Synthesis and antimicrobial activity of hybrid peptides as dual inhibitor of intracellular targets in ESKAPE pathogens

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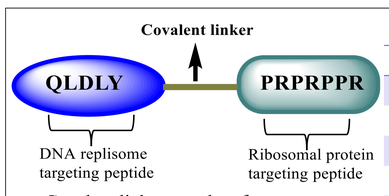
Background

❑ The infections caused by ESKAPE pathogens are a global challenge in clinical settings due to their multidrug resistance and even last-resort antibiotics ineffective for treatment.

❑Advances in science have shown that bacteria adapt and mutate to resist antibiotics. To combat this, new antibiotics with novel targets and mechanisms are needed.

❑Antimicrobial peptides (AMPs), which have evolved over millennia as natural antimicrobial agents and immune response mediators, are one of the promising candidates in this fight.

Rationale &Objectives



Covalent linkers can be of :-
(a) unnatural linkers (non-peptide alkyl chains) &
(b) natural amino acids

Proline &Arginine rich antimicrobial peptides (PrAMPs)

S.no	PrAMPs	Peptide Sequence
1.	Onc72	VDKPPYL**PRPRPPR**OIYNO
2.	Api88	Gu-ONNRPVYI**PRPRPP**HPRL
5.	Api134	Gu-ONNRPVYI**PRPRPP**HPOL
4.	A3-APO	(zRPDKPRPYL**PRPRPPR**PVR)2
5.	CheX1-R20	ZRPDKPRPYL**PRPRPPR**PVR
6.	Pyrrhocoricin	VDKGSYL**PRPTPPRP**IYNRN
7.	PR-39 (1-18)	RRRPRPPYL**PRPRPPP**FF

Objectives:

- Synthesis of the designed hybrid analogues of proline rich antimicrobial peptides.
- Purification & Characterization of the peptide through HPLC and LC-MS
- Determination of anti-bacterial potency of the peptides against ESKAPE pathogens.
- Evaluation of *in vitro* cell cytotoxicity and hemolytic activity of the peptides.

Methodology

❑ Design and synthesis of peptides

- Natural host defense cationic antimicrobial peptides (AMPs) are effective against multidrug-resistant (MDR) pathogens, but their membrane-disruptive action can be toxic to mammalian cells, limiting systemic use.
- However, intracellular-targeting AMPs, which can inhibit DNA or protein synthesis, bypass these restrictions. This study proposes the design of such AMPs to target enzymes involved in DNA replication and protein synthesis.
- The peptides were synthesized using standard solid-phase Fmoc chemistry and characterized by HPLC and ESI-MS.

❑ Antimicrobial potency

- Modified serial broth dilution method was used.
- The peptides were tested against a range of gram-negative and gram-positive pathogens, revealing lead sequences with potential against drug-resistant bacteria.

❑ Cell cytotoxicity assay

- Hemolytic activity of the designed peptides were determined on fresh human red blood cells (hRBC) and were found non-hemolytic even at the concentration of 0.5 mg/mL.
- We conducted cytotoxicity study of the lead peptides by MTT assay method against HEK-293T cell lines at higher concentrations of the calculated MIC value obtained from antibacterial assay (as depicted as in Figure 3.2).

❑ Drug resistance studies

- Resistance development studies of the peptides till 17 passages of bacteria. a) for *E. coli* ATCC 11775 and b) for *S. aureus* ATCC 33591.

❑ Mode of action studies

- Peptide internalization studies (using confocal microscopy).
- Bacterial membrane depolarization assay.

