115-9.
12. Chopra I, Storey C, Falla TJ, Pearce JH. Antibiotics, peptidoglycan synthesis and genomics: the chlamydial anomaly revisited. *Microbiology* 1998;144:2673-8.
13. Hatch TP, Allan I, Pearce JH. Structural and polypeptide differences between envelopes of infective and reproductive life cycle forms of *Chlamydia*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

spp. J Bacteriol 1984;157:13-20.

14. Hatch TP. Disulfide cross-linked envelope proteins: the functional equivalent of peptidoglycan in chlamydias? J Bacteriol 1996;178:1-5.

15. Pavelka MS Jr. Another brick in the wall. Trends Microbiol 2007;15:147-9.

16. Ghuysen JM, Goffin C. Lack of cell wall peptidoglycan versus penicillin sensitivity: new insights into the chlamydial anomaly. Antimicrob Agents Chemother 1999;43:2339-44.

17. Skilton RJ, Cutcliffen LT, Barlow D, et al. Penicillin induced persistence in Chlamydia trachomatis: high quality time lapse video analysis of the developmental cycle. PLoS One 2009;4:e7723.

18. Beatty WL, Morrison RP, Byrne GI. Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. Microbiol Rev 1994;58:686-99.

19. Hammerschlag MR. The intracellular life of chlamydias. Semin Pediatr Infect Dis 2002;13:239-48.

20. Hogan RJ, Mathews SA, Mukhopadhyay S, Summersgill JT, Timms P. et al. Chlamydial persistence: beyond the biphasic paradigm. Infect Immun 2004;72:1843-55.

21. Kern JM, Maass V, Maass M. Molecular pathogenesis of chronic *Chlamydia pneumoniae* infection: a brief overview. *Clin Microbiol Infect* 2009;15:36-41.
22. Gieffers J, Füllgraf H, Jahn J, et al. *Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment. *Circulation* 2001;103:351-6.
23. Gérard HC, Mishra MK, Mao G, et al. Dendrimer-enabled DNA delivery and transformation of *Chlamydia pneumoniae*. *Nanomedicine* 2013;9:996-1008.
24. Ossewaarde JM, Meijer A. Molecular evidence for the existence of additional members of the order Chlamydiales. *Microbiology* 1999;145:411-7.
25. Corsaro D, Venditti D, Valassina M. New parachlamydial 16S rDNA phylotypes detected in human clinical samples. *Res Microbiol.* 2002;153:563-7.
26. Niemi S, Greub G, Puolakkainen M. *Chlamydia*-related bacteria in respiratory samples in Finland. *Microbes Infect* 2011;13:824-7.
27. Haider S, Collingro A, Walochnik J, et al. *Chlamydia*-like bacteria in respiratory samples of community-acquired pneumonia patients. *FEMS*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Microbiol Lett 2008;281:198-202.

28. Greub G. Parachlamydia acanthamoebae, an emerging agent of pneumonia. Clin Microbiol Infect 2009;15:18-28.

29. Hammerschlag MR, Kohloff SA. Treatment of chlamydial infections. Expert Opin Pharmacother 2012;13:545-52.

30. Senn L, Hammerschlag MR, Greub G. Therapeutic approaches to Chlamydia infections. Expert Opin Pharmacother 2005;6:2281-90.

31. Baltch AL, Smith RP, Ritz WJ, et al. Effect of levofloxacin on the viability of intracellular Chlamydia pneumoniae and modulation of proinflammatory cytokine production by human monocytes. Diagn Microbiol Infect Dis 2004;50:205-12.

32. File TM. The science of selecting antimicrobials for community-acquired pneumonia (CAP). J Manag Care Pharm 2009;15:S5-11.

33. Thiem U, Heppner HJ, Pientka L. Elderly patients with community-acquired pneumonia: optimal treatment strategies. Drugs Aging 2011;28:519-37.

34. Gieffers J, Rupp J, Gebert A, et al. First-choice antibiotics at subinhibitory concentrations induce persistence of Chlamydia pneumoniae. Antimicrob

Agents Chemother 2004;48:1402-5.

