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ORIGINAL ARTICLE

Dibenzocyclooctadiene lignans from *Schisandra* spp. selectively inhibit the growth of the intracellular bacteria *Chlamydia pneumoniae* and *Chlamydia trachomatis*

Elina Hakala[1], Leena Hanski[1], Hanna Uvell[2], Teijo Yrjönen[1], Heikki Vuorela[1], Mikael Elofsson[2] and Pia Maarit Vuorela[1]

Lignans from *Schisandra chinensis* berries show various pharmacological activities, of which their antioxidative and cytoprotective properties are among the most studied ones. Here, the first report on antibacterial properties of six dibenzocyclooctadiene lignans found in *Schisandra* spp. is presented. The activity was shown on two related intracellular Gram-negative bacteria *Chlamydia pneumoniae* and *Chlamydia trachomatis* upon their infection in human epithelial cells. All six lignans inhibited *C. pneumoniae* inclusion formation and infectious progeny production. Schisandrin B inhibited *C. pneumoniae* inclusion formation even when administered 8 h post infection, indicating a target that occurs relatively late within the infection cycle. Upon infection, lignan-pretreated *C. pneumoniae* elementary bodies had impaired inclusion formation capacity. The presence and substitution pattern of methylenedioxy, methoxy and hydroxyl groups of the lignans had a profound impact on the antichlamydial activity. In addition our data suggest that the antichlamydial activity is not caused only by the antioxidative properties of the lignans. None of the compounds showed inhibition on seven other bacteria, suggesting a degree of selectivity of the antibacterial effect. Taken together, the data presented support a role of the studied lignans as interesting antichlamydial lead compounds.
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INTRODUCTION

Chlamydia spp. is a genus of Gram-negative intracellular bacteria that are significant agents of human infections. *Chlamydia pneumoniae* is a frequent cause of community-acquired pneumonia and other respiratory infectious diseases,[1] and *Chlamydia trachomatis*, in turn, causes sexually transmitted diseases. *C. pneumoniae* is the most common chlamydia in humans and nearly everyone is infected 2–3 times in a lifetime. Besides the acute illnesses of respiratory tract, *C. pneumoniae* has been associated in the pathogenesis of some chronic human diseases. These include chronic obstructive pulmonary disease and asthma,[2,3] cardiovascular disease,[4] reactive arthritis[5] and lung cancer.[6] *C. trachomatis* is considered the most prevalent causative agent of sexually transmitted diseases worldwide.[7] It is also an important causative agent of pelvic inflammatory disease, with complications including infertility[8]. Furthermore, trachoma, a disease caused by ocular *C. trachomatis* infection, is the leading infectious cause of preventable blindness in the world.[9]

Owing to their obligate intracellular nature *Chlamydiae* have a characteristic developmental cycle involving different morphological forms of the bacterium. In acute infection the life cycle includes two main forms, infectious extracellular elementary bodies (EBs) and replicating intracellular reticulate bodies. The well-acknowledged problem in the treatment of *C. pneumoniae* infections is insufficient response to the first line antibiotics, with problems in controlling persistent or relapsing symptoms, or both.[10,11] In addition to that the infection-induced pathological changes, such as cytokine and chemokine production, cannot be prevented by conventional antibiotics.

The fruits of plant *Schisandra chinensis* have long been used in traditional Chinese and other oriental medicine.[12] Recent studies have shown the lignans of *S. chinensis* fruit to possess various pharmacological activities, such as neuro-, cyto- and hepatoprotective, as well as anti-inflammatory effects.[13–15] The widely studied antioxidant properties of *Schisandra* lignans are considered one major mechanism mediating the cytoprotective effects.[13,16–18] Schisandrin B has been shown to inhibit the production of intracellular reactive oxygen species (ROS), which can at least partially be explained by nicotinamide adenine dinucleotide phosphate-oxidase inhibition.[19–21] *C. pneumoniae* infection induces ROS production in various cell