Results

1. Characterisation:

HPLC, UPLC & ESI-MS

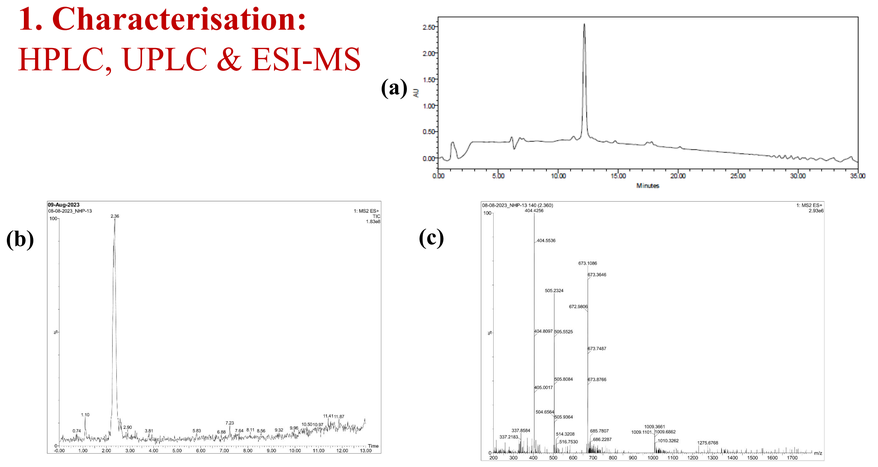


Fig 1: (a) HPLC, (b) UP-LC chromatogram, and (c) ESI-Mass spectra of peptides.

2. Antibacterial Assay

Table 1: Peptide name, sequence, and MIC of the synthesized peptides.

S.No	Sequence of peptides	Sample Code	S. aureus ATCC 33591	S. aureus ATCC BAA-44	MRSE ATCC 56625	B. subtilis ATCC 6693	S. epidermidis ATCC 51625	E. coli ATCC 11775	P. aureginosa ATCC BAA-427	E. feacium ATCC 35667	A. baumannii ATCC 19606	K. pneumoniae ATCC BAA-1705	E. faecalis ATCC 7080
1	Ac-PRPRPPR- $CONH_2$	Prn	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
2	Ac-QLDLY-R-PRPRPPR- $CONH_2$	NHP-11	500	500	500	500	500	500	>500	500	500	500	500
3	Ac-QLDLY-RR-PRPRPPR- $CONH_2$	NHP-12	250	500	500	>500	500	250	500	250	250	500	250
4	**Ac-QLDLY-RRR-PRPRPPR- $CONH_2$**	**NHP-13**	**31.2**	**31.2**	**62.5**	**62.5**	**31.2**	**15.1**	**62.5**	**31.2**	**31.2**	**125**	**125**
5	Ac-QLDLY-Spermine	Spn-1	62.5	31.2	62.5	62.5	31.2	250	250	250	250	250	250
6	**Lauric acid-QLDLY-Spermine**	**Spn-4**	**7.8**	**7.8**	**15.1**	**7.8**	**7.8**	**31.2**	**62.5**	**31.2**	**31.2**	**125**	**62.5**
7	Vancomycin	Van	0.4	1.9	1	ND	1	**62.5**	**125**	**62.5**	**125**	**ND**	**125**
8	Ciprofloxacin	Cipro	0.2	62.5	0.03	ND	0.03	**0.02**	**0.8**	**0.5**	**0.8**	**ND**	**0.8**

3. Cell cytotoxicity and Hemolytic assay

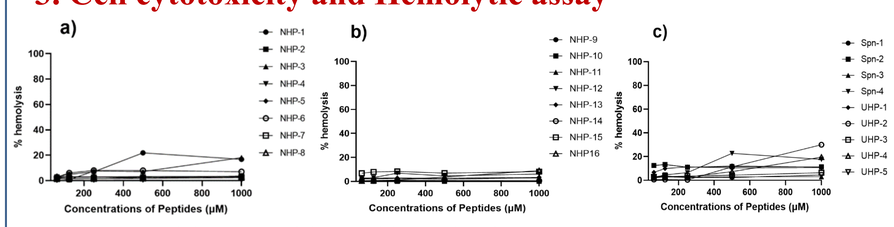


Fig 3.1: Cytotoxicity assay of the peptides against human RBCs.

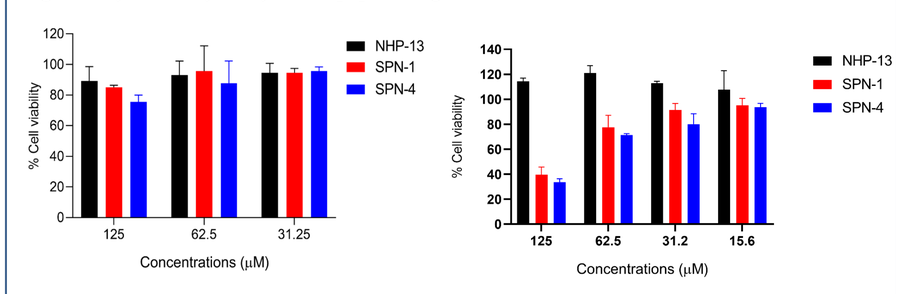


Fig 3.2: (a)Cytotoxicity assay of the peptides against HEK-293T cell lines.
(b) Cytotoxicity assay of the peptides against peripheral blood mononuclear cells (Primary cells). The assay was performed based on reduction of alamar blue dye.