35. Silver LL. Are natural products still the best source for antibacterial discovery? The bacterial entry factor. Expert Opin Drug Discov 2008;3:487-500.

36. Chopra I. The 2012 Garred lecture: discovery of antibacterial drugs in the 21st century. J Antimicrob Chemother 2013;68:496-505.

37. Tammela P, Alvesalo J, Riihimäki L, et al. Development and validation of a time-resolved fluorometric immunoassay for screening of antichlamydial activity using a genus-specific europium-conjugated antibody. Anal Biochem 2004;333:39-48.

***Describes the first assay applicable for high throughput screening on antichlamydial compounds.**

38. Alvesalo J, Vuorela H, Tammela P, et al. Inhibitory effect of dietary phenolic compounds on Chlamydia pneumoniae in cell cultures. Biochem Pharmacol 2006;71:735-41.

39. Keurulainen L, Salin O, Siiskonen A, et al. Design and synthesis of 2-arylbenzimidazoles and evaluation of their inhibitory effect against Chlamydia pneumoniae. J Med Chem 2010;53:7664-74.

40. Salin O, Alakurtti S, Pohjala L, et al. Inhibitory effect of the natural

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

product betulin and its derivatives against the intracellular bacterium
Chlamydia pneumoniae. *Biochem Pharmacol.* 2010;80:1141-51.

41. Bogdanov A, Endrsz V, Urbn S, et al. Application of DNA chip scanning
technology for the automatic detection of *Chlamydia trachomatis* and
Chlamydia pneumoniae inclusions. *Antimicrob Agents Chemother* 2013:
published online 4 November 2013, doi: 10.1128/AAC.01400-13

42. Marwaha S, Uvell H, Salin O, Lindgren A, Silver J, Elofsson M. Gylfe . et
al. N-acylated derivatives of sulfamethoxazole and sulfafurazole inhibit
intracellular growth of *Chlamydia trachomatis*. Unpublished manuscript.

43. Chirgwin K, Roblin PM, Hammerschlag MR. In vitro susceptibilities of
Chlamydia pneumoniae (*Chlamydia* sp. strain TWAR). *Antimicrob Agents
Chemother* 1989;33:1634-5.

44.- Azenabor AA, Chaudhry AU, Yang S. Macrophage L-type Ca²⁺ channel
antagonists alter *Chlamydia pneumoniae* MOMP and HSP-60 mRNA gene
expression, and improve antibiotic susceptibility. *Immunobiol* 2003;207:237-
45.

***Introduces the concept of persistent infection reactivation.**

45. Salin OP, Pohjala LL, Saikku P, et al. Effects of coadministration of natural
polyphenols with doxycycline or calcium modulators on acute *Chlamydia
pneumoniae* infection in vitro. *J Antibiot (Tokyo)* 2011;64:747-52.

46. Törmäkangas L, Vuorela P, Saario E, et al. In vivo treatment of acute Chlamydia pneumoniae infection with the flavonoids quercetin and luteolin and an alkyl gallate, octyl gallate, in a mouse model. *Biochem Pharmacol* 2005;70:1222-30.

47. Salin O, Törmäkangas L, Leinonen M, et al. Corn mint (*Mentha arvensis*) extract diminishes acute Chlamydia pneumoniae infection in vitro and in vivo. *J Agric Food Chem* 2011;59:12836-42.

48. Erkkilä L, Jauhiainen M, Laitinen K, et al. Effect of simvastatin, an established lipid-lowering drug, on pulmonary Chlamydia pneumoniae infection in mice. *Antimicrob Agents Chemother* 2005;49:3959-62.

***An illustrative example on successful drug repositioning approach combining antichlamydial and anti-inflammatory properties.**

49. Tirola T, Jauhiainen M, Erkkilä L, et al. Effect of pravastatin treatment on Chlamydia pneumoniae infection, inflammation and serum lipids in NIH/S mice. *Int J Antimicrob Agents* 2007;29:741-2.

