The cell-penetrating peptide, Pep-1, has activity against intracellular chlamydial growth but not extracellular forms of *Chlamydia trachomatis*

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Objectives: In the course of studies to identify novel treatment strategies against the pathogenic bacterium, *Chlamydia*, we tested the carrier peptide, Pep-1, for activity against an intracellular infection.

Methods: Using a cell culture model of *Chlamydia trachomatis* infection, the effect of Pep-1 was measured by incubating the peptide with extracellular chlamydiae prior to infection, or by adding Pep-1 to the medium at varying times after infection, and assaying for inhibition of inclusion formation.

Results: Pep-1 had a concentration-dependent effect on chlamydial growth with 100% inhibition of inclusion formation at 8 mg/L peptide. There was a window of susceptibility during the chlamydial developmental cycle with a maximal effect when treatment was begun within 12 h of infection. Pep-1 treatment caused a severe reduction in the production of infectious progeny even when started later, when the effect on inclusion formation was minimal. Furthermore, electron micrographs showed a paucity of progeny elementary bodies (EBs) in the inclusion. In contrast, pre-incubation of EBs with Pep-1 prior to infection did not affect inclusion formation. Taken together, these findings indicate that the antichlamydial effect was specific for the intracellular stage of chlamydial infection. By comparison, Pep-1 had no antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* or the obligate intracellular parasite, *Toxoplasma gondii*.

Conclusions: Pep-1 has antichlamydial activity by preventing intracellular chlamydial growth and replication but has no effect on extracellular chlamydiae.

Keywords: *Chlamydia* spp., antimicrobial peptides, antimicrobial activity, antimicrobial agents

Introduction

Diseases caused by *Chlamydia* spp. remain a significant public health problem, despite effective antibiotics to treat acute infection. *Chlamydia trachomatis* is the leading cause of bacterial sexually transmitted disease in the United Kingdom and the United States, and chlamydial genital infections are by far the most common infectious disease reported to the CDC.¹² In the underdeveloped world, *C. trachomatis* causes trachoma, one of the most prevalent forms of preventable blindness.³ A related species, *Chlamydia pneumoniae*, causes respiratory tract infections and has been linked to a role in atherosclerotic heart disease.⁴⁵

While conventional antibiotics have been used to treat chlamydial infections, there has been growing interest in antimicrobial peptides as antichlamydial agents that can be used as a topical microbicide, particularly for the treatment and prevention of genital infections.⁶ A number of natural and synthetic antimicrobial peptides have been shown to have activity against elementary bodies (EBs), which are the infectious, extracellular form of chlamydiae.⁷¹³ EBs, however, have a heavily
cross-linked protein coat for survival outside the cell,\textsuperscript{14} which might affect the access of antimicrobial peptides to their site of action on the bacterial membrane.\textsuperscript{15} We reasoned, instead, that reticulate bodies (RBs), as the intracellular, metabolically active form of chlamydiae, would be a more attractive target. To test known antimicrobial peptides for their activity against RBs, we have been developing a novel delivery strategy that utilizes the cell-penetrating peptide, Pep-1, as a carrier peptide to transport an antimicrobial peptide into a Chlamydia-infected cell.

In this study, we report our serendipitous finding that Pep-1 itself has activity against intracellular chlamydiae. This 21-residue synthetic peptide (N-acetyl-KETWETWWTWESQ PKKKRKV-OH) contains a hydrophobic tryptophan-rich motif for efficient targeting of cell membranes, and a hydrophilic lysine-rich domain derived from the nuclear localization signal of simian virus 40 large T antigen.\textsuperscript{16} Pep-1 has been commercialized as a non-cytotoxic transfection reagent for the rapid delivery of cargoes as diverse as proteins, peptides and antibodies. In the only published study to examine the antimicrobial activity of this peptide, Pep-1 was found to have modest activity against Bacillus subtilis but minimal activity against other Gram-positive and Gram-negative bacteria.\textsuperscript{17} We have found that Pep-1 has activity against an intracellular chlamydia-infected but not on EBs prior to infection and that the effect is most pronounced earlier in the chlamydial developmental cycle.

