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Antimicrobial activity of rationally designed amino terminal modified peptides

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Abstract—Series of short amino terminal modified cationic peptides were designed and synthesized. All of the synthesized compounds were tested against gram-positive as well as gram-negative bacterial strain. Some of the compounds exhibit potent antibacterial activity and no hemolytic activity even at high dose level (1000 μg/mL) in mammalian erythrocytes was observed.

The extensive use of antibiotics has resulted in an alarming increase in resistant pathogenic bacteria.[1,2] There is great need to develop new class of antibiotics possessing novel mode of action as well as different cellular targets compared to existing antibiotics in order to decrease the likelihood of development of resistance.[3–9] Cationic antimicrobial peptides represent their candidature for the same.[4,10,11] These peptides are believed to selectively target bacterial cells while remaining nontoxic to the host due to preferable charge interactions between the dense population of negatively charged lipids on bacterial cell surfaces and the cationic side chains of the peptides.[3,12] Although several studies have been reported on cationic antimicrobial peptides, their large size is matter of concern. It is thus need of the time to develop short antimicrobial peptide to make it cost effective.[13] It is evident from reported data that antimicrobial activity of peptides is governed by charge and hydrophobicity of residues and there should be a proper balance between cationic and hydrophobic residues to avoid eukaryotic toxicity.[14] Keeping this in mind, we systematically investigated various known cationic antimicrobial peptides and designed a series of short novel modified antimicrobial tetrapeptide. *p*-Hydroxy cinnamic acid (pHCA), acetic anhydride (Ac), cinnamic acid (CIN), and 3-(4-hydroxyphenyl) propionic acid (HPPA) were used to modify amino terminal of tetrapeptides.

Ornithine was chosen to represent the charged moiety as it is unnatural and incorporation of noncoded amino acid would provide stability against proteases.[13] Tryptophan which frequently occurs in many gene encoded antimicrobial peptides was picked up to represent properties of hydrophobic residues because it has a strong preference for the membrane interface as compared to other hydrophobic amino acid.[15,16] It is well known that cinnamic acid and its derivative possess moderate antibacterial activity.[17,18] *p*-Hydroxy cinnamic acid and other related moieties were used to modify amino terminal of tetrapeptides. To the best of our knowledge there is no report in the literature which shows conjugation of *p*-hydroxy cinnamic acid to peptides. Herein we report first synthesis of cinnamic acid modified peptides and evaluated them for antimicrobial activity. All modified peptides show potent activity against various gram-positive and gram-negative bacteria.

The modified peptides were synthesized by following solid phase synthesis technique on a solid support of Rink amide MBHA resin by standard protocol[19] using 1-hydroxy-benzotrizole (HOBT), diisopropyl-carbodiimide (DIPCDI) as a coupling reagent. The appropriate amino acid (4 equivalents) was preactivated with HOBT (4 equivalents) and DIPCDI (4 equivalents) in 2 ml of DMF for 2 min followed by coupling for 1.5 h. Coupling efficiencies were monitored by performing Kaiser

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Test.[20] Total eight peptides have been synthesized using this synthetic protocol. Crude peptides were purified by gel filtration chromatography on Sephadex G-15 using 20% acetic acid as eluent followed by reverse phase HPLC. Purity of peptides was assessed by analytical reverse phase HPLC on C-18 columns. The molecular mass of these peptides was determined by Maldi-Tof (Table 1). As for example peptide MP-8 shows molecular ion peak at *m*/*z* 535.430, and purity of this compound was found to be 95.45% as determined by HPLC (Table 1). The RP-HPLC analyses were performed on a Waters C_{18} column (4.6× 250 mm, 300 Å, 5-μm particle size) with linear gradients using acetonitrile (0.05% TFA) and water (0.05% TFA) as a mobile phase.