50. Kothe H, Dalhoff K, Rupp J, et al. Hydroxymethylglutaryl coenzyme A reductase inhibitors modify the inflammatory response of human macrophages and endothelial cells infected with Chlamydia pneumoniae. *Circulation* 2000;101:1760-3.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

51. Dechend R, Gieffers J, Dietz R, et al. Hydroxymethylglutaryl coenzyme A reductase inhibition reduces Chlamydia pneumoniae-induced cell interaction and activation. *Circulation* 2003;108:261-5.

52. Schmeck B, Beermann W, N'Guessan PD, et al. Simvastatin reduces Chlamydia pneumoniae-mediated histone modifications and gene expression in cultured human endothelial cells. *Circ Res* 2008;102:888-95.

53. Prochnau D, Rödel J, Prager K, et al. Induced expression of lectin-like oxidized ldl receptor-1 in vascular smooth muscle cells following Chlamydia pneumoniae infection and its down-regulation by fluvastatin. *Acta Microbiol Immunol Hung* 2010;57:147-55.

54. Yan Y, Silvennoinen-Kassinen S, Leinonen M, Saikku P. Rapamycin can inhibit the development of Chlamydia pneumoniae, which might partly contribute to the prevention of in-stent restenosis. *Cardiovasc Drugs Ther* 2010;24:189-95.

55. Yan Y, Silvennoinen-Kassinen S, Leinonen M, Saikku P. Inhibitory effect of heparan sulfate-like glycosaminoglycans on the infectivity of Chlamydia pneumoniae in HL cells varies between strains. *Microbes Infect* 2006;8:866-72.

56. Puolakkainen M, Lee A, Nosaka T, et al. Retinoic acid inhibits the infectivity and growth of Chlamydia pneumoniae in epithelial and endothelial

cells through different receptors. Microb Pathog 2008;44:410-6.

57. Mueller KE, Plano GV, Fields KA. New frontiers in type III secretion biology: The Chlamydia perspective. Infect Immun 2013: published online 14 October 20

58. Kauppi AM, Noredfelth R., Uvell H, et al. Targeting bacterial virulence: inhibitors of type III secretion in Yersinia. Chem Biol 2003;10:241-9.

59. Bao X, Beatty WL, Fan H. Exploration of chlamydial type III secretion system reconstitution in Escherichia coli. PLoS One 2012;7:e50833.

60. Muschiol S, Normark S, Henriques-Normark B, Subtil A. Small molecule inhibitors of the Yersinia type III secretion system impair the development of Chlamydia after entry into host cells. BMC Microbiol 2009;9:75:1-7.

61. Bailey L, Gylfe A, Sundin C, et al. Small molecule inhibitors of type III secretion in Yersinia block the Chlamydia pneumoniae infection cycle. FEBS Lett. 2007;581:587-95.

62. Ur-Rehman T, Slepentin A, Chu H, et al. Pre-clinical pharmacokinetics and anti-chlamydial activity of salicylidene acylhydrazide inhibitors of bacterial type III secretion. J Antibiot (Tokyo) 2012;65:397-404.

63. Stone CB, Bulir DC, Emdin CA, et al. Chlamydia pneumoniae CdsL

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

regulates CdsN ATPase activity, and disruption with a peptide mimetic prevents bacterial invasion. *Front Microbiol* 2011;2:21:1-9.

64. Soriani M, Petit P, Grifantini R, et al. Exploiting antigenic diversity for vaccine design: the chlamydia ArtJ paradigm. *J Biol Chem* 2010;285:30126-38.

65. Park SH, Chang JE, Hawkes HJ, et al. Structural analysis and serological test of arginine periplasmic binding protein 2 from *Chlamydomophila pneumoniae*. *Biochem Biophys Res Commun* 2012;418:518-24.

66. Mac TT, von Hacht A, Hung KC, et al. Insight into disulfide bond catalysis in *Chlamydia* from the structure and function of DsbH, a novel oxidoreductase. *J Biol Chem* 2008;283(2):824-32.

67. Stone CB, Sugiman-Marangos S, Bulir DC, et al. Structural characterization of a novel *Chlamydia pneumoniae* type III secretion-associated protein, Cpn0803. *PLoS One* 2012;7:e30220.