Materials and methods

Cell culture

Mouse fibroblast L929 cells were grown in a 48-well culture plate in RPMI 1640 cell culture medium with 25 mM HEPES, 5% fetal bovine serum and 10 mg/mL gentamicin sulphate at 37°C and 5% CO\textsubscript{2}. The L929 monolayer in each well was infected with C. trachomatis serovars L2 (L2/434/Bu), LGV Biovar by centrifugation at 2000 rpm (700 g) in a Beckman Allegra 6 centrifuge for 1 h at room temperature. After centrifugation, the inoculum was replaced by 180 μL of fresh cell culture medium and the monolayers were incubated at 37°C. Infections were performed at a multiplicity of infection ( moi) of 0.1 in order to produce ~40–50 infected cells per high-power field as viewed through the 40× objective of a Zeiss Axiovert 40 CFL microscope. Infections were carried out for 36 h, unless otherwise stated. At the end of the infection, the monolayer was fixed in methanol, and the chlamydial inclusion was stained with a 1:200 dilution of Mab E4 [mouse monoclonal antibodies to the VD 4 epitope of MOMP from C. trachomatis serovar E (BOUR)\textsuperscript{18} (gift of Dr Ellena Peterson, University of California, Irvine, CA, USA) and goat anti-mouse secondary antibody conjugated to horseradish peroxidase (MP Biomedicals, Solon, OH, USA).

Synthesis of Pep-1

Pep-1 (N-acetyl-KETWETWWTWESQPKKKRKV-OH) was synthesized using published protocols that we have used for the synthesis of other peptides.\textsuperscript{19–21} The peptide was synthesized at a 0.3 mM scale on an Fmoc (9-fluorenylmethoxycarbonyl)-Val-poly-ethyleneleglycol-polystyrene support using a Millipore 9050 Peptide Synthesizer (PerSeptive Biosystem, CA, USA). Fmoc deprotection was carried out with 2% piperidine and 2% DBU (1,8-diazabicyclo [5.4.0]undec-7-ene) in DMF (N,N-dimethylmethanamide). Amino acids were coupled with in situ HATU (O-(7-aza benzotriazole-1-yl)-N,N,N′,N′-tetramethyllumonium hexafluorophosphate/NMM (N-methylmorpholine) activation. Residues Lys1, Trp4, Trp5 and Lys16 were double coupled with in situ PyBOP (benzotriazol-1-yl-oxy-tri-pyrrolidinophosphonium hexafluorophosphate)/NMM activation. Glu2, Thr3, Thr7, Thr10 and Ser13 were double coupled using the pentafluorophenyl ester derivatives. The N-terminal acetylation was carried out with acetic anhydride/HOBt (1-hydroxybenzotriazole) in DCM (dichloromethane)–NMM after the removal of the final Fmoc group. The peptide was cleaved from the solid support by a 4 h treatment with reagent K (trifluoroacetic acid/pheno/water/thioanisole/ethanediol, 82.5:5.5:5:2.5) in the presence of indol (5% w/v) in the dark, under nitrogen. The crude synthetic product was obtained with acetic acid/dichloromethane extraction followed by lyophilization of the aqueous phase. The peptide was purified to homogeneity by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) and water–acetonitrile gradients containing 0.01% trifluoroacetic acid. Purified Pep-1 was characterized by acid–urea PAGE, analytical RP-HPLC and MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), and peptide concentrations were determined by UV absorbance measurement using the extinction coefficient for tryptophan. Preparations of Pep-1 were stored as a lyophilized powder, or resuspended in 0.01% acetic acid at a concentration of 800 mg/L and stored at 4°C under nitrogen.

