Peptide Nanonets as Antimicrobial and Anti-inflammatory Agents

Pui Lai Rachel Ee[a], Nhan Dai Thien Tram[a], Jian Xu[a], Dhanya Mahalakshmi Murali[a], Peiyan Yu[a], Wai Shiu Fred Wong[b], Lakshminarayanan Rajamani[a,c]

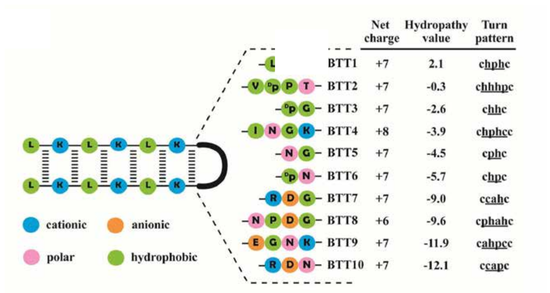
[a] *Department of Pharmacy and Pharmaceutical Sciences, National University of Singapore,*

[b] *Department of Pharmacology, National University Health System*

[c] *Ocular Infections and Anti-Microbials Research Group, Singapore Eye Research Institute*

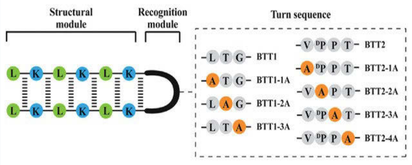
https://doi.org/10.17952/37EPS.2024.P2145

De novo design of beta-hairpin peptides



	Net charge	Hydropathy value	Turn pattern
BTT1	+7	2.1	chphc
BTT2	+7	-0.3	chhhpc
BTT3	+7	-2.6	chhc
BTT4	+8	-3.9	chphcc
BTT5	+7	-4.5	cphc
BTT6	+7	-5.7	chpc
BTT7	+7	-9.0	ccahc
BTT8	+6	-9.6	cphahc
BTT9	+7	-11.9	cahpcc
BTT10	+7	-12.1	ccapc

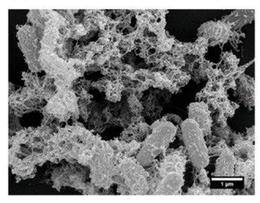
We have designed a series of de novo β-hairpin peptides with identical side strands but varied turns. We demonstrated that mutations of only 2 to 4 amino acids at the turn region could impart a wide range of antimicrobial profiles among synthetic β-hairpin AMPs.

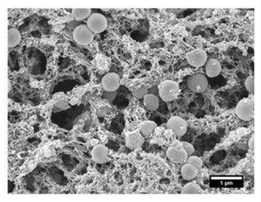


Through turn sequence modification by Ala scanning, we identified specific analogues capable of forming bacteria-responsive nanonets.

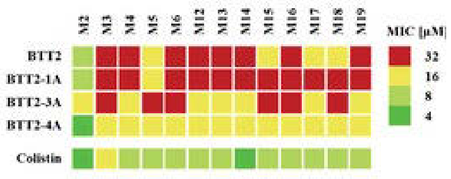
Nanonet forming analogues have anti-microbial activity

Peptide	MIC [μM]	
	E. coli	*S. aureus*
BTT1	4	8
BTT1-3A	8	16





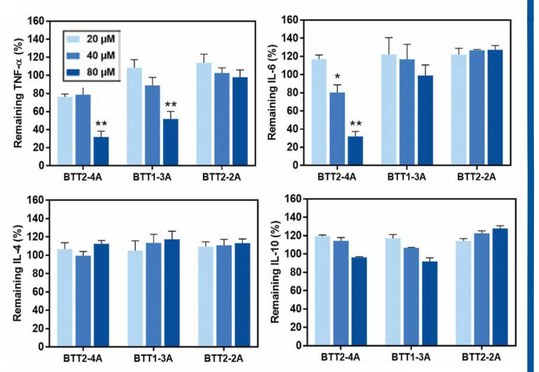
BTT1-3A was found to be the only analogue capable of forming bacteria-responsive nanonets. We successfully converted the kill-only BTT1 to the trap-and-kill BTT1-3A.

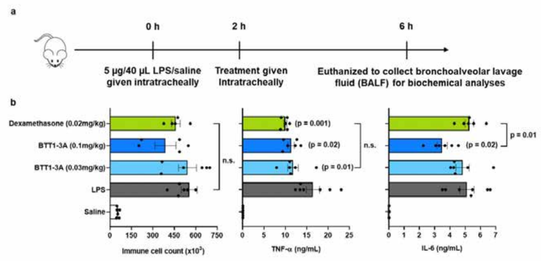


By replacing single amino acid at the run segment of BTT2, we obtained BTT2-4A with improved antimicrobial potency against clinically relevant antibiotic-resistant strains, while preserving the ability to form bacteria-trapping nonets.

