Design of hydrophobic hybrid tri peptides as broad spectrum potent antimicrobial agents with enhanced cell selectivity

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Abstract : In this study we mainly focus on structure-activity relationship aspects and important physiochemical properties that influence the antimicrobial and toxic properties of five hybrid peptides having one γ/β aminoacid. The contributions of individual amino acids with respect to the position in displaying antibacterial properties are presented. The mechanism of action of different peptides towards antimicrobial activity is also studied using SEM. The current study enhances knowledge on design strategies to illustrate the importance of antimicrobial peptide research in the development of next-generation antibiotics.

IndexTerms **:** Antimicrobial peptides; Mechanism of action; Hybrid peptides; γ- aminoacid; β- aminoacid; Scanning Electron Microscopy(SEM)

I. INTRODUCTION

II. Bacterial and fungal infections have been a major cause of death in humans from long period. In the late 19th century it was discovered that many common diseases were caused by microscopic pathogens, which led to introduction of antiseptic procedures, in order to minimize mortality related to post surgical infections.[1] Furthermore, sanitation and hygiene also contributed to reduction of the mortality caused by bacterial infections.[2] Antibiotics are undeniably one of the most important therapeutic discoveries of the 20th century that had effectiveness against serious bacterial infections.[3] The discovery of penicillin and streptomycin revolutionized the treatment of many bacterial infection diseases.[4,5] However, prolonged use of these compounds lead to development of resistance and in later years only one third of the infectious diseases known were successfully treated from these products.[6] Also, the current medicinal chemistry field is facing an increased problem of infections associated with drug resistance to conventional antibiotics.[7] This problem becomes even more pronounced during surgery, because bacteria can easily colonize the surface of living and non-living substrate including the raw patient's tissue, surgical devices, synthetic implants, orthopedics, and catheters etc.[8-10] However, this problem is not only limited to the clinic but also

extends to drinking water, food packaging and agriculture. Therefore, this leads us to focus on new synthetic compounds to develop novel kinds of antibacterial molecules and polymers.

III. While designing a new broad spectrum anti-bacterial material, the general structural feature usually followed by many researchers involves balance of proper charge, which draws the material close to the bacterial membrane, and hydrophobic residues, which interact with the phospholipid bilayer.[11] This is based on the natural defense mechanisms of many living organisms which synthesize cationic antimicrobial peptides. While each peptide is different and unique to the particular organism, they all generally consist of about 12-50 residues, in which approximately 50% of them are hydrophobic.[12-15] Recently, it has been shown that when a bacterial cell is approached by a peptide, hydrophobic interactions prevail and sink the peptide into the bacterial membrane, and thus places a torsional strain on the membrane bound phospholipids, which further lead to membrane disruption and subsequent cell lysis. Applying this theory to design a broad spectrum antimicrobial agent, we have developed hydrophobic short hybrid peptides, synthesized using both natural and unnatural amino acids. The use hybrid peptide will improve the overall physical and chemical behavior in physiological conditions. In this study, we designed and synthesized six peptides with 100% hydrophobic residues, to test the potential of hydrophobic interaction of these peptides with bacterial surface during antimicrobial activity

IV. Materials and Methods

V. *Design of Peptides*: The peptides were designed based on the following rules: (1) should not contain any charged amino acids, (2) should contain random structure, and (3) should contain only hydrophobic amino acids.

VI. *Bacterial Strains and Growth Conditions*: Three bacterial strains namely *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and three fungal strains namely *Aspergillus flavus*, *Chrysosporium keratinophilum* and *Candida albicans* were selected to measure antimicrobial activity of synthetic peptides **NC1-6**. The strains were obtained from Department of Studies in Microbiology, University of Mysore and were stored in refrigerator at 4^{o}C until use. The culture condition of these bacterial strains is summarized in Table 1.

Table 1: Bacterial and fungi stains used in the present study.

