Anti-chlamydial effect of plant peptides

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Abstract

Even in asymptomatic cases of *Chlamydia trachomatis* infection, the aim of the antibiotic strategy is eradication of the pathogen so as to avoid the severe late sequelae, such as pelvic inflammatory disease, ectopic pregnancy, and tubal infertility. Although first-line antimicrobial agents have been demonstrated to be predominantly successful in the treatment of *C. trachomatis* infection, treatment failures have been observed in some cases. Rich source of antimicrobial peptides was recently discovered in *Medicago* species, which act in plants as differentiation factors of the endosymbiotic bacterium partner. Several of these symbiotic plant peptides have proved to be potent killers of various bacteria *in vitro*. We show here that 7 of 11 peptides tested exhibited antimicrobial activity against *C. trachomatis* D, and that the killing activity of these peptides is most likely due to their interaction with specific bacterial targets.

Keywords: Chlamydia, antimicrobial, NCR peptide

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Introduction

Chlamydia trachomatis is a Gram-negative, obligate intracellular bacterium with a characteristic biphasic life cycle, forming metabolically inactive infectious forms (elementary bodies [EBs]) and metabolically active, non-infectious forms (reticulate bodies [RBs]). Serovars D to K cause urogenital infections that are often asymptomatic, but which can lead to severe complicated diseases [1].

C. trachomatis is of great public health significance because of the impacts of the untreated diseases on human reproduction. Cervicitis and urethritis commonly occur in women and about 40% of the untreated cases progress to pelvic inflammatory disease (PID). Infertility results in 20% of the PID cases, while 18% of the women with this disease experience chronic pelvic pain, and 9% may suffer an ectopic pregnancy [2].

At the individual level, *C. trachomatis* infection can generally be treated effectively with antibiotics, though antibiotic resistance appears to be increasing [2]. At the population level, public health control of the infection is rather problematic. With regard to the severe potential consequences of urogenital *C. trachomatis* infection in women, many countries offer screening. Vaccination, which is currently unavailable, would be the best way to reduce the prevalence of *C. trachomatis* infections, as it would be much cheaper and would have a greater impact on controlling *C. trachomatis* infections worldwide [3]. The development of new antimicrobial agents is required to overcome this problem.

Antimicrobial peptides (AMPs), natural antibiotics produced by nearly all organisms, from bacteria to plants and animals, are crucial effectors of innate immune systems, with different spectra of antimicrobial activity and with the ability to perform rapid killing. To date, more than 800 AMPs have been discovered in various organisms, including 270 from plants. It has become clear in recent years that these peptides are able not only to kill a variety of

pathogens, but also to modulate immune responses in mammals. However, their modes of action are poorly understood. In some species these peptides serve as the primary antimicrobial defense mechanism, whereas in others they serve as an adjunct to existing innate and adaptive immune systems [4]. Cationic AMPs interact with negatively charged microbial membranes and permeabilize the membrane phospholipid bilayer, resulting in lysis and the death of microbes [5, 6]. In view of their rapid and broad-spectrum antimicrobial properties, interest has emerged in AMPs as potential antibiotic pharmaceuticals with which to combat infections and microbial drug resistance [7, 8].

Most plant AMPs are cysteine cluster proteins. This group includes major plant immunity effectors such as defensins, and also symbiotic peptides, including the nodule-specific cysteine rich (NCR) peptides, which are produced in Medicago -*Sinorhizobium meliloti* symbiosis and provoke irreversible differentiation of the endosymbiont. The NCR family is composed of about 500 divergent peptides in *Medicago truncatula* [9, 10, 11]. Some cationic NCRs have been shown to possess genuine antimicrobial activities *in vitro,* killing various Gram-negative and Gram-positive bacteria highly efficiently [12].

In the present study, 7 of the 11 NCR peptides examined displayed dose- and time-dependent anti-chlamydial activity *in vitro*. NCR247 was also demonstrated to bind to the 60-kDa putative GroEL protein of *C. trachomatis* D.