Pep-1 antichlamydial titration assay

In separate wells, each infected monolayer was treated with Pep-1 by the addition of 20 μL of diluted Pep-1 to the cell culture medium. A range of final concentrations of Pep-1 from 1 to 32 mg/L was tested with Pep-1 added 1 h after infection (i.e. after the centrifugation step) and left in the cell culture medium until the end of the 36 h infection. Separate dose–response experiments were also performed for Pep-1 treatment initiated at different times after infection (6, 12, 18 or 24 h.p.i.). In each case, Pep-1 was left in the cell culture medium until the end of the 36 h infection. For each time of treatment, a control monolayer was treated with 20 μL of 0.01% acetic acid without Pep-1. The percentage inhibition was calculated by counting the average number of inclusions in each of 10 fields as viewed through the 40× objective of a Zeiss Axiovert 40 CFL microscope, and comparing this number with the respective untreated control. In these and the following experiments, each assay was performed in duplicate wells and repeated as three independent experiments, and the mean and SD were calculated.

Washout assay

These experiments were performed according to the Pep-1 titration assay described earlier with the addition of a washing step to remove Pep-1 after a period of treatment. Experiments were repeated as three independent experiments, with Pep-1 added at a final concentration of 8 mg/L to the infected monolayers at 1, 6 or 12 h.p.i. At each addition, Pep-1 was removed at 1, 2, 4, 8 or 16 h by aspirating the medium and washing three times with 1 mL of sucrose–phosphate–glutamic acid (SPG) buffer. After the wash steps, 200 μL of fresh cell culture medium lacking Pep-1 was added, and the incubation was continued until the usual 36 h.p.i. when each monolayer was fixed and stained with MAb E4 as described earlier.

Longer duration of infection

In some experiments, the duration of infection was extended to determine whether the antichlamydial effect of Pep-1 was due to a delay in the time course of the chlamydial infection. Infected
monolayers were treated at 1 h.p.i. with Pep-1 at a final concentration of 4 mg/L and incubated until 36, 48 or 72 h.p.i. before fixing and staining with MAb E4.

**Recovery assay**

Infected monolayers were treated with Pep-1 at a final concentration of 8 mg/L starting at 18 h.p.i. and continuing until 36 h.p.i. when they were washed three times with 1 mL of SPG. An aliquot of 200 μL of fresh cell culture medium without Pep-1 was added, and the incubation was continued for an additional 12, 24 or 36 h before each monolayer was fixed and stained with MAb E4.

**EB progeny assay**

Monolayers of 10⁵ cells each were infected at an moi of 0.15 and treated with Pep-1 to a final concentration of 16 mg/L at 1 or 18 h.p.i. At 36 h.p.i., the medium from each well was removed and the 48-well plate was frozen at −70°C for 15 min and then incubated at 37°C for 15 min. SPG buffer (0.5 mL) was added to each well. The cells were removed by scraping and washed with an additional 0.5 mL of SPG buffer, and the combined cell lysate was collected. Serial 10-fold dilutions of the lysate in a volume of 200 μL were used to infect fresh L929 cells in a 48-well culture plate. The infected cells were fixed at 36 h.p.i. and stained with MAb E4 as described earlier. For each Pep-1 treatment time, the average number of inclusion forming units (IFUs) recovered was compared with an untreated control to calculate the fold decrease in infectious progeny. The experiments were performed in duplicate, and the mean and SD were calculated.

**Transmission electron microscopy (TEM)**

For ultrastructural analysis, infected cells were treated at a final concentration of 2 mg/L Pep-1 beginning at 1 h.p.i. and fixed at 36 h.p.i. in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA, USA) in 100 mM phosphate buffer, pH 7.2, for 1 h at room temperature. Samples were washed in phosphate buffer and post-fixed in 1% osmium tetroxide (Polysciences Inc.) for 1 h. Samples were then rinsed extensively in dH₂O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA, USA) for 1 h. Following several rinses in dH₂O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL, USA), stained with uranyl acetate and lead citrate and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA, USA).

