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Development of next generation antimicrobial peptides as antimicrobial therapy

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14. ABSTRACT
The PI and team were successful in this research project. Briefly, the first aim of this project is to develop synthetic AMPs with good bactericidal properties, coupled with excellent biocompatibility, for potential in vitro and/or clinical translation. The second aim of this project is to develop a biological platform to cost effectively produce AMPs at reasonable scale.

Using a parent peptide that showed promising broad spectrum antimicrobial characteristics, rational engineering was performed to enhance cationicity, hydrophobic-to-charge ratio and amphipathicity of the peptide analogues. One of the peptide analogues, 23R, stood out as a promising candidate, demonstrating broad spectrum antimicrobial characteristics, with low minimum bactericidal concentration (MBC) values against most gram-positive and gram-negative bacteria. The adoption of an alpha-helical conformation when exposed to microbial membrane appears to be important to disrupt phospholipid arrangement and eventual disintegration of bacteria membrane. The rapid membrane targeting mode of action also minimizes the possibility of resistance development by the microbes. Safety and efficacy studies of 23R in an animal wounding model demonstrated that the candidate peptide is able to perform under the dynamic physiological condition, significantly inhibiting microbial manifestation at the wound site. This peptide is expected to have broad applicability in antimicrobial protection.

A peer reviewed manuscript is in preparation and a conference presentation is in preparation as well as a direct result of the grant funding. There were 1 research assistant and a student supported under this grant.

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USD $74,980

Executive Summary

The first aim of this project is to develop synthetic AMPs with good bactericidal properties, coupled with excellent biocompatibility, for potential *in vitro* and/or clinical translation. Using CP116A, a broad spectrum *de novo* designed peptide, as the parent peptide, rational peptide engineering to enhance cationicity, optimize hydrophobic-to-charge ratio and maximizing amphipathicity was performed to derive analogues. Amongst the analogues derived, 23R stood out as a promising candidate, showing potent bactericidal activities and outstanding biocompatibility. 23R is active against a broad spectrum of microbes, with low minimum bactericidal concentration (MBC) values against most gram-positive and gram-negative bacteria. In-depth structural analysis using circular dichroism and high-resolution 2D-NMR spectroscopy showed that 23R adopts an alpha-helical conformation when exposed to microbial membrane. Killing kinetics and SEM imaging highlighted that 23R is capable of killing 10^6 CFU/mL of inoculated microbes within 1.5 h upon initial exposure, through disruption of phospholipid arrangement and eventual disintegration of bacteria membrane. The rapid membrane targeting mode of action minimizes the possibility of resistance development by the microbes. Results from the resistance development assay support the aforementioned claim with 23R maintaining a constant MBC value (11 μM) against microbes exposed to a sub-bactericidal concentration of peptides, over 10 passages. *In vivo* testing on established animal wounding model demonstrated that the candidate peptide is able to perform under the dynamic physiological condition, significantly inhibiting microbial manifestation at the wound site.

The second aim of this project is to develop a biological platform to cost effectively produce AMPs at reasonable scale. To overcome constraints that are known to compromise peptide yield and/or stability in eukaryotic systems, fusion of two wild type AMP candidates to the human isoform of SUMO was studied. The candidate AMPs studied were not detrimental to *P. pastoris*' viability at 125 μg/ml peptide concentration. Post-expression analyses of the peptides, however, found that the peptides were undetected *via* Western Blot and reversed phase HPLC analyses. It is possible that AMP production at small culture volume was insufficient to compensate for downstream purification losses, resulting in failure to detect trace peptide production titers. A downstream purification strategy involving both cation exchange and RP-HPLC in succession are currently being adopted in place of IMAC. Matrix Assisted Laser Desorption / Ionization time-of-flight (MALDI-TOF) mass spectrometry is also being used to validate the molecular fingerprint of purified recombinant AMP with that produced by chemical synthesis. Quantitative transcriptomic analysis are ongoing to characterize the gene expression of both Bactrocerin-1 and Temporin-PTa in *P. pastoris*, in relation to the concentration of methanol inducer and growth environment factors.

1. Objectives

The specific aims of this project are detailed below, where the studies underpinning each specific aim are also outlined.

Specific aim 1. To engineer potent, broad spectrum antimicrobial peptides guided by peptide structure-function relationship studies

a) Characterization studies of selected natural AMP candidates to determine the peptide's general mode of antibacterial action and structure-activity relationship using high resolution physical and biochemical analytical assays.

b) Peptide engineering guided by rational design strategies based on findings from (a).

c) Determination of efficacy, safety and stability of the engineered AMPs in selected applications.