[1]Pharmaceutical Biology, CDR, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland and [2]Laboratories for Chemical Biology Umeå, Department of Chemistry, Umeå University, Umeå, Sweden
Correspondence: Professor PM Vuorela, Pharmaceutical Biology, CDR, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, P.Box 56 (Viikinkaari 5E) FI-00014, Finland.
E-mail: pia.vuorela@helsinki.fi
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types.[22,23] *C. pneumoniae*-infected macrophages use the induction of ROS as a defense response to eliminate the invader.[24] The ROS in macrophages generated through membrane-associated nicotinamide adenine dinucleotide phosphate-oxidase are known to affect the fate of *C. pneumoniae* life cycle in these cells.[25] Regarding the other cell types, it is unclear whether the ROS induction has a role in bacterial survival or reproduction or rather represents an unnecessary or even harmful side effect from the bacterial point of view.

In the current study we report antibacterial activity of six major lignans found in *Schisandra* spp. with emphasis on their anticlamydial activity.

MATERIALS AND METHODS

Chemicals

Schisandrin was purchased from Sigma-Aldrich (St Louis, MO, USA) and schisandrin A, schisandrin B, schisandrin C, schisandrol B and schisantherin A from Fine Tech Industries, London, UK (Table 1). All six lignans were dissolved in dimethyl sulfoxide. L-ascorbic acid, L-dithiothreito and *N*-acetyl-L-cysteine were dissolved in deionized sterile water.

Cell lines

Human respiratory tract epithelial HL cells were grown in Roswell Park Memorial Institute medium-1640 medium supplemented with 2 mM L-glutamine, 7.5% fetal bovine serum and 20 μg ml^{-1} gentamicin, at 37 °C, 5% CO_2 and 95% humidity to confluence in cell culture flasks.[26] HeLa-229 cells (CCL-2.1; ATCC, Manassas, VA, USA) were propagated in Roswell Park Memorial Institute medium-1640 medium supplemented with 10% fetal bovine serum, 20 mM HEPES (pH 8.0), 8 μg ml^{-1} gentamicin, 1 μg ml^{-1} amphotericin B and 2 mM L-glutamine at 37 °C, in a humified atmosphere in 5% CO_2. *C. pneumoniae* clinical isolate K7 was grown in HL cells and propagated as described earlier.[27] *C. trachomatis* serovar L2 (VR-902B; ATCC) was cultured in HeLa-229 cells and purified as previously described.[28] Both *C. pneumoniae* and *C. trachomatis* were stored in 0.2 M sucrose, 0.02 M sodium phosphate (pH 7.4) and 5 mM glutamic acid Sucrose-phosphate-glutamic acid (buffer) at −80 °C until use.

Infections

HL cells were seeded into 24-well plates with coverslips at 4×10^5 cells per well and incubated for 24 h at 37 °C, 5% CO_2 and 95% humidity. The cell monolayers were inoculated with a suspension of *C. pneumoniae* EBs diluted in cell culture medium supplemented with 0.5 μg ml^{-1} cycloheximide with the multiplicity of infection 0.2. The inoculated cells were centrifuged at 550 g for 1 h and incubated at 37 °C for 1 h to allow the EBs to internalize into the host cells. Medium supplemented with 1 μg ml^{-1} cycloheximide containing the samples or controls at indicated concentrations was added. Non-treated infections, infections treated with 0.01 μg ml^{-1} rifampicin and non-infected cells served as controls. In each well dimethyl sulfoxide was adjusted to 0.25%. Plates were incubated at 37 °C, 5% CO_2 and 95% humidity. Seventy-two hours post infection, the wells were fixed with methanol. The chlamydia inclusions were stained with Pathfinder *Chlamydia* culture confirmation system (Bio-Rad Laboratories, Berkeley, CA, USA) and inclusion counts were determined under fluorescent microscope with a ×200 magnification.