Materials and Methods

Inoculum preparation

C. trachomatis D (ATCC) was propagated on HeLa cells as described earlier [13]. The partially purified and concentrated EBs were aliquoted and stored at -80 °C until use. A mock preparation was prepared from an uninfected HeLa cell monolayer processed in the same way as the infected cells. The titer of the infectious EBs was determined by indirect

immunofluorescence assay. Serial dilutions of the EB preparation were inoculated onto tissue culture monolayers and, after a 48-h culture, cells were fixed with acetone and stained with murine monoclonal anti-*Chlamydia* LPS antibody (AbD Serotec, Oxford, UK) and FITC-labeled secondary anti-mouse IgG (Sigma, St. Louis, MO, USA). The number of inclusions was counted under a UV microscope, and the titer was expressed in inclusion forming units/ml (IFU/ml).

Measurement of in vitro antibacterial activity of NCR peptides

First, the toxicity of the NCR peptides was tested on non-infected HeLa cells in the highest concentration (10 μg/ml) used during our experiments. The toxic peptides were excluded from the further experiments.

EBs of *C. trachomatis* D (4×10^4 IFU/ml) were incubated with chemically synthesized mature NCR030 (AFLPTSRNCITNKDCRQVRNYIARCRKGQCLQSPVR pI=10,37); NCR044 (AFIQLSKPCISDKECSIVKNYRARCRKGYCVRRRIR pI=10,32); NCR055 (VNDCIRIHCKDDFDCIENRLQVGCRLQREKPRCVNLVCRCLRR pI=9,21); NCR095 (ELVCDTDDDCLKFFPDNPYPMECINSICLSLTD pI=3,62); NCR137 (MTLRPCLTDKDCPRMPPHNIKCRKGHCVPIGKPFK pI=9,7); NCR168 (YPFQECKVDADCPTVCTLPGCPDICSFPDVPTCIDNNCFCT pI=3,61); NCR169 (EDIGHIKYCGIVDDCYKSKKPLFKIWKCVENVCVLWYK pI=8,45); NCR183 (ITISNSSFGRIVYWNCKTDKDCKQHRGFNFRCRSGNCIPIRR pI=10,1); NCR192 (MKNGCKHTGHCPRKMCGAKTTKCRNNKCQCVQL pI=9,54); NCR247 (RNGCIVDPRCPYQQCRRPLYCRRR pI=10,15); or NCR280 (MRVLCGRDGRCPKFMCRTFL pI=9,8) (Proteogenix Oberhausbergen, France) at various concentrations (10, 5, 2.5, or 1.25 μg/ml) in sucrose-phosphate-glutamic acid buffer (SPG) for 2 h at 37 °C. As control, *C. trachomatis* D was incubated in buffer alone. The time courses of

the anti-chlamydial effects of the NCR peptides were tested after incubation periods of 15, 30, 60 and 120 min. To quantify the anti-chlamydial effects of the NCR peptides, HeLa cells were seeded in 24-well tissue culture plates with 13-mm cover glasses. After 24 h, the confluent cells were infected with NCR-treated *C. trachomatis* D or the control. After 48 h, the cells were fixed with acetone at −20 °C for 10 min. Fixed cells on cover glasses were stained by the indirect immunofluorescence method described in "Inoculum preparation" section. The number of recoverable inclusions was counted under a UV microscope, and the titre was expressed in IFU/ml.

Far-Western blot assay for identification of NCR-binding Chlamydia proteins

Concentrated *C. trachomatis* (2×10^5 IFU) (prepared as described earlier) and a mock preparation were heated at 95 °C for 5 min in sample buffer, and polyacrylamide gel electrophoresis (PAGE) was performed. The proteins were separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel in duplicate, and half of the gel carrying the separated proteins of the *C. trachomatis* or the mock samples was blotted onto a polyvinylidene difluoride membrane (SERVA, Heidelberg, Germany). The membrane was blocked overnight at 4 °C with 5% skimmed milk and 0.05% Tween 20 containing PBS. The membrane was probed for 4 h with a buffer [1% bovine serum albumin in PBS with 0.05% Tween 20 (PBST)] containing 10 μg/ml NCR247. After washing 3 times with PBST, the filter was incubated with anti-NCR247 rabbit IgG for 4 h and further incubated after washing 3 times with HRP-conjugated anti-rabbit antibody (Sigma). A control lane with separated *C. trachomatis* EBs was also incubated with anti-NCR247 and HRP-conjugated anti-rabbit antibody without prior treatment with NCR247 peptide. Following 3 further washings, the colour was developed by using diaminobenzidine tetrahydrochloride (Sigma) with hydrogen peroxide in 10 mM Tris at pH 7.5. The second half of the gel with the separated proteins of *C.*

trachomatis or the mock preparation was stained with PageBlue Protein Staining Solution (Fermentas).