Antimicrobial susceptibility testing was performed by a modification of the method proposed by the Hancock Laboratory (MIC Determination for Cationic Antimicrobial Peptides by Modified Micro Titer Broth Dilution Method).[21] Briefly, the bacterial strains were grown in Mueller-Hinton broth (HIMEDIA) until exponential growth was achieved. Test compound was dissolved in water/DMSO to make a series of twofold dilution. Ninty microliters of $2–7 \times 10^5$ CFUs/ml of bacterial sample per ml of Mueller-Hinton broth (HIMEDIA) was dispensed into 96-well polypropylene microtiter plate (SIGMA). Then 10 μl of serially diluted peptides in 0.01% acetic acid and 0.2% bovine serum albumin of desired concentration were added. The microtiter plates were incubated overnight at 37 °C and the absorbance was read at 630 nm. Cultures without the peptides were used as positive control. Uninoculated Mueller-Hinton broth was used as negative control. The tests were carried out in triplicate. The minimum inhibitory concentration (MIC) is taken as the lowest concentration of compound that inhibits 80% growth of microorganism.

The minimum inhibitory concentrations (MIC) of synthesized peptide are summarized in Table 2. The tetrapeptide amide MP-1 (H_2N-Orn-Orn-Trp-Trp-NH_2) showed moderate activity against gram-positive and gram-negative bacteria studied. The peptide MP-2 (pHCA-Orn-Orn-Trp-Trp-NH_2) was eightfold more active than MP-1 (Table 2) which suggests the importance of pHCA at N-terminal in displaying antibacterial activity. Peptides MP-2 (pHCA-Orn-Orn-Trp-Trp-NH_2) and MP-6 (HPPA-Orn-Orn-Trp-Trp-NH_2) both exhibit potent activity against different bacterial strains implying that *trans* configuration across double bond is not playing any role rather the molecule as a whole is indispensable at N-terminal of tetrapeptides for antimicrobial activity.

Acetylation of MP-1 improves its antibacterial activity against *Escherichia coli*, *Stahpylococcus aureus*, and *Bacillus subtilis*. It is already known that *N*-acylation

Table 1. Sequence, charge, molecular weight, and % area (HPLC) of synthesized compounds

Name	Sequence	Q^c	Molecular weight[a]		Purity[b] (% area)
			Calc.	Obs.	
MP-1	NH_2-O-O-W-W- NH_2	+3	617.726	618.236	91.87
MP-2	pHCA-O-O-W-W-NH_2	+2	763.726	764.291	88.56
MP-3	Ac-O-O-W-W- NH_2	+2	659.726	660.204	95.64
MP-4	pHCA-O-O-W-W-COOH	+1	764.726	765.292	<98
MP-5	CIN-O-O-W-W- NH_2	+2	746.726	747.288	99.45
MP-6	HPPA-O-O-W-W- NH_2	+2	765.926	766.725	<98
MP-7	pHCA-O-O-F-F- NH_2	+2	685.592	685.592	98.65
MP-8	pHCA-W-W- NH_2	0	535.430	535.430	95.45

[a] Molecular weights were analyzed by Maldi-Tof.
[b] % purity of peptides was obtained by using HPLC.
[c] Net charge.

Table 2. Minimum inhibitory concentration (MIC) of modified tetrapeptide

Name of Comp.	MIC (μg/ml)				
	Pseudo. aeruginosa (MTCCB, 741)	*E. coli* (MTCCB, 1610)	*S. aureus* (MTCCB, 96)	*B. subtilis* (NCIM, 2063)	*E. coli* (Clinical Isolate)
MP-1	256	512	128	512	256
MP-2	2	16	2	1	8
MP-3	128	32	2	2	128
MP-4	64	64	128	128	128
MP-5	16	2	2	32	4
MP-6	32	2	1	2	4
MP-7	2	4	32	2	8
MP-8	<512	<512	2	1	<512
Genta	1	0. 25	0. 062	0. 03	0. 5
Tetra	1	0. 5	0. 25	0. 125	2

Tetracycline and Gentamycin were used as standard antibiotics.

stabilizes structure and improves antimicrobial properties of short dermaseptin derivative and other antimicrobial peptides.[24] The C-terminal amide seems to be essential for biological activity as MP-4 (pHCA-Orn-Orn-Trp-Trp-COOH) which has C-terminal carboxylic acid demonstrates significantly high MIC value as compared to MP-2.