68. Alvesalo JK, Siiskonen A, Vainio MJ, et al. Similarity based virtual screening: a tool for targeted library design. *J Med Chem* 2006;49:2353-6.

69. Siiskonen A, Keurulainen L, Salin O, et al. Conformation study of 2-arylbenzimidazoles as inhibitors of *Chlamydia pneumoniae* growth. *Bioorg*

1
2
3 Med Chem Lett 2012;22:4882-6.
4
5

6
7 70. Mäurer AP, Mehltitz A, Mollenkopf HJ, Meyer TF. Gene expression profiles
8 of Chlamydomonas reinhardtii during the developmental cycle and iron
9 depletion-mediated persistence. PLoS Pathog 2007;3:e83.
10
11

12
13 71. Klos A, Thalmann J, Peters J, et al. The transcript profile of persistent
14 Chlamydomonas (Chlamydia) reinhardtii in vitro depends on the means by
15 which persistence is induced. FEMS Microbiol Lett 2009;291:120-6.
16
17

18
19 ****Describes the heterogenic nature of persistent infection.**
20
21

22
23 72. Gandahl BB, Birkelund S, Christiansen G. Genome and proteome
24 analysis of Chlamydia. Proteomics 2004;4:2831-42.
25
26

27
28 73. Molestina RE, Klein JB, Miller RD, et al. Proteomic analysis of
29 differentially expressed Chlamydia reinhardtii genes during persistent
30 infection of HEp-2 cells. Infect Immun 2002;70:2976-81.
31
32

33
34 74. Wehrl W, Meyer TF, Jungblut PR, et al. Action and reaction:
35 Chlamydomonas reinhardtii proteome alteration in a persistent infection
36 induced by iron deficiency. Proteomics 2004;4:2969-81.
37
38

39
40 75. Vandahl BB, Birkelund S, Demol H, et al. Proteome analysis of the
41 Chlamydia reinhardtii elementary body. Electrophoresis 2001;22:1204-23.
42
43

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

76. Shaw AC, Vandahl BB, Larsen MR, et al. Characterization of a secreted Chlamydia protease. *Cell Microbiol* 2002;4:411-24.

77. Vandahl BB, Stensballe A, Roepstorff P, et al. Secretion of Cpn0796 from Chlamydia pneumoniae into the host cell cytoplasm by an autotransporter mechanism. *Cell Microbiol* 2005;7:825-36.

78. Coombes BK, Mahony JB. cDNA array analysis of altered gene expression in human endothelial cells in response to Chlamydia pneumoniae infection. *Infect Immun* 2001;69:1420-7.

79. Eickhoff M, Thalmann J, Hess S, et al. Host cell responses to Chlamydia pneumoniae in gamma interferon-induced persistence overlap those of productive infection and are linked to genes involved in apoptosis, cell cycle, and metabolism. *Infect Immun* 2007;75:2853-63.

80. Alvesalo J, Greco D, Leinonen M, et al. Microarray analysis of a Chlamydia pneumoniae-infected human epithelial cell line by use of gene ontology hierarchy. *J Infect Dis* 2008;197:156-62.

81. Savijoki K, Alvesalo J, Vuorela P, et al. Proteomic analysis of Chlamydia pneumoniae-infected HL cells reveals extensive degradation of cytoskeletal proteins. *FEMS Immunol Med Microbiol* 2008;54:375-84

82. Binet R, Maurelli AT. Transformation and isolation of allelic exchange mutants of *Chlamydia psittaci* using recombinant DNA introduced by electroporation. *Proc Natl Acad Sci USA* 2009;106:292-7.

****Describes a technical breakthrough in genetical modification of chlamydia.**

83. Nguyen BD, Valdivia RH. Virulence determinants in the obligate intracellular pathogen *Chlamydia trachomatis* revealed by forward genetic approaches. *Proc Natl Acad Sci USA* 2012;109:1263-8.

