Recent advances in technologies for developing drugs against Chlamydia pneumoniae

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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

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Journal:	*Expert Opinion On Drug Discovery*
Manuscript ID:	Draft
Manuscript Type:	Review
Keywords:	Chlamydia pneumoniae, drug discovery, gram-negative bacterium, persistance, respiratory pathogen, atypical pneumonia

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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 pneumonia*e from atheroschlerotic arteria in 1990s [1[, the role of this obligate intracellular bacterium as a risk factor for atheroschlerosis 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 atheroschlerosis 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 theused 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 atheroschlerosis has received immense attention and been the subject of various human, animal and molecular level studies [5], atheroschlerosis 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

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

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

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 antibacyerial 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 riles on physicochemical requirements for active compounds can be drawn based on either classical antibiotics or the nonconventional antichlamydial agents, and considering the compounds

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 oe rifampin improved the susceptibility of *C. pneumonia*e 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 atheroschlerosis 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

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.

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

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

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

4.2. Targeting type 3 secretion system

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. pneumonia*e 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

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 ^{35}S 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].

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*

strain via the plaque purification method, which cannot be applied to non-cytotoxic *C. pneumoniae* strains.

Another recent advancement in genetic modification of *C. trachomatis* was the delivery of a plasmid shuttle vector into a plasmid-free strain of the bacterium, allowing the generation of a green fluorescent protein (GFP) containing strain [85]. One major genetic difference between *C. trachomatis* and *C. pneumoniae* is the absence of this 7.5 kb plasmid [endogenous to most *C. trachomatis* strains) from *C. pneumoniae*. However, the first report on delivering the plasmid artificially into *C. pneumoniae* has newly been described [23].

Even though most of the newly described techniques may not directly enable targeted *C. pneumoniae* mutant generation, they are expected to have a deep impact on the molecular level studies of related chlamydial species and will therefore improve our understanding on the biology of also *C. pneumoniae* via comparative genetic analyses.

7. Expert Opinion

During the past decade, our understanding on the biology of *C. pneumoniae* infections has dramatically increased, and potential molecular targets for therapeutic intervention have been suggested based on molecular biology, in silico and omics studies. Inspired by both bacteria host cell interaction studies and the general trends in intracellular pathogen therapy approaches, such targets could include both *C. pneumoniae* and host cell proteins. These proteins have not, however, been subjected to comprehensive target

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

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 atheroschlerosis by antibiotics, evaluating such hypotheses requires not only the careful selection of therapeutic agents used and enpoints 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

trials, as well as the knowledge on the specific requirements on lead molecules set by an intracellular gram-negative bacterium.

Acknowledgements

The authors thank researchers related to the topics presented in this paper for informative correspondence.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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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.

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 devekiomental 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.

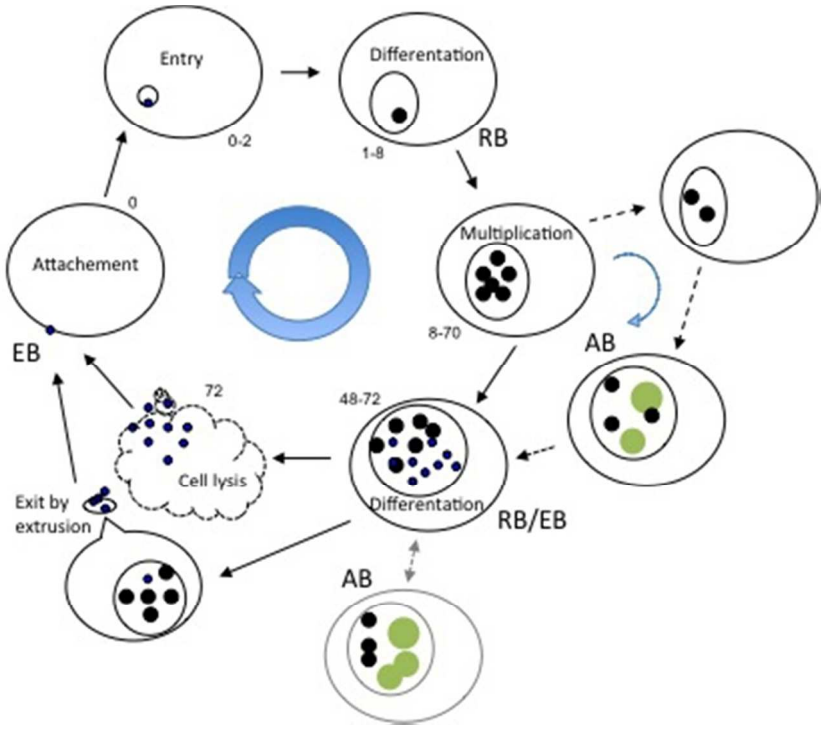

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Table 1 Nonconventional antichlamydial agents presented in the literature within past 10 years

Compound class	MW	Log P	Notes	Refs
Approved drugs				
Statins	418-470	1.4-4.4	Suppress *C pneumonia* infection and related inflammation	48-51
heparins	>5000	N/A	Prevent *C. pneumonia* entry into epithelial cells	55
rapamycin	914	3.54	Macrolide from *Streptomyces hygroscopicus*	54
Investigational compounds				
Plant polyphBenolics	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 *C. pneumonia 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 vittro and in vivo	60-62
Amino acid derivatives	400-450	3.4	Mimic cationic antimicrobial peptides	89

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