HeLa-229 cells were seeded in 96-well flat-bottom cell culture plates (1×10^4 cells per well) in Roswell Park Memorial Institute medium-1640 medium supplemented as above. Confluent cells were infected with *C. trachomatis* serovar L2 diluted in Hank's balanced salt solution at multiplicity of infection 0.3. After 1 h incubation at 37 °C, Hank's balanced salt solution was replaced with growth medium including test compounds or solvent alone (1% dimethyl sulfoxide). Cell cultures were fixed at 24 h, immunostained (Pathfinder) and examined at ×200 magnification with fluorescent microscopy.

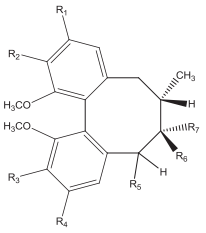
Evaluation of infectious progeny

HL cells were seeded into 24-well plates either with or without coverslips, infected and treated with the samples as described above. After 72 h infection (first passage) the wells with coverslips were fixed and stained to calculate the inclusions. Cell lysates from the wells without coverslips were used to infect fresh HL cell monolayers. After another 72 h infection (second passage) these samples were fixed and stained to determine the amount of infectious progeny.

Effects on other bacterial species

Growth inhibition on other bacterial species was investigated by turbidity measurements and resazurin staining.[29] Gram-negative bacteria; *Enterobacter aerogenes, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and Gram-positive bacteria; *Bacillus subtilis, Staphylococcus aureus* and *Staphylococcus epidermis* (ATCC reference strains) were grown on agar (Bacteriological agar Type A), inoculated to 10 ml of nutrient broth and grown overnight at 37 °C in a shaker at 100 rpm. A 500× dilution was made in the broth and this suspension was seeded into a 96-well plate. The lignans were added at 50 μM concentration and nontreated cultures, cultures treated with either 12 nM (0.01 μg ml^{-1}) rifampicin or 20 μg ml^{-1} ampicillin as well as only nutrient broth served as control samples. Starting point turbidity was measured as light absorbance at 590 nm with VarioSkan Flash plate reader (Thermo Fischer Scientific, Vantaa, Finland). The plates were incubated for 24 h on a shaker at 37 °C, 100 rpm. The change in turbidity was measured at 590 nm. Resazurin at 20 μM in phosphate-buffered saline was added and incubated for 5 min at 37 °C, on a shaker in darkness and the fluorescence was measured with VarioSkan flash plate reader at 560/590 nm (ex/em).

Table 1 Names and chemical structures of the assayed *schisandra* lignans



Schisandra lignan	R_1	R_2	R_3	R_4	R_5	R_6	R_7	*Synonyms*[a]
Schisandrin	OCH_3	OCH_3	OCH_3	OCH_3	H	OH	CH_3	Schizandrin, Schisandrol A, wuweizi alcohol A, wuweizichun A
Schisandrin A	OCH_3	OCH_3	OCH_3	OCH_3	H	H	CH_3	Deoxyschisandrin, dezoxischizandrin, wuweizisu A, dimethylgomisin J
Schisandrin B	-O-CH_2-O		OCH_3	OCH_3	H	H	CH_3	(±)-γ-schizandrin, deoxygomisin A, wuweizisu B
Schisandrin C	-O-CH_2-O		-O-CH_2-O		H	H	CH_3	Wuweizisu C
Schisandrol B	-O-CH_2-O		OCH_3	OCH_3	H	OH	CH_3	Gomisin A, wuweizi alcohol B, wuweizichun B, TJN 101
Schisantherin A	-O-CH_2-O		OCH_3	OCH_3	O-C-O-Bz	CH_3	OH	Gomisin C, schizandrer A, wuweizi ester A

[a]Synonyms, Opletal *et al*.[17]

Intracellular ROS detection

To determine changes in intracellular ROS levels, HL cells were seeded into 96-well plates to 60 000 cells per well, incubated overnight and loaded with 20 μM 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min at 37 °C. The solution was removed from the wells and washed once with Hank's balanced salt solution prior to the exposure of the cells to the lignans for 1 h at 37 °C. The fluorescence (relative fluorescence units) was measured at 503/523 (ex/em) with VarioskanFlash plate reader. 1 mM hydrogen peroxide and *N*-acetylcysteine were used as positive and negative controls. To determine the possible direct interaction of the lignans with the dye, a similar experiment was conducted with no cells (compounds and DCFH-DA). The potential interference on dye de-esterification or efflux was excluded with a 2'7'-dichlorofluorescein diacetate (DCF-DA) assay.