4. In-vitro antiendotoxin neutralization assay

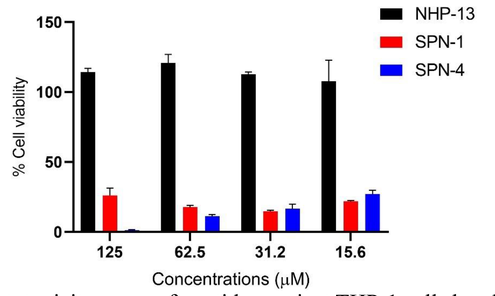


Fig 4.1: (b) Cytotoxicity assay of peptides against THP-1 cells by alamar blue assay.

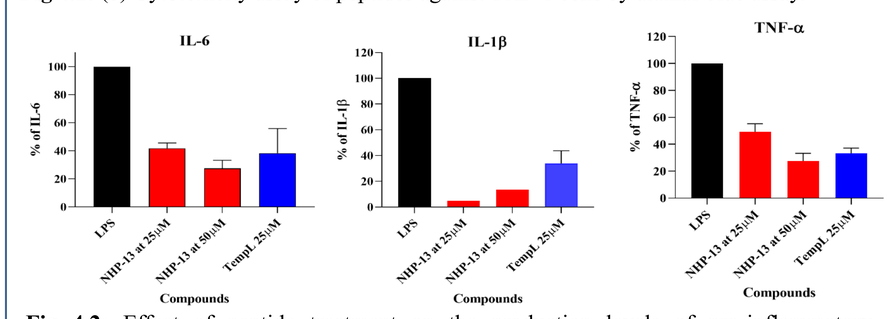


Fig 4.2: Effect of peptide treatment on the production levels of pro-inflammatory cytokines in THP-1 cells by ELISA experiments.

Results

5. Drug resistance studies

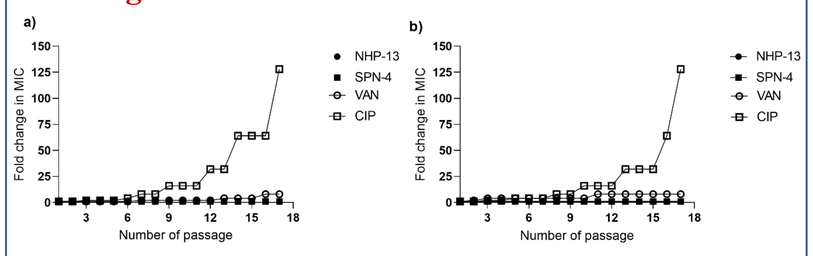


Fig 5: Resistance development studies of the peptides till 17 passages of bacteria. a) for E. coli ATCC 11775 and b) for S. aureus ATCC 33591.

6. Mode of action studies

Fig 6.1: Peptide internalization studies by Confocal Microscopy.

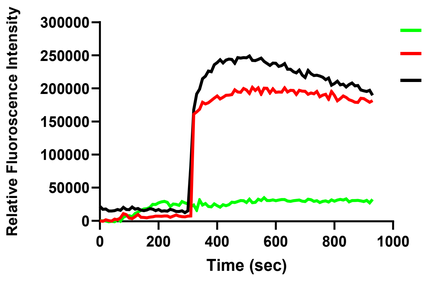


Fig 6.2: Depolarization assay of NHP-13 peptide in live MRSA cells (at 2 × MIC).

Key findings

❑The peptide templates, PRPRPPR and QLDLY were employed to design for dual inhibition of protein and DNA synthesis, respectively.

❑Peptide NHP-13 and SPN-4 was found to be active in the MIC range of 7.8-125 μM concentrations against tested strains with no hemolytic activity observed up to 500 μM concentrations.

❑Further studies was performed to evaluate peptide internalization in bacteria by labelling with FITC.

❑The most active peptide (NHP-13) was hybrid of above two templates with linker of triple arginine, highlighting arginine's role in cell penetration and enhancing antibacterial activity.

❑In vitro LPS neutralization activity of NHP-13 shown potential effects on reduction in inflammatory cytokines in human monocytes.

❑Further studies are going on for evaluation of protein synthesis by interaction with 70s ribosomes and DNA replisomes.

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