****Opens future opportunities to identify and validate drug targets for *C. pneumoniae*.**

84. Nguyen BD, Valdivia RH. Forward genetic approaches in *Chlamydia trachomatis*. *J Vis Exp* 2013;80:e50636.

85. Wang Y, Kahane S, Cutcliffe LT, et al. Development of a transformation system for *Chlamydia trachomatis*: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog* 2011;7:e1002258. 13, doi:10.1128/IAI.00917-13

86. Swinney DC, Anthony J. How were new medicines discovered? *Nat Rev Drug Discov* 2011;10:507-19.

87. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. Drugs for bad bugs:

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

confronting the challenges of antibacterial discovery. Nat Rev Drug Discov
2007;6:29-40.

88. Lewis K. Platforms for antibiotic discovery. Nat Rev Drug Disc
2013;12:371-87

***Highlights the current trends in antibiotic discovery.**

89. Pohjala, L., Ausbacher, D., Strøm, M., Vuorela, P. Inhibition of Chlamydia
pneumoniae infectivity by β 2,2-amino acid derivatives mimicking cationic
antimicrobial peptides. Proceedings / oral presentation, 7th Meeting of the
European Society for Chlamydia Research. Amsterdam. The Netherlands,
July 1 – 6, 2012.

Article highlights.

- Microbial burden theory is the motivation for the attempts on discovering C. pneumoniae specific antibiotics.
- Lack of validated targets and general trends in bioactivity screening support phenotypic assays for hit/lead discovery.
- Nature of the bacterium poses particular demands on the physicochemical properties of the chemical libraries, favoring natural products.
- Evaluation of antichlamydial properties of clinically approved drugs alone and in combinations offers one strategy for decreasing the bacterial burden.
- Omics techniques and genetic tools are expected to provide means for validating bacterial and host cell targets for chemical intervention.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure legend.

Figure 1. The life cycle of *C. pneumoniae* involves successive conversion of the infective elementary bodies (EB) to the intracellular, replicating reticulate bodies (RB) and maturation of new EBs. EB, as the extracellular form, contains a cell wall with extensively disulfide-cross-linked proteins, the machinery needed for host cell invasion, but with no or very little metabolic activity until it attaches to a host cell. The multiplying RB form can only survive inside the inclusion, the parasitophorous vacuole formed upon the host cell entry. Depending on the environmental conditions intracellular forms of chlamydia may enter a non-replicative state of persistence (aberrant bodies, AB) leading to chronic infection. As regards to exit from the host cell, release of mature EBs via lysis and extrusion has been described for related *Chlamydia* species, but details on *C. pneumoniae* exit have not been studied. In principle, any of the developmental phases can be targeted with antichlamydial compounds. Conventional antibiotics mostly target the multiplication phase, which may not, however, represent the optimal target since it easily triggers persistence. Applying new strategies to combat microbial infections should result in more specific inhibitors and tailored treatments instead of broad-spectrum antibiotics.

144x126mm (72 x 72 DPI)

Table 1 Nonconventional antichlamydial agents presented in the literature within past 10 years

Compound class	MW	Log P	Notes	Refs
Approved drugs				
Statins	418-470	1.4-4.4	Suppress <i>C pneumonia</i> infection and related inflammation	48-51
heparins	>5000	N/A	Prevent <i>C. pneumonia</i> entry into epithelial cells	55
rapamycin	914	3.54	Macrolide from <i>Streptomyces hygroscopicus</i>	54
Investigational compounds				
Plant polyphenolics	270-300	1.8-2.4	Activity shown in vitro and in vivo	38, 46, 47
Betulin derivatives	450-490	8.5-9.7	Triterpenoids isolated from birch bark	40
Retinoic analogues	300-350	7.4-8.6	Prevent <i>C. pneumonia</i> entry and replication	56
2-arulbenzimidazoles	330-380		Identified via in silico homolog modeling	39, 68, 69
Salicylidene acylhydrazides	320-350	2.7-5.3	Putative T3SS inhibitors; studied in vitro and in vivo	60-62
Amino acid derivatives	400-450	3.4	Mimic cationic antimicrobial peptides	89

MW = molecular weight g/mol; log P = water – octanol partition coefficient, predicted log P value is given in cases where an experimentally determined value is not available.