**Pre-incubation assay**

About 1.5 × 10⁸ IFU of EBs from *C. trachomatis* LGV serovar L2 diluted in 180 μL of SPG were pre-incubated with Pep-1 diluted in 20 μL of 0.01% acetic acid so that the final Pep-1 concentration was 2, 8, 32 or 128 mg/L. The pre-incubations were performed at 4°C or room temperature for 1 h, in parallel with a mock treatment using 20 μL of 0.01% acetic acid instead of Pep-1. After the pre-incubation, the EB/Pep-1 mixture was used to infect L929 cells in a 48-well culture plate based on an expected moi of 0.15 if there was no effect with Pep-1 treatment. Infected monolayers were centrifuged for 1 h at 2000 rpm (700 g) in a Beckman Allegra 6 centrifuge at room temperature. After centrifugation, the inoculum was replaced with 200 μL of fresh cell culture medium lacking Pep-1, and the incubation was continued for 36 h.p.i. before fixing and staining with MAb E4.

**Pep-1 assays against other bacteria**

The antimicrobial activities of Pep-1 against *Escherichia coli* ML35 and *Staphylococcus aureus* 502a were measured with an agar diffusion assay, as described previously.²⁰ Wells of 10 μL diameter were bored in a 9 cm² plate of agarose, buffered with 10 mM 1,4-piperazine-bis(ethanesulfonic acid) (PIVES), pH 7.4, containing 5 mM glucose and seeded with 1 × 10⁶ mid-log phase cells. Pep-1, diluted in 0.01% acetic acid to a final volume of 5 μL, was added to each well and incubated at 37°C for 3 h. We tested a range of final concentrations of Pep-1 from 10 to 300 mg/L. As a positive control, we performed parallel experiments over the same peptide concentrations with a cyclic antimicrobial peptide, RTD-1, with known activity against *E. coli* and *S. aureus.*²⁹ Negative control experiments were performed with 0.01% acetic acid without Pep-1. After this incubation, the seeded agar was overlaid with molten agarose containing 6% trypticase soy broth. Plates were incubated at 37°C for 18–24 h and the antimicrobial activity was determined by measuring the diameter of the clearing around each well.

**Cytotoxicity assays**

The cytotoxicity of Pep-1 was measured using a WST-1 mitochondrial dehydrogenase assay (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s instructions. L929 cells were grown in a 96-well plate overnight. The medium was replaced with fresh medium containing 8 or 32 mg/L Pep-1 and incubated for 36 h at 37°C and 5% CO₂. WST-1 (10 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added to each well and allowed to incubate for an additional 1 h. The optical density at 450 nm was determined using a Multiscan RC plate reader (Thermo Electron, Milford, MA, USA). Toxicity from Pep-1 treatment was calculated by comparing Pep-1-treated cells with control cells treated with 0.01% acetic acid and reported as a percentage, with 0% being the absence of an effect.
To measure the effect of Pep-1 on cell proliferation, L929 cells were seeded in individual wells of a 48-well plate and grown overnight to form a subconfluent monolayer. The medium was replaced with fresh medium containing Pep-1 at a final concentration of 8 or 32 mg/L and incubated for a further 36 h at 37°C. Cells were then washed with PBS, removed with trypsin and resuspended in PBS containing 0.02% Trypan Blue. Viable cells were counted using a haemocytometer. The total number of viable cells after treatment with Pep-1 was reported as a percentage compared with a control monolayer treated with 0.01% acetic acid alone. These WST-1 cytotoxicity and cell proliferation assays were each performed in duplicate wells, repeated as three independent experiments, and reported as mean ± SD.