Host cell viability assay

Effect of the lignans on host cell viability was determined by a resazurin assay as recently described[30] in two phases of cell growth. The effect on logarithmically dividing cells and confluent cell cultures were assayed with a density of 5×10^4 and 6×10^5 cells per well, respectively. In brief, HL cells seeded into a 96-well plate were incubated overnight and exposed to the test compounds for 72 h at 37 °C. Viability was detected with a profluorescent probe, resazurin and the fluorescence was measured with VarioSkan Flash plate reader at 570/590 nm (ex/em). Concentration of dimethyl sulfoxide in the sample wells was 0.25%. Triton X was used as a positive control.

Data analysis

The average inclusion counts were determined by counting altogether 12 eye fields for each sample (four eye fields per coverslip from experiments with three coverslips). The reductions in inhibition counts were calculated by comparing the average inclusion counts in a treated sample to the average inclusion counts in the untreated infection controls. Data were expressed as means ± s.e.m., and statistical analyses were performed by the Student's *t*-test using SPSS Statistics 21.0 software. *P*-values below 0.05 were considered statistically significant.

Table 2 MIC values of *Schisandra* lignans against *C. pneumoniae* and *C. trachomatis*

Schisandra *lignan*	*MIC (μM) on* C. pneumoniae	*MIC (μM) on* C. trachomatis
Schisandrin	>100	—
Schisandrin A	50	100
Schisandrin B	50	100
Schisandrin C	25	50
Schisandrol B	>100	—
Schisantherin A	50	100

Human epithelial HL cells were infected with *C. pneumoniae* strain K7 and the effect of the lignans on *C. pneumoniae* inclusion counts after the 72 h infection was determined using immunofluorescence with a fluorescein isothiocyanate-conjugated genus-specific anti-LPS antibody *C. trachomatis* inclusion count in HeLa cells was determined after 24 h infection. To determine the MIC-value the lignans were assayed using seven different concentrations: 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 μM. ($n=12$; mean ± s.e.m).

RESULTS

Chlamydia growth inhibition and host cell viability

Schisandrin B was identified as a hit compound in our recent high-content screening study (Hanski *et al.*, unpublished data) using a novel screening platform to identify antichlamydial compounds.[31] Based on these findings, six dibenzocyclooctadiene lignans originating from *Schisandra* spp. (Table 1) were assayed against *C. pneumoniae* and *C. trachomatis* at varying concentrations. MIC values of the lignans ranged from >100 to 25 μM when assayed against *C. pneumoniae*, and from no inhibition to 50 μM when assayed against *C. trachomatis* (Table 2). All of the lignans showed inhibition in a dose-dependent manner (Figure 1a). The estimated IC_{50}- values for the lignans against *C. pneumoniae* were 52, 10, 5, 5, 6 and 20 μM for schisandrin, schisandrin A, schisandrin B, schisandrin C, schisandrol B and schisantherin A, respectively.

The effect of the lignans on the host cell viabilities assayed on confluent cells (60 000 cells per well) ranged from 90.3 to 122.0% The corresponding numbers when assayed with growing cells (5000 cells per well) were from 72.7 to 97.4%. Lignans did not significantly reduce the viability of either confluent or growing cells except schisandrin, which reduced the viability of the growing cells by 18.9% ($P<0.01$).

Impact of administration time on inhibitory activity

Schisandrin and schisandrin B were administered to *C. pneumoniae*-infected cell cultures 2, 8 and 24 h after the bacterial inoculation at a concentration of 50 μM (Figure 1b). Delaying the administration of schisandrin B and schisandrin to 8 h did not affect their ability to inhibit *C. pneumoniae* inclusion formation, and partial inhibitory activity remained also upon administration of the lignans at 24 h post infection.