Strain	Culture condition (^{o}C)	Medium
Escherichia coli,	37	Luria-Bertani
Staphylococcus aureus	37	Luria-Bertani
Pseudomonas aeruginosa	37	Luria-Bertani
Aspergillus flavus,	37	Martin Broth
Chrysosporium Keratinophilum	37	Martin Broth
Candida albicans	37	Martin Broth

Peptide Synthesis and characterization:

Synthesis of γ^4-aminoacid

Literature procedures[16,17] was followed to synthesize β^3(*R*)- phenyl alanine. γ^4(*R*) –valine, γ^4(*R*) –lucine and γ^4(*R*)–Phenylalanine were synthesized by following previously reported procedure[18] with minor modification. (*S*)-pregabaline was purchased from Sigma-Aldrich and Boc protection was done using standard protocol.

Peptides **NC1-6** were synthesized by conventional solution phase methods, by means of a fragment condensation strategy. The Boc-group was used for N-terminal protection, and the C-terminals was protected as a methyl ester. N-terminal deprotections were performed with 98% formic acid (HCOOH) and saponification was carried out for C-terminal deprotection. Couplings were mediated by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxy-1H-benzotriazole (HOBt) (1.01 equiv.). The final peptides were obtained as pure products after washing with hexane/ether mixtures. All the peptides and intermediates were characterized by ESI-MS and by 700-MHz.

Table 2: The sequences and characterization data of six hybrid peptides.

Peptide Name	Sequence	Molecular weight	
		Calculated	Observed
NC1	Boc-Leu-Phe-Val-OH	477.28	478.35
NC2	Boc-Leu-$^{\beta}$**Phe**-Val-OH	491.30	492.41
NC3	Boc-Leu-$^{\gamma}$**Phe**-Val-OH	505.32	506.43
NC4	Boc-$^{\gamma}$**Leu**-Phe-Val-OH	505.32	506.52
NC5	Boc-**Pgb**-Phe-Val-OH*	505.32	506.31
NC6	Boc-Leu-Phe-$^{\gamma}$**Val**-OH	505.32	506.42

*Pgb= (*S*) pregabalin

Measurement of Antibacterial Activity:

Antimicrobial activity of hybrid peptides **NC1-6** was evaluated using an inhibition zone assay, as well as or determination of the minimal inhibitory concentration (MIC) value followed by the method described previously.[19]

1) *Inhibition zone assay*: agar plates were seeded with strains (~100 cells in 10 mL of 1% agar medium). Wells (3 mm in diameter) were made equidistantly on the medium using cork borer and 5μL peptide samples dissolved in buffer were loaded to these wells, along with controls loaded with only buffer. The plates were incubated at 37^{o}C for overnight. Later the diameter of the inhibition zone was determined.
2) *Determination of the MIC value*: Using the standard serial dilution method, MICs were determined in appropriate medium for each bacterial strain as follows. 1 mL of different peptides dilution solutions was poured into a series of sterile plates, and then 9 mL appropriate medium that was preincubated at 37

°C was added into a plate and was mixed gently to reach various concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, and 1.9 μg/mL). Bacteria were added to the 96-well plates. The amount of bacteria for each spot is approximately 100 to 110 CFU/mL. After incubating the inoculated plate at 37°C for 16 to 18 hours, MICs were determined as the lowest peptide concentration that inhibited bacterial growth. All MICs were determined in three independent experiments performed in duplicate.

Measurement of Hemolytic Activity (MHC):

Hemolytic activity was determined by following the reported procedure of Yoshida et al.[20] with adoption of slight modification. Fresh human blood (1 mL) was centrifuged at 1000 ×g for 5 min and the precipitates were collected and then washed 3 times with phosphate buffered saline (PBS) (pH 7.4). Precipitates were resuspended in 4-fold volumes of PBS. Then, 995 μL of PBS solution containing serial diluted peptides was added to a human erythrocyte solution of 5 μL. The resulting solution was incubated at 37°C for 1 h and was centrifuged at 1000 ×g for 5 min. The supernatant was diluted 5-fold with PBS and was monitored at 415 nm using a UV spectrophotometer. Zero hemolysis (blank) and 100% hemolysis were determined in PBS and 1%Triton X-100, respectively.