Results

Pep-1 inhibits chlamydial inclusion formation

To determine whether Pep-1 has any activity against Chlamydia, we tested the effect of the peptide in a cell culture model of chlamydial infection. We infected mouse L929 cells with C. trachomatis for 36 h and performed a titration assay adding Pep-1 at 1 h.p.i. over a range of final concentrations from 1 to 32 mg/L (equivalent to 0.36–11.4 μM). At 4 mg/L (=1.4 μM) of Pep-1, there was an 82% reduction in the number of chlamydial inclusions compared with an untreated control infection (Figure 1a and b). At 8 mg/L and higher concentrations of the peptide, there was a complete absence of chlamydial inclusions. We have obtained similar results with different batches of Pep-1 synthesized in our laboratory. However, two different commercial preparations, Chariot (Active Motif, Carlsbad, CA, USA) and Pep-1 (GenScript, Piscataway, NJ, USA), did not inhibit chlamydial inclusion formation when tested with the same protocol (data not shown). These results show that our synthesized Pep-1 molecule contains an N-acetyl group that was described in the original publication, but that is absent in the two commercial sources of the peptide.

The antichlamydial effect of Pep-1 is dependent on the time of addition during the chlamydial infection

We next varied the time at which Pep-1 was added after initiation of the infection to determine whether Pep-1 could
inhibit inclusion formation throughout the chlamydial developmental cycle or whether there was a window of susceptibility. Treatment with Pep-1 beginning at 6 or 12 h.p.i. inhibited chlamydial inclusion formation in terms of number and size to a similar extent as treatment at 1 h.p.i. (Figure 1c and d). In contrast, when Pep-1 treatment was delayed until 18 h.p.i., there was a decreased antichlamydial effect and the maximum inhibition of inclusion formation was 30% at 32 mg/L Pep-1 (Figure 2b). Nonetheless, both the number of inclusions and the size of the remaining inclusions were still reduced compared with a control infection with no Pep-1 treatment (Figure 2a). Later treatment starting at 24 h produced almost no inhibition of chlamydial inclusion formation (Figure 2c and d). These results demonstrate that the inhibitory effect of Pep-1 on chlamydial inclusion formation is limited to early stages of the chlamydial developmental cycle.

The antichlamydial effect of Pep-1 requires a minimum duration of treatment

To determine whether Pep-1 inhibition requires a minimum duration of treatment, we treated a chlamydial infection with Pep-1 for a period of time and then replaced the peptide-containing medium with fresh medium. We performed this washout experiment with treatments begun at 1, 6 or 12 h.p.i. using a Pep-1 concentration that caused almost complete inhibition of inclusion formation (8 mg/L). For each treatment start time, the Pep-1-containing medium was removed after incubation for 1, 2, 4, 8 or 16 h and replaced with fresh medium lacking Pep-1. We found that the full antichlamydial effect of Pep-1 required exposure for at least 4 h (Figure 3). For example, with treatment begun at 1 h.p.i., there was reduced inhibition of inclusion formation with exposure to Pep-1 for 1 h (13% inhibition) or 2 h (59% inhibition). Only after 4 h of Pep-1 exposure was there 98% inhibition comparable to the full antichlamydial effect at this Pep-1 concentration when the peptide was left in the medium for the duration of the infection (compare with Figure 1b). The results were similar for treatment started at 6 h.p.i., which required Pep-1 exposure for 4 h to produce 84% inhibition comparable to the full antichlamydial effect at this Pep-1 concentration (compare with Figure 1c). Treatment with Pep-1 started at 12 h.p.i. required a longer exposure period, as removal of the Pep-1-containing medium after treatment for 8 h or less reduced the antichlamydial effect. These results suggest that a minimal treatment time of ~4 h is required for Pep-1 to
inhibit chlamydial inclusion formation and that the antichlamydial effect can be prevented or abrogated if Pep-1 is removed from the medium before this duration of treatment.