Identification of proteins by mass spectrometry

The gel slices containing the polypeptides of the concentrated *C. trachomatis* EBs corresponding to proteins exhibiting NCR247 positivity in the blotting assay were cut out from the gel and analyzed by mass spectrometry. Briefly, protein bands were diced and washed with 25 mM NH_4HCO_3 in 50% (v/v) acetonitrile/water. Disulphide bridges were reduced with dithiothreitol (DTT), and free sulphydryls were alkylated with iodoacetamide. Proteins were digested with modified porcine trypsin (Promega Madison, WI, USA) for 4 h at 37 °C. Samples were analysed on liquid chromatography-tandem mass spectrometry (LC-MSMS) instruments. LC-MSMS raw data were converted into a Mascot generic file with Mascot Distiller software (v2.1.1.0). The resulting peak lists were searched by using the Mascot Daemon software (v2.2.2) against the NCBI non-redundant database without species restriction (NCBInr 20080718, 6833826 sequences). Monoisotopic masses with a peptide mass tolerance of ±0.6 Da and a fragment mass tolerance of 1 Da were submitted. Carbamidomethylation of Cys was set as a fixed modification, and acetylation of protein N-termini, methionine oxidation, and pyroglutamic acid formation from peptide N-terminal Gln residues were permitted as variable modifications. Acceptance criteria were at least 2 individual peptides with a minimum peptide score of 55 per protein.

Detection of NCR peptide binding to Chlamydia EBs by FACS

Chlamydia EBs (1×10^6 IFU) were treated with 1 µg of FITC-labelled NCR247 or FITC-labelled NCR035 peptide containing PBS for 2 h at 37 °C. As controls, untreated Chlamydia

EBs were used. After 3 times washing with PBS, cells were analyzed with the FACS StarPlus (Becton Dickinson) device.

Results

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To determine whether they possess anti-chlamydial activity, 11 NCR peptides (NCR030, NC0R44, NCR055, NCR095, NCR137, NCR168, NCR169, NCR183, NCR192, NCR247 and NCR280) were co-incubated individually with *C. trachomatis* EBs at 10 μg/ml for 2 h at 37 °C. Counting of the number of viable *C. trachomatis* inclusions demonstrated that 7 of the 11 peptides (NCR044, NCR055, NCR095, NCR183, NCR192, NCR247 and NCR280) were effective killers of *C. trachomatis in vitro*, while NCR030 and NCR168 displayed weaker activity and NCR137 and NCR169 did not exert an anti-chlamydial effect (Fig. 1A). *C. trachomatis* inclusions were then treated for 2 h with concentrations of the peptides ranging from 1.25 μg/ml to 10 μg/ml (Fig. 1B). NCR044, NCR055 and NCR183 were found to exert the strongest anti-chlamydial activities by reducing the viability to 95%, 78% and 85%, respectively, at 1.25 μg/ml, whereas the other peptides revealed no effect at 1.25 μg/ml concentration. NCR192 and NCR247 had significant anti-chlamydial effects at 2.5 μg/ml concentration. The time course of killing was investigated in the cases of NCR044, NCR055, NCR183 and NCR247 at 5 μg/ml concentration (Fig. 1C). NCR044 elicited the fastest effect, achieving an 80% reduction in the number of viable Chlamydia inclusions after a 15-min co-incubation with *C. trachomatis* EBs. The other three peptides required longer times to attain the killing effect. Of the tested peptides therefore, NCR044 exhibited the strongest anti-chlamydial activity, acting at the lowest concentration and most rapidly.

Identification of the chlamydial ligand responsible for NCR247 binding

Further investigations were carried out with NCR247, which displayed anit-chlamydial activity in the previous tests.