MP-8 (pHCA-Trp-Trp-NH_2) surprisingly showed strong activity against gram-positive bacteria (*S. aureus*, *B. subtilis*) studied, however it did not show any effect on gram-negative bacterial strains (*E. coli*, *Pseudo. aeruginosa*) up to 512 μg/ml. pCHA at N-terminal of peptides may be responsible for the observed antimicrobial activity. Moreover it is well known that tryptophan is unique amino acid having hydrophobic as well as polar character. The two tryptophan moieties at C terminal of MP-8 may provide it enough polarity and hydrophobicity to interact with bacterial membrane of gram-positive bacteria. MP-5 (CIN-Orn-Orn-Trp-Trp-NH_2) is found to be active against all bacterial strains studied but its MIC against *B. subtilis* is comparatively higher. This result indicates that hydroxyl group is playing a role in killing of *B. subtilis*. MP-7 displayed antimicrobial activity against gram-negative bacteria equivalent to MP-2 but it showed (2–4) fold increase in MIC against gram-positive bacteria. This result indicates that Trp residues at C-terminal are crucial for antimicrobial activity against gram-positive bacteria. On studying antimicrobial data it was observed that antimicrobial activity is associated with *p*-hydroxycinnamic acid at amino terminal of tetrapeptide. There was slight variation in antimicrobial activities on modification of amino terminal with similar moiety like cinnamic acid and *p*-hydroxyphenylpropionic acid.

Toxicity to erythrocytes was investigated using human red blood cells (hRBC).[22,23] Results demonstrated that concentration up to 1000 μg/ml of these peptides lysed only 0–2% of mammalian erythrocytes (Fig. 1). Compared to tetracycline, these modified peptides are less toxic to human erythrocytes. Mechanistic study of these peptides may reveal whether they kill microbes by rupturing bacterial surface or have some intracellular target.

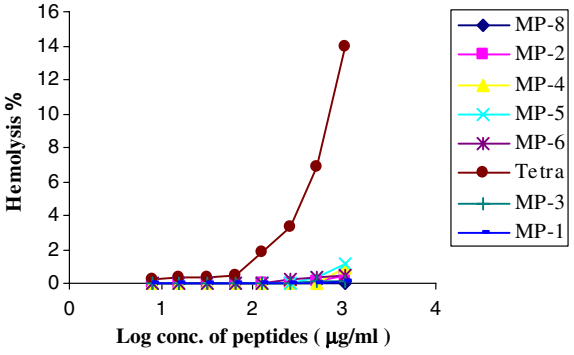

Figure 1. Hemolytic activity of modified peptides.

In conclusion, we report the synthesis and antimicrobial activity of modified tetrapeptide. These peptides specially MP-2, MP-5, and MP-6 were found to be more active as compared to many cationic antibacterial peptides of far larger size, such as the protegrins, Indolicidin, and Tritrpticin.[22] The mode of action of these peptides is under intense investigation and may lead to new peptide antibiotics. As all the synthesized peptides were nontoxic, these can be used for development of suitable antimicrobial drug.

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23. The hemolytic activities of the peptides were determined using human red blood cells (hRBC). The hRBC were centrifuged for 15 min to remove the buffy coat and washed three times with phosphate-buffered saline (PBS: 35 mM phosphate buffer, pH 7.0/150 mM NaCl). One hundred microliters of the hRBC suspended 4% (v/v) in PBS was plated into sterilized 96-well plates and then 100 μl peptide solution was added to each well. The plates were incubated for 1 h at

37 °C and centrifuged at 1000*g* for 5 min. Aliquots (100 μl) of the supernatant were transferred to 96-well plates, where hemoglobin release was monitored using ELISA plate reader by measuring the absorbance at 414 nm. Percent hemolysis was calculated by the following formula: % HEMOLYSIS = [($Abs_{414\ nm}$ in the peptide solution-$Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100-$Abs_{414\ nm}$ in PBS)] × 100. Zero percentage and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively.

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