Inhibition of infectious progeny

The six lignans were assayed for their effect on *C. pneumoniae* infectious progeny production at 25 and 50 μM concentrations (Figure 2). Consistent with previous experiments above, the inhibition of inclusion formation at the first passage at 25 and 50 μM were lowest at 8.1% and 26.3%, respectively with schisandrin. Inhibition percentages were highest with schisandrin C (99.5%, 25 μM), schisandrin B, schisandrin C and schisantherin A (100.0%, 50 μM), each. Regarding the progeny production (inclusion counts in the passaged samples), the inhibition percentages at 25 and 50 μM were lowest with

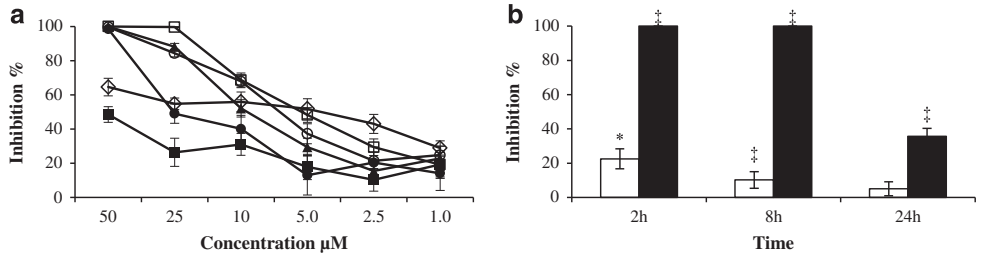


Figure 1 A Suppression of *C. pneumoniae* inclusion counts by the *Schisandra* lignans. The growth inhibition of *C. pneumoniae* is expressed as % using the infected nontreated control as 0% and the uninfected control as 100% The data represent mean ± s.e.m., $n=12$. ▲ = schisandrin; ■ = schisandrin A; ◆ = schisandrin B; ○ = schisandrin C; ◇ = schisandrol B; □ = schisantherin A. (**b**) The impact of delayed administration of schisandrin (white bars) and schisandrin B (black bars) on *C. pneumoniae* inclusion counts. The lignans were administered at 50 μM concentration at either 2 h, 8 h or 24 h post infection. Data represent mean ± s.e.m., $n=12$. (*$P<0.05$, †$P<0.01$, ‡$P<0.001$).

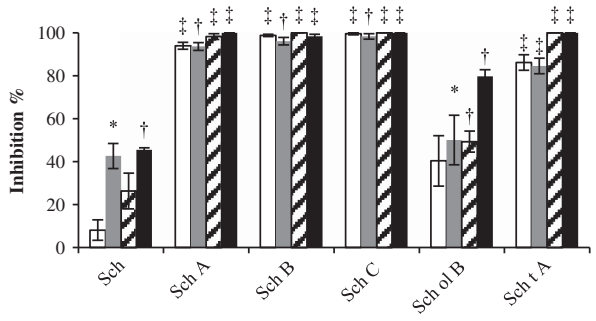


Figure 2 Effect of the *Schisandra* lignans on C. *pneumoniae* infectious progeny production. The inclusion counts were determined at the first and the second passage of the infection (see section 2.4.). The lignans were assayed at concentrations of 25 and 50 μM. Sch = schisandrin, Sch A = schisandrin A, Sch B = schisandrin B, Sch C = schisandrin C, Sch ol B = schisandrol B, Sch t A = schisantherin A. First passage 25 μM: white bars, second passage 25 μM: gray bars, first passage 50 μM: bars with downward diagonal lines, second passage 50 μM: black bars. Data represent mean ± s.e.m., $n = 4$. (*$P<0.05$, †$P<0.01$, ‡$P<0.001$).