To identify the chlamydial ligand responsible for NCR peptide binding, concentrated *C. trachomatis* EB preparations and mock control preparations were separated by SDS-PAGE. After blotting, the membranes were probed with synthetic NCR247 peptide and incubated with anti-NCR247 IgG and then with HRP-labeled anti-rabbit antibody. The control lane with Chlamydia EBs was stained with anti-NCR247 IgG and HRP-labeled anti-rabbit antibody without incubation with synthetic NCR247 peptide. The synthetic NCR247 peptide was bound to a 60-kDa protein band in the Chlamydia lysate (Fig. 2A, lane 4). The synthetic NCR247 did not react with the mock lysate (lane 2), and the Chlamydia EB lysate did not react with the HRP-conjugated anti-rabbit antibody (lane 3). The gel slice containing the corresponding polypeptide of the concentrated *C. trachomatis* EBs associated with the synthetic NCR247 peptide was cut out from the gel and analyzed by LC-MSMS. A 60 kDa putative GroEL protein of Chlamydia was indicated by LC-MSMS and confirmed by post source decay analysis (Fig. 2B).

FACS analysis for the detection of NCR247 binding to the whole C. trachomatis EBs

To show that NCR247 is able to bind not only to the degraded Chlamydia particles but to the native, viable Chlamydia EBs, a FACS analysis was carried out. Fig. 3 reveals that Chlamydia EBs interacted with FITC-conjugated NCR247 peptide. Untreated or FITC-labeled NCR035 peptide-treated (this peptide showed no anti-chlamydial effect earlier) Chlamydia EBs did not demonstrate increased fluorescence.

Discussion

C. trachomatis is the leading cause of sexually transmitted bacterial diseases in both developed and developing countries, with more than 90 million new cases of genital infections occurring annually. The development of effective new antimicrobial compounds is indispensable if the late severe sequelae of the infections, such as ectopic pregnancy and infertility, are to be avoided [14]. AMPs appear to be potentially promising candidates for this purpose. Although their antimicrobial activity against bacteria, fungi and protozoa has been extensively tested [15], their anti-chlamydial action has not yet been tested. In the present study, therefore, we investigated the *in vitro* activity of 11 NCR peptides against *C. trachomatis*. Seven of these peptides exerted significant anti-chlamydial activity at a 10 μg/ml concentration. A number of synthetic NCR peptides from *Medicago truncatula* have been reported to be potent killers of various Gram-negative (*Escherichia coli*, *Salmonella* Typhimurium, *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa* and *Xanthomonas campestris*) and Gram-positive (*Bacillus megaterium*, *Bacillus cereus*, *Clavibacter michiganensis*, *Staphylococcus aureus* and *Listeria monocytogenes*) bacteria, including human/animal and plant pathogens [12]. Furthermore, AMPs were effective against *Staphylococcus epidermidis* in *in vivo* mouse model, and they also displayed anti-inflammatory activity [8].

Our LC-MSMS experiment identified the GroEL protein of *C. trachomatis* as the chlamydial ligand of the NCR247 peptide. The GroEL protein is one of the few proteins that have so far been confirmed as relevant in chlamydial pathogenesis; it is also referred to as heat shock protein 60 (Hsp60) [16]. This protein belongs to group I chaperones produced by almost all prokaryotic and eukaryotic cells, which assist as intracellular proteins, in the correct folding of nascent or denatured proteins under both normal and stress conditions [17]. Several reports have indicated that molecular chaperones produced by pathogenic bacteria,

can function as intracellular, cell surface, or extracellular signals in the course of infection processes [18]. The immune responses to chlamydial GroEL correlate significantly with disease sequelae in humans, and 80 to 90% of patients infected with *C. trachomatis* have antibodies directed against GroEL [19]. The high degree of antigenicity of GroEL in patients implies that the protein is easily accessible to the immune system, perhaps because it is localized on the surface of the chlamydial particles. Early studies on isolated outer membrane complexes from *C. trachomatis* and *Chlamydophila psittaci* EBs had indeed pointed to the possibility that GroEL might be associated with chlamydial membranes [20]. Taken together, GroEL is accessible for the binding of the NCR247 peptide.

