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The Synthesis and Biological Investigation of New Potent Chimeric Antimicrobial NCR247 Derivatives

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Introduction

The worldwide spread of antibiotic-resistant bacteria and the increasing mortality rate by untreatable microbial infections makes the development of new antibiotics with novel modes of action an urgent topic. Plant peptides, produced only in Rhizobium bacterium-legume symbiosis, in the symbiotic cells of root nodules, represent a rich source of so far unexplored biological activities and antimicrobial agents. The infected nodule cells contain thousands of bacteria that are encapsulated by plasma membrane-derived vesicles. In the host cells, the bacteria adapt to the intracellular lifestyle, microaerobic conditions and differentiate progressively into nitrogen-fixing bacteroids. In many legumes, this differentiation process is irreversible and manifested by extreme cell growth, altered morphology and physiology, genome amplifications and definitive loss of cell division.

In *M. truncatula*, 700 genes code for nodule-specific cysteine-rich NCR. The NCR genes usually consist of two exons; the first coding for a relatively conserved signal peptide, while the second one for the mature peptide. The mature NCR peptides exhibit extreme differences in their physicochemical properties due to their highly divergent amino acid compositions and sequence where only the position of 4 or 6 cysteines and a few neighbouring amino acids are conserved. The NCR peptides enter the endoplasmic reticulum during their translation where the signal peptidase complex cleaves the signal peptide, and the mature peptides reach the symbiosomes. By entering the cells, (Figure 1) NCR peptides can interact with the bacterial cell envelope, the bacterial membranes, and specific targets in the bacterial cytosol. NCR247, which is the smallest peptide of the NCR family, is composed of 24 amino acids, four of which are cysteines. This cationic peptide (pI 10.15) is a self-penetrating peptide entering the bacterial cytosol without pore formation, which has exceptionally high protein binding ability and interacts with many bacterial proteins. NCR247 interacting with many ribosomal proteins inhibits translation and downregulates ribosomal gene transcription. Accordingly, treatment of various pathogenic bacteria with synthetic NCR247 provoked the efficient killing of many of them.

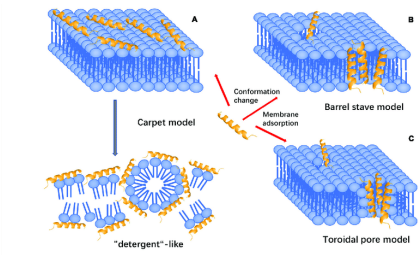

Fig. 1. The role and modes of action of AMPs.

Results and Discussion

The antimicrobial peptides (AMPs) listed in Table 1 were synthesized according to the standard procedure of the solid-phase peptide synthesis (SPPS) by using an automatic peptide synthesizer (CEM Liberty Blue) with TentaGel S RAM resin (loading of amino groups 0.23 mmol/g). The applied chemistry utilized the Fmoc amino protecting group and diisopropylcarbodiimide/oxyma coupling with a fivefold excess of reagents. Removal of the fluorenyl-9-methoxycarbonyl (Fmoc) group was carried out with 10% piperazine and 0.1 mol 1-hydroxy-benzotriazole (HOBt) dissolved in 10% ethanol and 90% DMF in two cycles. After completion of the synthesis, peptides were detached from the resin with a 95:5 (v/v) trifluoroacetic acid (TFA)/water mixture containing 3% (w/v) dithiothreitol (DTT) and 3% (w/v) triisopropylsilane (TIS) at room temperature (RT) for 3 h. The resin was removed by filtration and the peptides were precipitated by the addition of ice-cold diethyl ether. Next, the precipitate was filtered, dissolved in water and lyophilized. The identity of

Table 1. List of antimicrobial peptides.

Code	*Name*	*Amino acid sequence*	*pI*
A	NCR247	RNGCIVDPRCPYQQCRRPLYCRRR	10.15
B	NCR247C	QQCRRPLYCRRR	11.5
C	NCR247C-StrepII	QQCRRPLYCRRRWSHPQFEK	11.05
D	X1-NCR247C	RPLNFKMLRFWGQQQCRRPLYCRRR	11.99
E	X1-NCR247C-StrepII	RPLNFKMLRFWGQQQCRRPLYCRRRWSHPQFEK	11.8
F	NCR247C-X2	QQCRRPLYCRRRKALAALAKKIL	11.55
G	NCR247C-X2-StrepII	QQCRRPLYCRRRKALAALAKKILWSHPQFEK	11.4
H	X2-NCR247C	KALAALAKKILQQCRRPLYCRRR	11.65
I	X2	KALAALAKKIL	10.98
J	Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	10.77

the peptides was verified by ESI-MS using a Waters SQ detector. The crude peptides were analyzed and purified by reverse-phase high-performance liquid chromatography (RP-HPLC). Peptides were purified using a C18 column with a solvent system of (A) 0.1% (v/v) TFA in water and (B) 80% (v/v) acetonitrile and 0.1% TFA (v/v) in water at a flow rate of 4.0 mL/min. The absorbance was detected at 220 nm. The appropriate fractions were pooled and lyophilized. The purity of the peptides was characterized by analytical RP-HPLC at a flow rate of 1.0 mL/min.