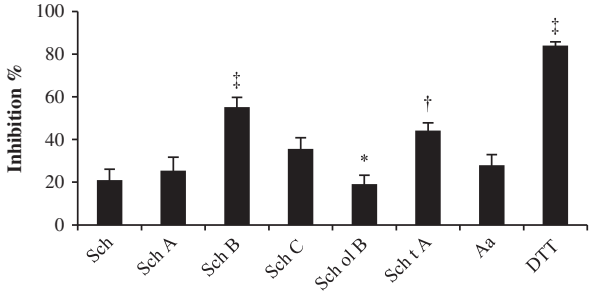


Figure 3 To evaluate direct effects of the lignans on chlamydial elementary bodies, *C. pneumoniae* stocks were diluted in the infection medium to yield a 400 000 IFU ml^{-1} suspension. The elementary bodies in the suspension were pretreated at +4 °C with 50 μM lignans for 1 h prior to the inoculation on HL cell monolayers. Sch = schisandrin, Sch A = schisandrin A, Sch B = schisandrin B, Sch C = schisandrin C, Sch ol B = schisandrol B Sch t A = schisantherin A, Aa = ascorbic acid 250 μM, DTT = 10 mM dithiotreitol. Data represent mean ± s.e.m., $n = 12$. (*$P<0.05$, †$P<0.01$, ‡$P<0.001$).

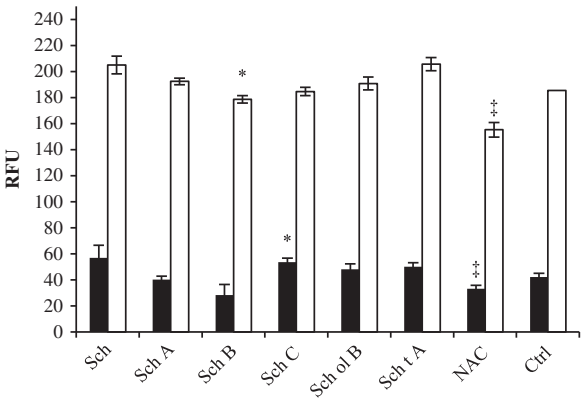


Figure 4 The impact of the *Schisandra* lignans on basal and infection-associated intracellular ROS levels. For basal experiments (black bars): HL cells were loaded with the ROS probe DCFH-DA for 1 h, after which the cells were incubated with 50 μM lignans for an additional hour before the fluorescent readout. For infection-associated experiments (white bars): HL cells were loaded with DCFH-DA and infected with *C. pneumoniae* MOI 1 simultaneously with 50 μM lignans. Intracellular ROS levels were measured after 2 h. Sch = schisandrin, Sch A = schisandrin A, Sch B = schisandrin B, Sch C = schisandrin C, Sch ol B = schisandrol B, Sch t A = schisantherin A. 10 mM *N*-acetylcystein (NAC) was used as a positive control. Data represent mean ± s.e.m., $n = 4$. (*$P<0.05$, †$P<0.01$, ‡$P<0.001$).

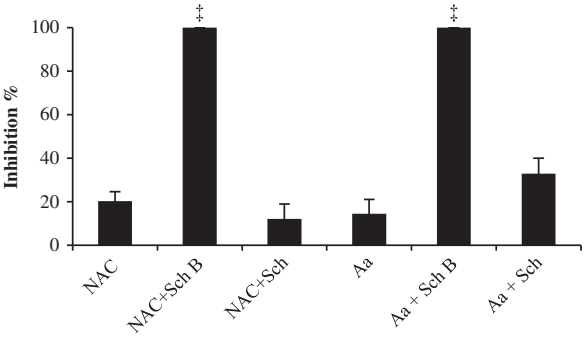


Figure 5 Effect of ascorbic acid and *N*-acetylcysteine on *C. pneumoniae* inclusion counts alone and in combination with schisandrin and schisandrin B. *C. pneumoniae* infections were treated with either 250 μg ml^{-1} ascorbic acid (Aa), 10 mM *N*-acetylcysteine (NAC) or Aa in combination with 50 μM lignans. Data represent mean ± s.e.m., $n = 12$. (*$P<0.05$, †$P<0.01$, ‡$P<0.001$).

schisandrin, 42.6% and 45.6%, respectively, and highest with schisandrin C, 98.3%, and schisandrin A, schisandrin C and schisantherin A 100.0%, respectively. The highest inhibition percentages were reached with schisandrin C at both concentrations and both passages.