Scanning Electron Microscopy.

A mid-logarithmic phase culture of *Escherichia coli* was exposed to KW-13 (256 μg/mL) or water at 37°C for 2 h. Bacteria were precipitated by centrifugation at 5000 rpm for 5 min and washed 3 times in PBS (pH 7.4). The supernatants were removed and the pellets were fixed in 800 μL of 2.5% glutaraldehyde in 0.1 M PBS at 4°C for 24 h. The fixed bacteria were centrifuged at 5000 rpm for 10 min, washed 2 times with 0.1 M PBS, and then step-dehydrated with 70%, 80%, 90%, and 100% ethanol. After drying and gold coating, the samples were examined by scanning electron microscopy.

Results

We synthesized the following six short hybrid peptides (Figure 1) containing β and γ amino acids with Leu, Ile and Phe hydrophobic side chains.

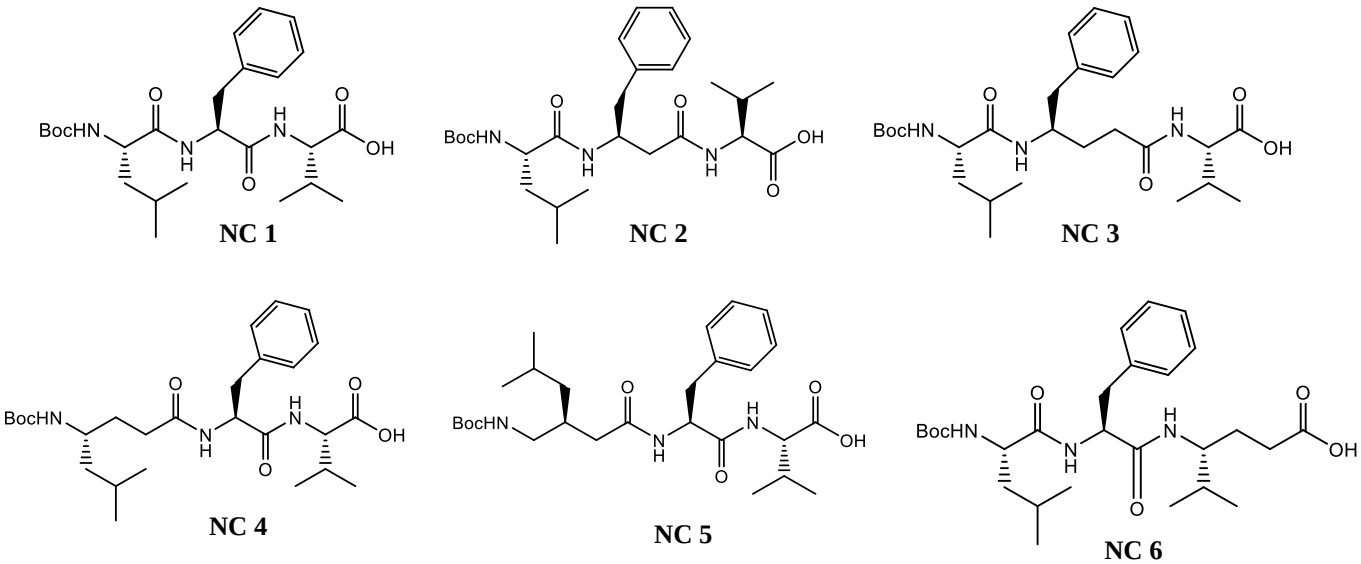

Figure 1: Structures of hybrid peptides used in the present study

Short hybrid peptides with five or more than five residues are very well known for attaining regular secondary structure, therefore we restricted to tripeptides, since these peptides exhibit random structure. This is because a random core structure is expected to facilitate the interaction with the bacterial membranes through hydrophobic forces. The random core was also designed to limit the packing density of the phenylalanine rings, which allows for a more overall hydrophobicity of the compound.