Previously we have shown bactericidal activity of the synthetic NCR247 peptide in PBS on several human and plant pathogen bacteria. Our present work is focused on the activity of NCR247 and its various derivatives (Table 1) on the ESKAPE strains as well as on *L. monocytogenes* and *S. enterica*. Bacterial cultures were treated with 2-fold dilution series of synthetic NCR247 (peptide A, Table 1) starting with 25 mM concentration. The minimal bactericidal concentration (MBC) was the lowest concentration of the tested molecules where no viable bacteria remain after the treatment and therefore the growth of bacteria could not be detected (Table 2). The killing activity of NCR247 was the strongest with an MBC of 3.1 mM on *P. aeruginosa* while the MBC was 6.3 mM in the case of *S. aureus* and *E. coli*, 12.5 mM for *A. baumannii* and 25 mM for *S. enterica*. *K. pneumoniae* and *L. monocytogenes* were resistant at 25 mM to NCR247 (peptide A, Table 2).

Table 2. Minimal bactericidal concentrations (MBC; in mM) of the studied peptides and antibiotics against different pathogens.

Peptides/ Antibiotics	*E. f.*	*S. a.*	*K. p.*	*A. b.*	*P. a.*	*E. c.*	*L. m.*	*S. e.*
A	>25	6.3	>25	12.5	3.1	6.3	>25	25
B	>25	>25	>25	25	25	6.3	>25	>25
C	>25	6.3	>25	25	6.3	6.3	>25	>25
D	6.3	3.1	12.5	3.1	3.1	3.1	3.1	1.6
E	12.5	3.1	6.3	3.1	3.1	3.1	3.1	3.1
F	25	3.1	6.3	3.1	3.1	1.6	1.6	1.6
G	12.5	1.6	3.1	1.6	3.1	1.6	3.1	1.6
H	3.1	3.1	6.3	3.1	3.1	3.1	3.1	3.1
I	>25	25	>25	25	6.3	>25	25	>25
J	3.1	3.1	3.1	1.6	3.1	3.1	3.1	1.6
Cb	5120	640	>10240	5120	10240	1280	80	640
Lvx	160	2.5	320	20	1.3	5.0	320	1.3

Then we tested which region of NCR247 is responsible for the antimicrobial properties. From the 24 amino acid long NCR247, the 12 amino acid long *N*-terminal and the 12 amino acid long *C*-terminal halves were synthesized. The *N*-terminal part (RNGCIVDPRCPY) was inactive on the tested bacteria, while the *C*-terminal part (peptide B: NCR247C, Table 1) retained the antimicrobial activity of NCR247 on *E. coli* (peptide B, Table 2) but was ineffective to kill the other bacteria at 25 mM concentration. The synthetic, reduced and even oxidized forms of NCR247 seem to be unstructured. However, it cannot be excluded that NCR247 might be properly folded by interacting with the bacterial membranes, which may not be possible if the peptide is only 12 amino acids long. Therefore, we synthesized derivatives of the NCR247C peptide adding either *C*- or *N*-terminal extension. First, the neutral, 8 amino acid long StrepII tag (WSHPQFEK) was added to the *C* terminus (peptide C: NCR247C-StrepII, Table 1). NCR247CStrepII became more active than NCR247C killing in addition to *E. coli*, *S. aureus*, and *P. aeruginosa* (peptide C, Table 2). Then the *N*-terminus of NCR247C was extended with 13 amino acids (X1) deriving from the cationic *N*-terminal part of the unusual double-size NCR335 which part lacks cysteines and characteristics of NCRs but increased the pI from 10.15 to 11.99 (peptide D, Table 1). This chimeric peptide (X1-NCR247C) turned out to be very effective on *S. enterica* (MBC 1.6 mM), *S. aureus*, *A. baumannii*, *P. aeruginosa, E. coli*, and *L. monocytogenes* (MBC 3.1 mM) and became able to kill *E. faecalis* (MBC 6.3 mM) and *K. pneumoniae* (MBC 12.5 mM) (peptide D, Table 2). The addition of StrepII to the *C* terminus of X1-NCR247C (peptide E: X1-NCR247C-StrepII, Table 1) slightly improved the bactericidal property against *K. pneumoniae* but became somewhat less efficient against *E. faecalis* and *S. enterica* (peptide E, Table 2). Thereupon, we investigated how the attachment of another AMP at the *C*- or *N*-terminus of NCR247C could influence the bactericidal efficiency and spectrum. We used the KALAALAKKIL sequence (peptide I: X2, Table 1) from the membranolytic, anti-cancer mastoparan peptide toxin from wasp venom (INLKALAALAKKIL), which is also present in the *C*-terminus of the 27 amino acid long cell-penetrating cationic peptide, transportan (peptide J: Transportan, Table 1) Attachment of KALAALAKKIL to the *C*-terminus Plant Peptides Kill ESKAPE Bacteria of NCR247C (peptide F: NCR247C-X2, Table 1) reduced the MBC to 1.6 mM in the case of *E. coli*, *L. monocytogenes*, and *S. enterica* (peptide F, Table 2). Further elongation of NCR247CX2with StrepII (peptide G: NCR247C-X2-StrepII, Table 1) made this derivative even more effective against *S. aureus* and *A. baumannii* (MBC 1.6 mM) (peptide G, Table 2). The addition of X2 to the *N*-terminal of NCR247C (peptide H: X2-NCR247C, Table 1) drastically increased the killing of *E. faecalis* and was the most active one (MBC 3.1 mM) among all tested peptide derivatives (peptide H, Table 2). X2 alone was incomparably less active on all tested bacteria (peptide I, Table 2). On the other hand, transportan effectively killed all bacteria with MIC in the range of 1.6–3.1 mM (peptide J, Table 2). The antimicrobial activity of cationic peptides is generally attenuated by the presence of divalent cations and higher salt concentrations. Therefore, we determined the MBC and the minimal inhibitory concentration (MIC) values of all peptides (A-J) in MHB used for the cultivation of *S. aureus*, *A. baumannii*, and *E. coli* (Table 3). Peptides A-C, corresponding to NCR247, NCR247C and NCR247C-StrepII were ineffective against the three bacteria cultivated for 3 or 20 h at 25 mM concentration. Growth inhibition was only detectable in the case of A: NCR247 at 25 mM on *E. coli* cultivated for 20 h. Unlike A-C, the D-H peptides retained their activity in MHB. The action of D and E was, however, slowed down, as their MBC values were significantly higher at 3 h than at 20 h. In contrast, the MBC values of peptides F, G and H were identical or almost the same at 3 and 20 h. Moreover, in line with the fast action of these peptides, the MIC and MBC values were practically identical.