**Pep-1 does not exert its antichlamydial effect by slowing down the chlamydial growth cycle**

To determine whether Pep-1 slowed down the time course of chlamydial infection, we tested whether extending the time of infection could mitigate the inhibition of inclusion formation. We treated infected cells with 4 mg/L Pep-1 at 1 h.p.i. (which produced an 82% reduction in inclusion number in the preceding experiments) and allowed the infection to proceed for a longer time, stopping the infection at 48 or 72 h instead of the standard 36 h. We observed the same decrease in inclusion number despite the longer infection time (data not shown), which suggests that the antichlamydial effect of Pep-1 could not be accounted for by a delay in inclusion formation.

**Pep-1 blocks chlamydial growth in a non-reversible manner**

We performed a recovery assay to determine whether Pep-1 treatment left viable chlamydiae that could replicate upon removal of Pep-1. To increase our chance of detecting any recovery, we chose a Pep-1 treatment condition that prevented the detection of inclusion formation. Three separate sets of experiments were performed with Pep-1 added to the cell culture medium at 1, 6 or 12 h.p.i. For each starting time of treatment, Pep-1 was incubated with the infected monolayer for 1, 2, 4, 8 or 16 h before the Pep-1-containing medium was removed and replaced with fresh medium lacking Pep-1. The average number of inclusions for each Pep-1 treatment was compared with an untreated control to calculate the percentage inhibition. Each assay was performed in duplicate wells and repeated as three independent experiments. The results are reported as a mean with SDs marked by error bars.

**Pep-1 inhibits the production of infectious progeny**

As Pep-1 had a negative effect on chlamydial inclusions, we tested if Pep-1 could also inhibit the production of infectious chlamydiae. We performed an EB progeny assay in which we treated a chlamydial infection with Pep-1 and then passaged the progeny in fresh L929 cells to measure the infectious yield. At a Pep-1 treatment condition that prevented the detection of inclusion formation (16 mg/L Pep-1 started at 1 h.p.i.), we measured a 5.4 $\times 10^3$-fold reduction in infectious progeny compared with a negative control infection with no Pep-1 treatment (Figure 4). In this experiment, we recovered 303 IFU, which is a small fraction of the $1.5 \times 10^4$ IFU used for the infection or the number of cells ($10^8$) that could have sustained a productive infection. We measured a large reduction in infectious progeny ($3.6 \times 10^2$-fold decrease compared with no treatment) even when treatment caused partial inhibition of inclusion formation (16 mg/L Pep-1 started at 18 h.p.i., which produced a 22% reduction in inclusion number and a decrease in inclusion size) (Figure 4). These results indicate that Pep-1 has an antichlamydial effect in inhibiting the production of infectious chlamydiae and that the magnitude of this effect exceeds the reduction in the number of chlamydial inclusions.

**Pep-1 causes a reduction in the number of EBs within the inclusion**

To further examine the effect of Pep-1 on the production of chlamydial progeny, we performed an ultrastructural analysis of the Pep-1-treated inclusion. In order to visualize these inclusions, we chose Pep-1 treatment conditions that caused partial inhibition of inclusion formation (2 mg/L Pep-1 started at 1 h.p.i.). At 36 h.p.i., the monolayers were fixed and examined by TEM. Inclusions from an untreated control sample showed many more EBs than RBs, which is typical of a late-stage chlamydial inclusion where most of the RBs have already differentiated into EBs (Figure 5a). In contrast, inclusions in the Pep-1-treated cells contained mostly RBs and very few EBs.
Pep-1 activity against *Chlamydia trachomatis*

Even at higher magnification, chlamydiae within treated cells did not show any morphological abnormalities such as the large aberrant RBs characteristic of IFN-γ-induced persistent infection. This paucity of EBs is consistent with the severe reduction in infectious progeny that we have measured after Pep-1 treatment.

**Pep-1 does not have a direct inhibitory effect on the extracellular form of chlamydiae**

To test whether Pep-1 has a direct effect on EBs, we pre-treated EBs with Pep-1 at 4°C or room temperature for 1 h and performed the subsequent cell culture infection in the absence of Pep-1 treatment. We found that Pep-1 pre-incubation at either temperature had no measurable effect on the number and size of chlamydial inclusions compared with a control experiment with mock treatment of EBs (data not shown). These results indicate that Pep-1 does not appear to affect the extracellular form of chlamydiae prior to infection, which is in contrast to its inhibitory effect during a chlamydial infection.