Peptide **NC1** consists of three α amino acid residues, peptide **NC2** contains β-Phe and peptide **NC3** contains γ-phe residue at the centre respectively. Peptide **NC4** has γ-Leu and peptide **NC5** has pregabalin residue at N-terminal. Peptide **NC6** consist γ-Val at C-terminal. The N- terminal of all peptides were protected by Boc protecting group in order to prevent the charge due to protonation of free amine group at buffer condition and C-terminal have free carboxylic acid in order to improve the solubility of peptides in aqueous buffer.

Antimicrobial Activity

Antimicrobial activities of six hybrid peptides (**NC1-6**) was tested for determining the MICs against broad model microbes (listed in table 1) and the results are summarized in Table 3.

Table 3: Antimicrobial activities (MIC) of peptide against different bacteria and fungi

	MIC (mg/liter)[a]					
Strain	NC1	NC2	NC3	NC4	NC5	NC6
Escherichia coli	ND	500	31.5	500	125	ND
Staphylococcus aureus	ND	125	3.9	125	15.62	125
Pseudomonas aeruginosa	ND	125	15.62	125	62.5	125
Aspergillus flavus	>1000	31.25	7.81	31.25	15.62	31.25
Chrysosporium keratinophilum	>1000	31.25	7.81	31.25	15.62	31.25
Candida albicans	>1000	7.81	1.9	7.81	3.9	7.81

[a] The data are average of three independent experiments performed in triplicate. ND – not detected.

Peptide **NC1** with all α-amino acids showed weak activities against fungi and did not show activity against bacteria. Interestingly, peptide **NC2** in which α-Phe residue is replaced with β-Phe showed activity against both bacteria and fungui. The MICs of **NC2** for *Staphylococcus aureus* and *Pseudomonas aeruginosa* were 3 fold better compared to *Escherichia coli*. Similarly, the MICs *for Aspergillus flavus* was 2 fold better compared to *Chrysosporium keratinophilum* and *Candida albicans*. Surprisingly, peptide **NC3** with γ-Phe in place of β-Phe residue strongly inhibited the growth of both bacteria and fungui and exhibited 8 fold more activity than **NC2** for all bacterial and fungal strains, indicating that NC**3** was more powerful than **NC2**. However, **NC2** was less effective against bacteria compared to fungi. Other hybrid peptides **NC4** and **NC6** showed moderate antimicrobial activity.

Cytotoxicity Assay against human cells

Among the five hybrid peptides, **NC3** and **NC5** were determined to be the most effective antimicrobial peptides. Next, we tested the cytotoxicities of **NC3** and **NC5** against human cells. These two peptides were subjected to hemolytic assay against human red blood cell (RBC) at 1mg/mL, 10 mg/mL, and 100 mg/mL concentrations. Release of hemoglobin was monitored to estimate the degree of erythrocyte lysis caused by the peptides. No significant hemolysis was observed for peptides **NC3** (< 2%) and **NC5** (< 3%) even at 6mg/mL concentration (Fig 2a), indicating that these peptides are less cytotoxic and therefore, safer with regard to human erythrocytes. Then, the cytotoxicities of **NC3** and **NC5** to HEK 293 cells and human epithelial fibroblast cells were assessed. Viability remained at the same level when cells were treated with **NC3** or **NC5** up to concentration lower than 80 mg/liter. Tt higher peptide concentrations, **NC3** had lower cytotoxicity than **NH5** (Fig. 2b and 2c).