The present study indicates that certain of the NCR peptides possess substantial *in vitro* activity against *C. trachomatis* D. Studies of chlamydial infection in animal models are clearly needed to establish whether they have parallel *in vivo* results and whether these peptides can be useful lead compounds for the development of anti-chlamydial drugs.

Acknowledgements

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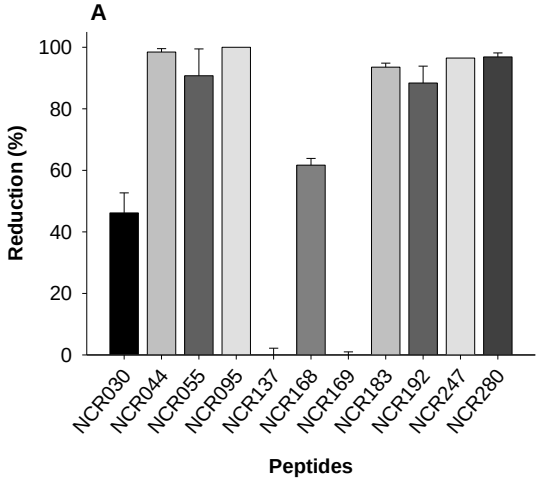
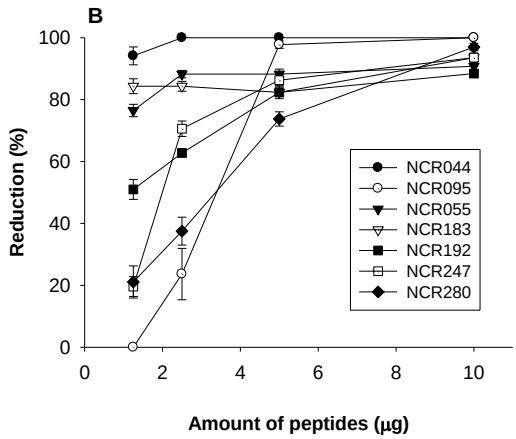
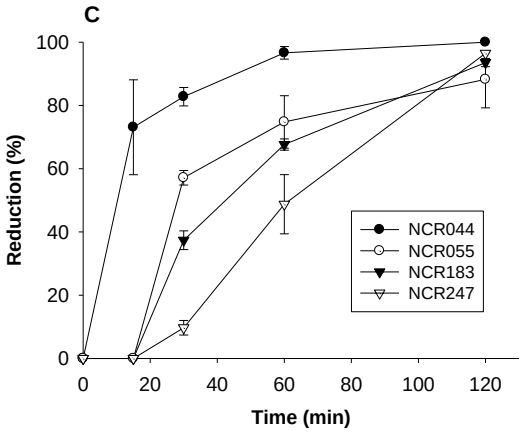
Figures

Fig. 1. Concentration and time dependences of the anti-chlamydial effects of NCR peptides. *C. trachomatis* at $4x10^4$ IFU/ml was incubated with 10 μg/ml of synthetic NCR peptide for 2 h at 37 °C (A). *C. trachomatis* EBs were incubated with different quantities of synthetic NCR peptides for 2 h at 37 °C (B) *C. trachomatis* was co-incubated individually with different NCR peptides (5 μg/ml) for 0, 15, 30, 60 or 120 min (C). The infectivity of the NCR peptide-treated *C. trachomatis* was determined by inoculating the mixture onto confluent HeLa cells on cover glasses. After a 24-h incubation, the fixed cells were stained with anti-chlamydia LPS antibody and the number of inclusions was counted under a UV microscope. All the data are representative of three separate experiments.