In this work, we show that NCR247 is capable of killing several, but not all of the most problematic bacteria causing incurable infections. Therefore, we aimed to further improve its bactericidal properties. Testing the antimicrobial activity of the synthetic *N*-terminal and *C*-terminal halves of NCR247 revealed that antimicrobial activity resided mostly in the *C*-terminal region (NCR247C). While the positive charge of the AMPs is essential for killing, in the case of NCR247C the increased positive charge (pI = 11.50) did not result in the improvement of the antimicrobial activity, or the antimicrobial spectrum compared to NCR247 (pI = 10.15). NCR247C had even a much weaker antimicrobial activity which may be due to the lack of its proper folding as a result of its short length and/or its failure to interact with the bacterial membranes. Adding the neutral StrepII tag to the NCR247C peptide improved slightly the antibacterial properties. Extending the *N*-terminus of NCR247C with the 13 amino acid long cationic peptide from NCR335 (D) increased the pI to

11.99 and resulted in significantly lower MBCs (1.6–12.5 mM) and a broader spectrum, killing all the eight bacterium strains. In summary, we successfully designed and generated several chimeric peptides from the symbiotic antimicrobial plant peptide NCR247 with fast killing action, low MBC values, and numerous bacterial targets without toxicity on human cells. These peptides represent an extremely promising novel generation of highly powerful antimicrobial candidates [1].

Table 3. Minimal bactericidal concentrations (MBC) and minimal inhibitory concentrations (MIC) of the studied peptides in Mueller Hinton Broth on selected pathogens.

	(A)				***(B)***				***(C)***		
	S.a.	*A.b.*	*E.c.*		*S.a.*	*A.b.*	*E.c.*		*S.a.*	*A.b.*	*E.c.*
A	>25	>25	>25	**A**	>25	>25	>25	**A**	>25	>25	>25
B	>25	>25	>25	**B**	>25	>25	>25	**B**	>25	>25	>25
C	>25	>25	>25	**C**	>25	>25	>25	**C**	>25	>25	>25
D	25	6.3	12.5	**D**	3.1	3.1	3.1	**D**	3.1	3.1	3.1
E	25	12.5	25	**E**	6.3	6.3	6.3	**E**	6.3	6.3	6.3
F	3.1	3.1	3.1	**F**	3.1	3.1	3.1	**F**	3.1	3.1	3.1
G	6.3	3.1	3.1	**G**	6.3	3.1	3.1	**G**	3.1	3.1	3.1
H	6.3	6.3	6.3	**H**	3.1	3.1	6.3	**H**	3.1	3.1	6.3
I	>25	>25	>25	**I**	>25	>25	>25	**I**	>25	25	>25
J	6.3	3.1	6.3	**J**	3.1	3.1	6.3	**J**	3.1	3.1	3.1

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