Nanonet forming analogues are anti-inflammatory

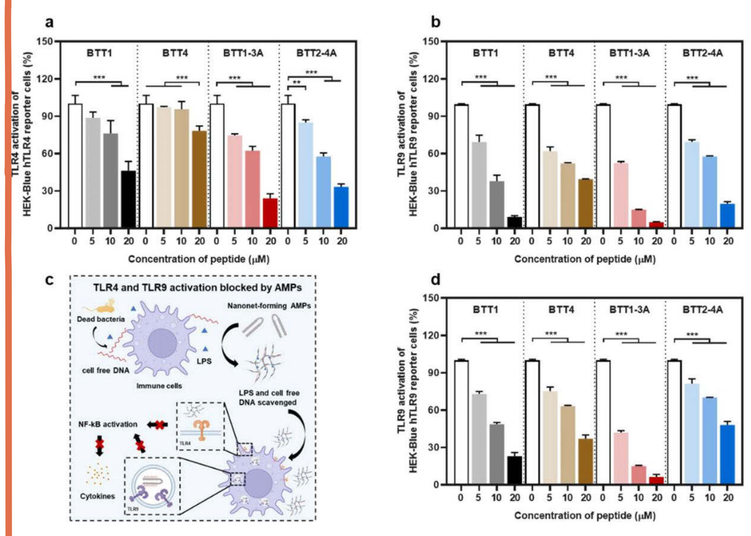
BTT2-4A and BTT1-3A nanonets also displayed selective trapping activity toward pro-inflammatory cytokines while not affecting anti-inflammatory cytokines



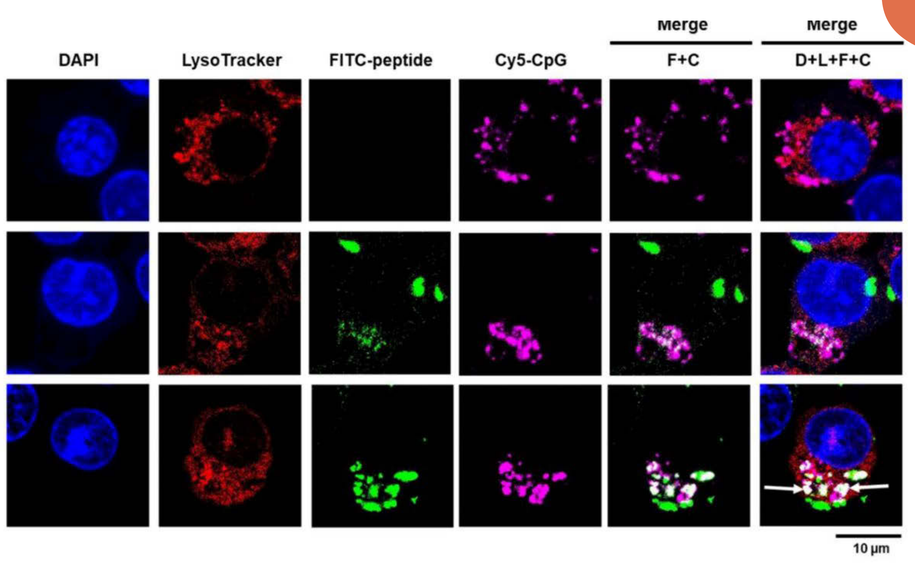


In endotoxin-induced acute lung injury mice model, BTT1-3A via intratracheal administration, significantly lowered the level of pro-inflammatory cytokines.

Nanonet-forming peptides inhibit TLR4 and TLR9 activations in cells by inflammatory mediators



(a) BTT peptides inhibited TLR4 activation in HEK-Blue™ hTLR4 cells by entrapping extracellular LPS. (b, d) BTT peptides inhibited TLR9 activation in HEK-Blue™ hTLR9 cells by scavenging extracellular (b) and intracellular (d) CpG DNA. (c) Scheme of nanonet-forming AMPs blocking TLR4 and TLR9 activations and the downstream NF-κB pathway.



Co-localization of BTT peptides with intracellular CpG DNA in RAW264.7 cells. Most of FITC-BTT1-3A but less of FITC-BTT4 intracellularly co-localized with CpG DNA and endo-lysosomes, demonstrating that BTT1-3A had stronger capacity of entering cells and competitively binding with intracellular CpG DNA than BTT4.

1 Objective: To explore anti-inflammatory activity via inhibition of TLR4 and TLR9 pathways

Methodology:

- CpG DNA binding assay

CpG DNA was incubated with PicoGreen and peptide solutions. Fluorescence intensity was measured.

- Cellular co-localization of peptides with CpG DNA

Overnight culture of RAW 264.7 cells were incubated with 1 μg/mL of Cy5-CpG DNA for 1 hour. FITC-peptides were then added in the medium and incubatied for 4 hours. The cells were stained by LysoTracker Red DND-99, followed by fixation with 4% formaldehyde in PBS and staining with DAPI.