C
Reduction (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (min)
NCR044
NCR055
NCR183
NCR247

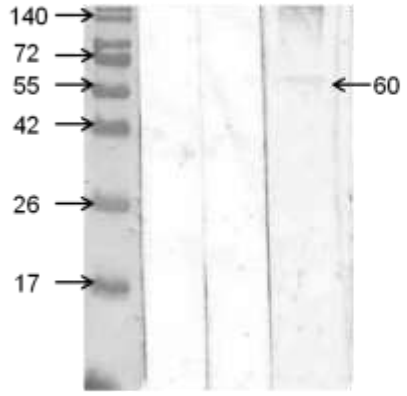
328

329

330

Fig. 2. Interaction of NCR247 peptide and *C. trachomatis* EBs. Far-Western blot analysis of the chlamydial ligands responsible for NCR247 peptide binding. (A) Concentrated *C. trachomatis* and mock control preparations were separated by SDS-PAGE. After blotting, the membrane (lane 2,4) was probed with synthetic NCR247 peptide and incubated with anti-NCR247 IgG and HRP-labelled anti-rabbit antibody. (lane 1- molecular weight marker, lane 2 - mock preparation, lane 4 - Chlamydia EBs lysate). A control lane (lane 3) with separated *C. trachomatis* EBs was also incubated with anti-NCR247 and HRP-conjugated anti-rabbit antibody without prior treatment with the NCR247 peptide. Identification of the *C. trachomatis* proteins by LC-MSMS (B). Peptide fragments that match the defined protein sequences are to be found in the Table.

A



B

D7DCT1 Uniprot ID: D7DCT1_CHLTD Species: CHLTD Name: 60 kDa chaperonin
Organism: Chlamydia trachomatis serovar D (strain D-EC) Gene: groEL Existence: Inferred from homology Version: 1
Protein MW: 58147.4 Protein pI: 5.3 Protein Length: 544

Num Unique	% Cov	Best Disc Score	Best Expect Val
17	36.9	4.84	8.1e-8

m/z	z	Da	Peptide	RT	Start	End	Score	Expect
643.0846	3	0.040	EVELADKHENMGAQMVK	13.5501	59	75	18.6	0.015
615.7597	2	-0.051	NVTAGANPMDLK	15.3489	106	117	36.5	1.9e-5
911.0538	3	-0.066	EIAQVATISANNDAEIGNLIAEAM(Oxidation)EK	27.2683	143	168	26.1	7.9e-6
997.1231	3	-0.016	NGSITVELAKGFETVLDIVEGMNFNR	35.3565	172	197	20.3	0.0027
928.9320	2	-0.014	GFETVLDIVEGM(Oxidation)NFNR	29.8399	182	197	46.0	2.4e-7
920.9175	2	-0.031	GFETVLDIVEGMNFNR	33.6069	182	197	49.1	8.1e-8
1122.8075	3	0.011	GYLSSYFATNPETQEC(Carbamidomethyl)VLEDALVLIYDKK	34.1365	198	226	22.8	1.6e-6
826.1337	3	0.028	KAM(Oxidation)LEDIAILTGGQLISEELGMK	31.5225	286	308	30.6	1.1e-6
820.7403	3	-0.034	KAMLEDIAILTGGQLISEELGMK	33.2856	286	308	57.2	4.5e-7
1173.6437	3	0.034	AM(Oxidation)LEDIAILTGGQLISEELGMKLENANLAM(Oxidation)LGK	35.7915	287	319	20.4	7.4e-4
1168.3364	3	0.058	AMLEDIAILTGGQLISEELGM(Oxidation)KLENANLAMLGK	37.7253	287	319	29.5	1.0e-6
1166.8025	2	-0.0081	AMLEDIAILTGGQLISEELGMK	36.0232	287	308	47.2	6.3e-7
595.2812	2	-0.035	LENANLAM(Oxidation)LGK	15.5306	309	319	34.9	4.2e-6
665.3108	3	0.0022	EDTTIVEGM(Oxidation)GEKEALEAR	14.4267	328	345	19.8	0.0080
763.8327	2	0.0044	QrGln ~pyro-Glu)EDSSSDYDKEK	11.5751	352	364	18.1	0.0078
524.7479	2	-0.023	VGAATEIEMK	13.1456	381	390	35.2	3.5e-5
861.8609	2	-0.055	DAYTDMLEAGILDPAK	27.4021	485	500	31.1	1.8e-4

Fig. 3. FACS analysis of NCR247 peptide binding to whole *C. trachomatis* EBs. Untreated and unstained Chlamydia EBs (A). FITC-labelled NCR247 peptide treated *C. trachomatis* EBs (B). Chlamydia EBs treated with FITC-labelled NCR035 peptide (C).