Inhibition of EB-infectivity

To study the impact of the lignans on *C. pneumoniae* extracellular form and its entry to the host cell, a pretreatment of *C. pneumoniae* EBs was carried out. When the chlamydial EB particles were pretreated with the lignans at 50 μM a partial inhibition of inclusion formation was observed (Figure 3). The inhibition percentages were lowest with schisandrin, 11.2% and highest with schisandrin B, 50.2%.

Effects on other bacteria

To evaluate the sensitivity of bacteria, other than *Chlamydiae*, to the lignans, the compounds' inhibition of the bacteria was assayed at 50 μM of the lignan against both Gram-negative (*P. aeruginosa*, *E. aerogenes*, *P. mirabilis*, *E. coli*) and Gram-positive (*S. epidermis*, *S. aureus*, *B. subtilis*) bacteria. The investigated lignans did not show growth inhibition of these bacteria (Supplementary Information S1).

Intracellular ROS levels

To evaluate the impact of the lignans on cellular basal ROS levels, HL cells preloaded with the ROS probe DCFH-DA were exposed to each of the lignans. In these experiments, 25 μM schisandrin A and schisandrin B reduced the amount of intracellular ROS with 30.5% and 32.3%, respectively ($P<0.01$). At 50 μM only schisandrin C caused slight change in ROS level (Figure 4, black bars). When 1 mM H_2O_2 was applied to induce an increase in intracellular ROS levels, none of the lignans at 25 or 50 μM either increased or decreased the amount of ROS (data not shown). In *C. pneumoniae*-infected cells (multiplicity of infection 1.0), only 50 μM schisandrin B reduced the amount of ROS by 6.5% ($P<0.05$, Figure 4, white bars). The amount of ROS as

such was not significantly increased upon *C. pneumoniae* infection compared with uninfected controls.

None of the lignans at 25 or 50 μM concentration either increased or decreased the amount of fluorescence produced by the DCFH-DA probe in a control experiment conducted without HL cells (data not shown). When incubated with the oxidized form of probe (DCFH-DA), only schisandrin B at 25 μM had a diminishing effect on the fluorescent signal (37.6% of control, $P<0.05$), indicating that the observed effects may partially result from altered probe distribution or deacetylation by the sample. The effect was not observed at 50 μM.

The effect of other antioxidants on *C. pneumoniae* inclusion formation

To evaluate the ability of other known antioxidants to inhibit *C. pneumoniae* inclusion formation and affect the inhibitory activity of *Schisandra* lignans, ascorbic acid and *N*-acetylcysteine were assayed alone and in combination with schisandrin and schisandrin B. Ascorbic acid and *N*-acetylcysteine yielded a small, statistically non-significant effect on *C. pneumoniae* inclusion formation at concentrations generally known effective in ROS scavenging and diminishing oxidative stress (Figure 5). Furthermore, neither ascorbic acid nor *N*-acetylcysteine affected the inhibitory activity of schisandrin or schisandrin B when they were administered concomitantly to the infected HL cell cultures.

DISCUSSION

Although widely studied, there are no previous reports considering *Schisandra* lignans' antibacterial effects. In the current work, we report that the dibenzocyclooctadiene lignans from *Schisandra* spp. are potent chlamydia inhibitors with dose-dependent effects on the growth of *Chlamydia in vitro*. The inhibition was established with two different chlamydial strains, *C. pneumoniae* clinical pneumonia isolate K7 and *C. trachomatis* serovar L2, a lymphogranuloma venereum strain with invasive properties. Complete eradication of chlamydial inclusions was achieved on both species but the lignans did not have an inhibitory effect on seven other studied bacteria, indicating a degree of selectivity for the antichlamydial effect. Besides reducing chlamydial inclusion counts in infected cell cultures, all six lignans inhibited formation of infectious progeny at concentrations of 25 and 50 μM (Figure 2). This indicates that the inhibitory activity of these compounds is not likely to involve any drastic effects on events occurring late during the replication cycle, such as reticulate body to EB maturation or exit of EBs from the host cell.