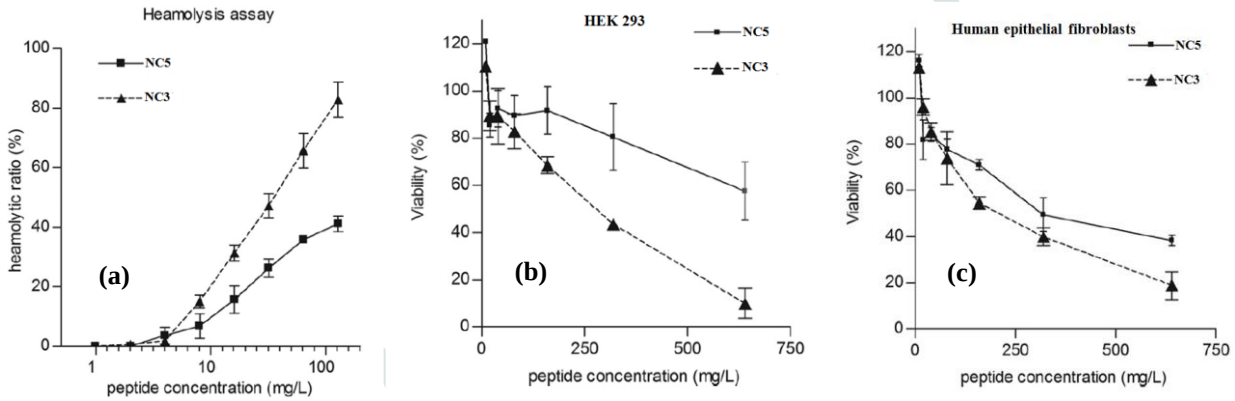

Figure 2: Hemolytic activity (a) and cytotoxicity (b and c) of **NC3** and **NC5** peptides.