**Pep-1 does not have significant antimicrobial activity against *E. coli* and *S. aureus***

To determine whether Pep-1 has activity against other bacteria, we used an agar diffusion assay to measure the activity against *E. coli* and *S. aureus*. Pep-1 had no effect on *E. coli* and only a modest effect against *S. aureus* (data not shown). For comparison, we performed a positive control experiment with RTD-1, a cyclic antimicrobial peptide from the rhesus monkey, which showed activity against both bacteria. These results indicate that Pep-1 does not have a general antibacterial activity.

**Pep-1 does not inhibit invasion or growth of the intracellular parasite, *Toxoplasma***

To determine whether the effect of Pep-1 against intracellular growth is *Chlamydia*-specific, we tested Pep-1 against another obligate intracellular parasite, *T. gondii*, which also grows inside an intracytoplasmic vacuole. Over a range of concentrations from 4 to 16 mg/L, Pep-1 had no effect on the ability of *Toxoplasma* parasites to infect HFF monolayers, as measured with invasion and intracellular growth assays (data not shown). These results demonstrate that Pep-1 does not have a general inhibitory effect on the ability of a host cell to sustain an intracellular infection.

**Measurement of Pep-1 cytotoxicity**

We directly measured the cytotoxicity of Pep-1 with two assays even though it is marketed as a non-cytotoxic transfection reagent. In a WST-1 mitochondrial dehydrogenase assay, Pep-1 showed almost no cytotoxicity to L929 cells at 8 mg/L (mean: 0.8 ± 14.4%) and slight cytotoxicity at 32 mg/L (mean: 18.2 ± 7.8%). In a cell proliferation assay, Pep-1 had little effect on a subconfluent L929 monolayer compared with an untreated control at 8 mg/L (mean: 96.3 ± 10.9%). However, cell proliferation was decreased by treatment with 32 mg/L Pep-1 for 36 h compared with no treatment (mean: 51.1 ± 14.2%). Thus, Pep-1 did not cause cytotoxicity at concentrations that produced a significant antichlamydial effect, although cellular proliferation could be inhibited by prolonged exposure at high concentrations.

**Figure 5.** TEM showing that Pep-1 reduces the number of EBs within the chlamydial inclusion. Uninfected cells (a) and cells treated with 2 mg/L Pep-1 (b) were fixed at 36 h.p.i. and examined by TEM. Representative electron micrographs are shown at 6000× magnification (scale bar represents 2 µm). (c) Higher magnification (20 000×) of chlamydiae within a Pep-1-treated cell (scale bar represents 0.5 µm). In each image, an RB is indicated with an arrow and an EB is marked by an arrowhead.

(Figure 5b). Even at higher magnification, chlamydiae within treated cells did not show any morphological abnormalities (Figure 5c), such as the large aberrant RBs characteristic of IFN-γ-induced persistent infection. This paucity of EBs is consistent with the severe reduction in infectious progeny that we have measured after Pep-1 treatment.
Discussion

This study demonstrates that Pep-1, a peptide used as a carrier to transport cargo molecules into eukaryotic cells, inhibits the intracellular growth of Chlamydia but does not affect the infectivity of the extracellular form of this bacterium. The time course of Pep-1 activity, with the greatest effect when treatment was initiated within the first 12 h of infection, is consistent with a time in the chlamydial developmental cycle when RBs are present and dividing. In contrast, Pep-1 had no effect when pre-incubated with EBs prior to infection and a diminishing effect when added at 18 h post-infection or later, corresponding to a stage when RBs have replicated and conversion to EBs has begun. Our progeny assays and EM studies indicate that Pep-1 treatment inhibited the formation of EBs, although it is not clear whether the block was at the actual conversion stage or at a prior step in chlamydial growth. We have not been able to test the activity of Pep-1 against RBs directly as purified RBs are no longer infectious, and thus the effect of incubating RBs with Pep-1 cannot be assayed.