In delayed administration time experiments, schisandrin and schisandrin B maintained their inhibition on *C. pneumoniae* growth when they were added into the infected cell cultures 8 h after the initiation of the infection and schisandrin B partially inhibited the inclusion formation even when added at 24 h post infection (Figure 1b). These results indicate that the observed inhibitory effects are not caused by events related to *C. pneumoniae* entry into the host cell or early stages of its replication cycle, such as establishment of inclusions or EB to reticulate bodies differentiation.

The small differences in the chemical structures of the studied lignans resulted in differential antichlamydial activities (Table 2). The most active lignin schisandrin C harbors a methylenedioxy bridge at R3 and R4, whereas the schisandrin B has methoxy groups at R3 and R4. A pairwise comparison of schisandrin and schisandrol B, as well as schisandrin A and schisandrin B revealed a similar structure-activity pattern. Also earlier studies suggest the methylenedioxy group to be a structural determinant of biological activity of these lignans.[32,33] Another structural determinant identified in this study was that schisandrin and schisandrin A differ from each other only by a hydroxyl group at R6 (present in schisandrin, missing in schisandrin A), schisandrin A being significantly more active than schisandrin. A similar pattern is seen also between schisandrol B and schisandrin B. Consistent with various earlier studies comparing these compounds[13,17,18,34] schisandrin B appears to bear higher biological activity than schisandrin.

We also examined whether the lignans have a direct inhibitory effect on the chlamydial EB infectivity. Pretreatment of *C. pneumoniae* EBs with the *Schisandra* lignans resulted in inhibition of chlamydial inclusion formation in the following infection (Figure 3). These effects may not be directly associated with the observed growth inhibition when treating the cellular infection (described above). As the inhibitory effect is maintained when the compound is administered 8 h post infection, a time point reflecting a stage in which majority of EBs have differentiated into reticulate bodies,[35] these effects cannot be explained by the potential interference of EB integrity by the compounds.

As *Schisandra* lignans have been widely studied for their antioxidant and pro-oxidant properties,[18,22,36,37] and *C.* pneumoniae infection is known to induce an increase in host cell ROS levels,[22,23] the relevance of this aspect in the antichlamydial effect of the lignans was studied. However, the results indicated that the antichlamydial activity and effects on cellular ROS levels were not directly correlated and coadministration of the lignans with ascorbic acid or *N*-acetylcysteine did not affect their antichlamydial activity. Also the time course of *C. pneumoniae*-induced increase in ROS levels and that of the antichlamydial effect of schisandrin B does not support the role of ROS in the mechanism of the inhibitory effect. *C. pneumoniae*-induced ROS production has been reported to occur shortly after the entry into host cell, typically within 1 h after the initial contact between the bacterium and the host cell, whereas the results from the experiment with delayed administration of schisandrin B demonstrate an efficient inhibition even at 8 h administration (Figure 1b).

Toxicology of *Schisandra* lignans and extracts has been studied *in vitro* and *in vivo* and they are generally considered non-toxic.[15,22,36,38,39] Consistent with the literature, in this study the lignans did not affect the host cell viabilities at the studied concentrations. Besides safety, gastrointestinal absorption is a key requirement for a successful lead compound. Earlier studies have demonstrated *in vivo* bioactivities of oral doses of schisandrin B or C,[32,40] thus indicating the bioavailability of these compounds when orally administered.

Taken together, these data suggest that the antichlamydial activities of *Schisandra* lignans are targeted to the mid-phase of the intracellular replication cycle and are not solely based on their impact on cellular redox-status. Instead, the lignans might affect more explicit biochemical pathways vital to chlamydial infection, and *Schisandra* lignans have previously been shown to affect many host-cellular pathways that are activated upon chlamydial replication.[13,16,41,42] *Schisandra* lignans are promising antichlamydial natural products that can be used as tools to further understand the *Chlamydia* life cycle and starting points for discovery of novel antibacterial agents.

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