2 Objective: To improve the proteolytic stability of β-hairpin antimicrobial peptide

Methodology:

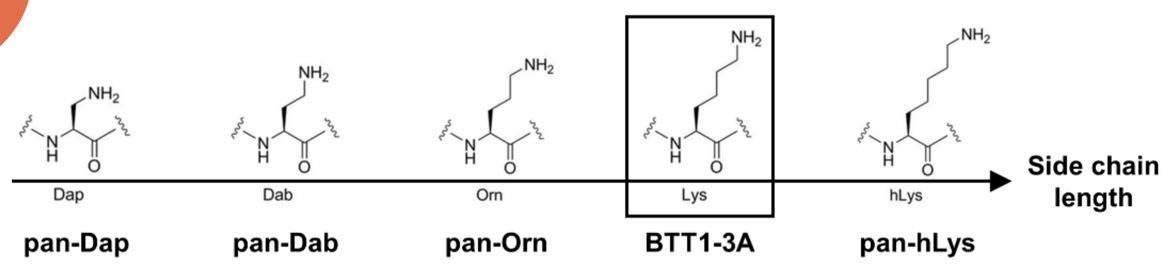
- Minimum inhibitory concentration (MIC) determination

MIC of peptides was determined by broth microdilution method. The MIC presented the lowest peptide concentration at which at least 90% of OD600 was reduced compared to control without peptide treatment.

- Field-emission scanning electron microscope (SEM) was employed for the observation of bacteria-trapping nanostructures.

- NPN (N-phenylnaphthalen-1-amine) uptake assay for outer membrane permeability; SYTOX green uptake assay for indication of inner membrane permeability.

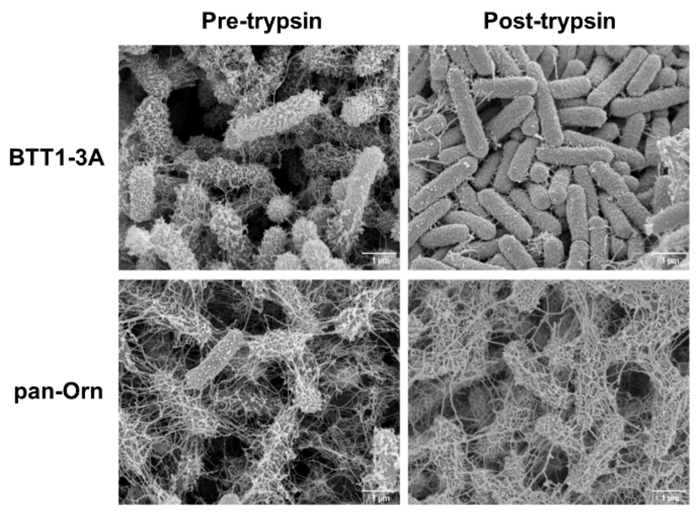
Pan-substitution with Ornithine confer robust enzymatic stability to BTT1-3A while retaining activity



Design scheme for pan-substitution of Lysine residue in BTT1-3A using unnatural amino acids with unaltered stereochemistry but different side chain chemistry.

Peptide	MIC against *E. coli* (μM)	
	Pre-trypsin	Post-trypsin
pan-hLys	128	> 128
BTT1-3A	16	> 128
pan-Orn	8	8
pan-Dab	64	> 128
pan-Dap	>128	> 128

Without trypsin treatment, only pan-Orn displayed better antimicrobial potency relative to BTT1-3A; following trypsin treatment, pan-Orn retains antimicrobial activity with unchanged MIC, suggesting excellent trypsin stability.



In contrast to BTT1-3A whose nanonet-forming capacity drastically degraded post-trypsin, pan-Orn formed expansive bacteria-trapping nanonets both pre- and post-trypsin, further strengthening the claim on superior proteolytic stability of pan-Orn.

References:
1. Tram, N. D. T., et. al., Bacteria-Responsive Self-Assembly of Antimicrobial Peptide Nanonets for Trap-and-Kill of Antibiotic-Resistant Strains. *Adv. Funct. Mater.* 2023, 33, 2210858.
2. Nhan D.T. Tram, et. Al., Manipulating turn residues on de novo designed β-hairpin peptides for selectivity against drug-resistant bacteria, Acta Biomaterialia, Volume 135, 2021
3. N. D. T. Tram, Q. T. N. Tran, J. Xu, J. C. T. Su, W. Liao, W. S. F. Wong, P. L. R. Ee, Multifunctional Antibacterial Nanonets Attenuate Inflammatory Responses through Selective Trapping of Endotoxins and Pro-Inflammatory Cytokines. Adv. Healthcare Mater. 2023, 12, 2203232.

Contact Information
Associate Professor: Pui Lai Rachel EE
Email: phaeplr@nus.edu.sg