Scanning Electron Microscopy study

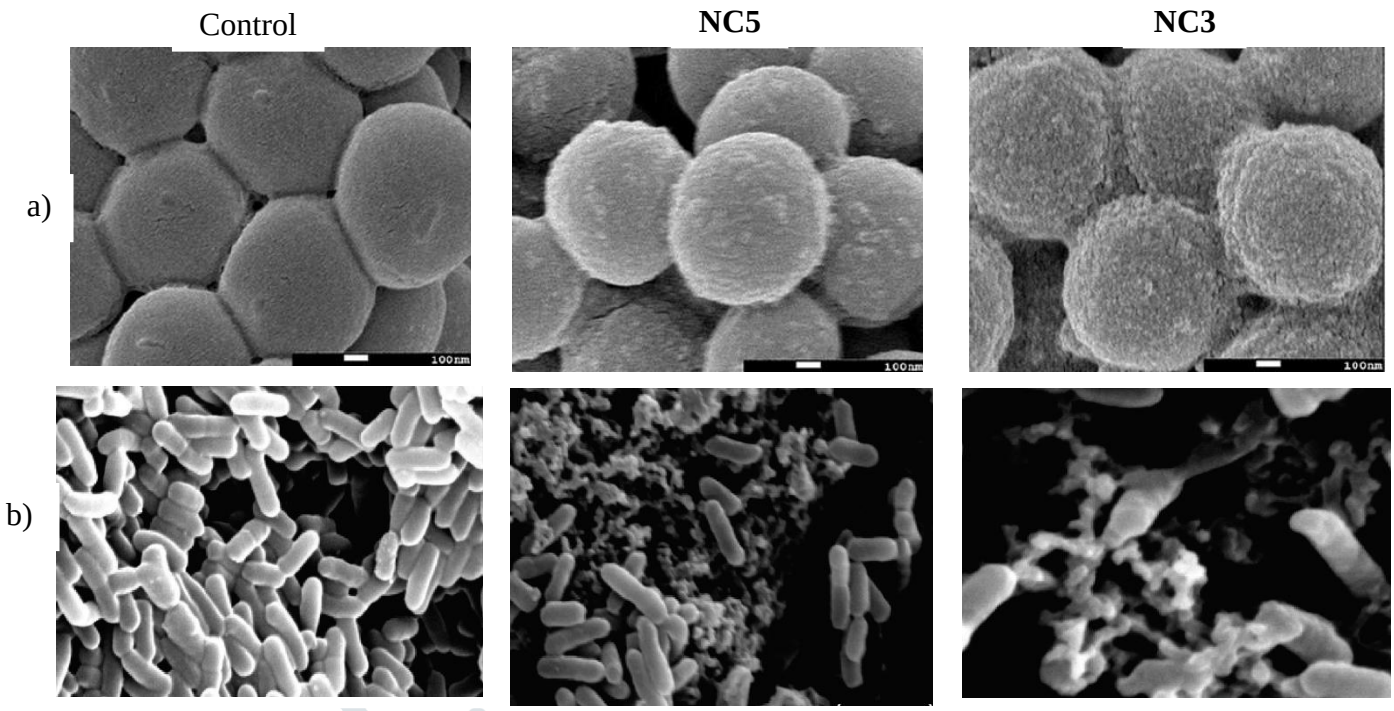

Figure 3: SEM image of a) *Candida albicans* and b) *Staphylococcus aureus* treated with 1X MIC concentrations of peptides NC5 and NC3. The smooth surface of *Candida albicans* cells became plicated and some cracks appeared (in panel a). Untreated *Staphylococcus aureus* showing a normal intact cell surface, when treated with peptides the cell membranes were disrupted (in panel b).

We examined the morphological changes that occur in *Candida albicans* and *Staphylococcus aureus* by scanning electron microscopy (SEM) to elucidate how the hybrid peptides **NC3** and **NC5** affect the outer layers of bacteria and fungi strains. Both species were treated with 1 X MIC concentrations of **NC3** and **NC5** for 1 h and then visualized by SEM. The result observed is shown in Figure 3. **NC3** and **NC5** peptides largely destroyed the cellular surface of both bacterial as well as fungal strains. Furthermore, membrane formation of dividing cells was affected by these peptides protruding from the cells.

These results indicate that the antimicrobial mechanism of hybrid peptides **NC3** and **NC5** is distinct from that of most cationic peptides. The wrinkled surface observed with SEM indicates that the cell shape is maintained but possessed a few cracks. This leads to release of DNA or protein into the cytoplasm, their by causing cell death.

DISCUSSION

The design of effective AMPs plays an important role to meet the demand of efficient antimicrobial therapies. In this study, to test the significance of hydrophobicity over chare, we constructed a model hybrid AMPs with highly hydrophobic residues. Our design is based on the clear principle that each type of amino acid possesses a different level of importance with respect to peptide activity. Our study focused on changing only a few of amino acids from the model peptide to improve antimicrobial activity, allowing for comparisons between peptides with just one amino acid difference. In this study, we developed five short hybrid AMPs. In an antimicrobial activity assay, we found that most of our designed peptides possessed

higher to moderate antimicrobial activities. In particular, **NC3** and **NC5** show the strongest antimicrobial activity to both bacterial and fungal standards and display lower cytotoxicity to human erythrocytes, HEK 293 cells, and human epithelial fibroblast cells. Unlike most AMPs, which form pores in the cytoplasmic membrane that lead to cell lysis or leakage of the cytoplasm, it appears that our peptides operate via a distinct mechanism.

The synergistic action of AMPs with other AMPs or antibiotics can reduce the need for high dosages or minimize side effects, both of which are beneficial attributes for therapeutic strategies to fight multidrug-resistant bacteria.[21-23] The novel AMPs developed in this study suppress bacterial growth via an outer membrane disruption mechanism. This finding could potentially be applied to enhance the antimicrobial activities of AMPs with different sequences or structures,[23] and our novel AMPs may show some synergistic effects with antibiotics that display alternative antimicrobial mechanisms. Such issues will be investigated in future studies.

Conclusion

In summary, our study provides a new method for AMP design and prediction. Peptides generated by our method were found to possess effective antibacterial functions both *in vitro* and *in vivo*. In addition, our findings provide insight into the antimicrobial mechanism of the AMP **NC3** and **NC5**, which may have important implications for future studies into the development and application of novel antimicrobial therapies.

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