A number of studies have shown that naturally occurring and synthetic antimicrobial peptides have activity against Chlamydia spp., although the usual means of testing has been to assay the effect on EBs. Cecropins, melittin and cathelicidins, such as protégirin, are among the antimicrobial peptides that have been shown to have antichlamydial activity when incubated with EBs prior to a cell culture infection. In some cases, the antimicrobial peptide has been shown to have a direct effect on EBs with electron micrographs showing damaged EBs appearing as outer membrane shells lacking cytoplasmic contents. In fact, EBs, as the extracellular, infectious form of chlamydiae, have been proposed as the preferred target for a topical microbicidal, and antimicrobial peptides have been tested in gel formulations against EBs for potential use as a topical microbicidal.

In contrast, there is only limited evidence from published studies that antimicrobial peptides have activity against intracellular chlamydiae. Antimicrobial peptides have not inhibited chlamydial inclusion formation when incubated with Chlamydia-infected cells. However, it is not known whether these negative results were due to the lack of an effect against intracellular chlamydiae or failure of the antimicrobial peptide to enter infected cells. In one instance, however, an antimicrobial peptide has been shown to have an antichlamydial effect when expressed inside an infected cell. Using an innovative plasmid transfection approach, Lazarev et al. showed that expression of melittin in C. trachomatis-infected cells, under the control of a tetracycline-responsive promoter, resulted in a 75% reduction in inclusion number. In a subsequent study, they also tested their transfection approach against a murine genital infection with intravaginal administration of the melittin-expressing plasmid and found that there was a reduction in chlamydial shedding. Therapeutic approaches based on the inhibition of intracellular chlamydial growth offer the potential for both preventing transmission and interrupting an established chlamydial infection.

Prior to this report, only one published study had examined Pep-1 for antimicrobial activity, but Pep-1 itself was found to have poor antibacterial activity. That study by Zhu et al. was prompted by the observation that the cationic nature, and the amphipathic and α-helical structure of Pep-1 were reminiscent of antimicrobial peptides in general. By altering the Pep-1 sequence and replacing three glutamic acid residues with lysines, the authors created a peptide called Pep-1-K with antibacterial activity against both Gram-positive and Gram-negative bacteria. To the best of our knowledge, Pep-1-K has not been tested against intracellular bacteria such as Chlamydia spp.

In our studies, we detected activity against chlamydial growth with different batches of Pep-1 that we had synthesized but not with two commercial preparations of Pep-1 [Chariot (Active Motif) and Pep-1 (GenScript)]. The Pep-1 molecule that we synthesized is based on the original description of Pep-1, which has an acetylated N-terminus. In contrast, both commercial preparations of Pep-1 lack this N-acetyl group, which may account for the absence of antichlamydial activity in them. Our Pep-1 preparation was highly purified, as assayed by mass spectrometry and the appearance of a single band on acid–urea PAGE (data not shown), making it highly unlikely that its antichlamydial activity was due to a contaminant.

In conclusion, Pep-1 is a small molecule that inhibits the growth and replication of intracellular chlamydiae but has no effect on extracellular chlamydiae. This effect appears to be specific to chlamydial growth as Pep-1 had no activity against other bacteria such as E. coli and S. aureus, or another intracellular parasite, T. gondii. We believe that this unusual activity of Pep-1 may be due to a combination of the ability of this peptide to enter a Chlamydia-infected cell and its activity against intracellular chlamydial growth. This antichlamydial activity may be the result of a direct effect on chlamydiae or an indirect effect through targeting of the chlamydial inclusion or host cell processes that are essential for chlamydial growth.

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Transparency declarations

None to declare.

References

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