Specific aim 2. To develop a bioproduction platform for cost effective large scale production of antimicrobial peptides

a) Genetic manipulation of *P. pastoris* as cell factory for high yield secretion of candidate antimicrobial peptides.

b) High density fermentation studies of *P. pastoris* culture at pilot scale.

2. Materials and Methods

2.1. Materials

2.1.1. Peptides

Pure peptides were synthesized by Bio Basic Inc. (Canada) using commercial FMOC chemistry to a purity of >90%. Chemicals were purchased from Sigma Aldrich (Singapore), unless otherwise stated. Human uropathogenic *Escherichia coli* strain UTI89 (*E. coli*) and the fluorescent protein-expressing strain (*E. coli*-GFP), *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and *Pseudomonas aeruginosa* PAO1 (*P. aeruginosa*) were used for respective antimicrobial and anti-biofilm studies.

2.1.2. Strains and plasmids

Pichia pastoris X-33 was employed for expression of our peptides of interest. *Escherichia coli* DH5α (Invitrogen) was used as the host strain for propagation of our vectors. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 were used for determination of the peptides' Minimum Inhibitory Concentration (MIC). The pGAPαZA (Invitrogen) and pAOXαZA (created by switching GAP to AOX) were used for cloning and expression (Figure 1). pGAPZαA vector contains the constitutive promoter of GAPDH protein while pAOXαZA contains the inducible promotor of alcohol oxidase 1 protein. Although GAP is a strong promoter, continuous high levels of expression needs to be modulated as AMPs may be toxic to *P. pastoris*. On the other hand, AOX1 promoter allows for a more

regulated expression by allowing the cells attain a desired density using glycerol as a carbon source. Step-wise addition of methanol was employed to induce expression of AMPs. Both vectors contained the alpha factor signal sequence, AOX translation terminator and gene for resistance to Zeocin. The signal sequence for *Saccharomyces cerevisiae* α-mating factor drives protein targeting the secretory pathway.

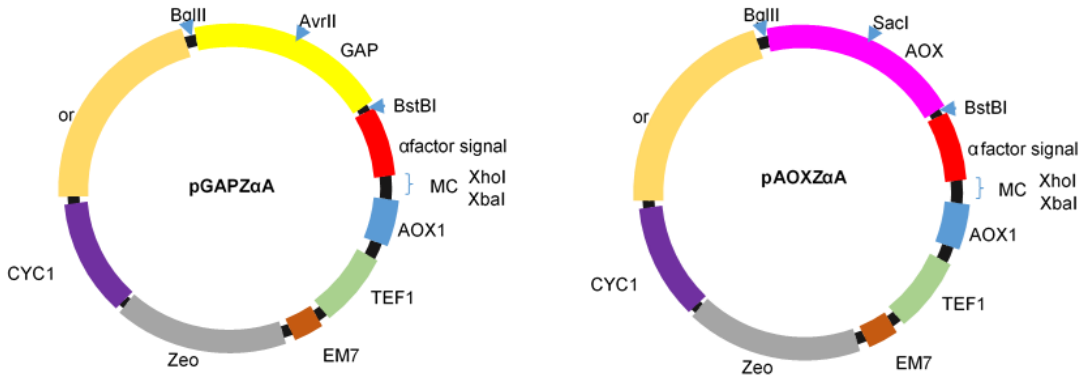

Figure 1. Plasmid maps of pGAPZαA (left) and pAOXZαA (right) integrative shuttle vector. Design constructs encoding for the AMP and Sumo carrier proteins were assembled into the plasmid vectors using the XhoI and XbaI restriction sites. Positive clones were selected by Zeocin resistance and verified by PCR. GAP and AOX promoters were used to drive constitutive and methanol-inducible expression of the gene of interest, respectively.

2.1.3. Media

E. coli cells were grown in Luria-Bertani (LB) (Sigma Aldrich) media liquid cultures. For selection, 50 μg/mL of Zeocin was added. The cultures were grown overnight at 37°C while shaking at 200 rpm. *P. pastoris* cells were grown in YPD medium (1 % (w/v) yeast extract, 2 % (w/v) Peptone, and 2 % (w/v) D-glucose. Bio Basic). For selection, 100 μg/mL Zeocin was added. The cultures were grown overnight at 30°C while shaking at 200 rpm. For both organisms, solid media were prepared as above with addition of 2% (w/v) agar (Sigma Aldrich). BMGY (10g/L Yeast Extract, 20g/L Peptone, 10g/L Glycerol, 13.4g/L Yeast Nitrogen Base without Amino Acids, 3.013g/L Potassium phosphate dibasic, 11.8g/L Potassium phosphate monobasic, 0.04mg/L D-Biotin. Sigma Aldrich) was used for growing *P. pastoris* transformants containing the AOX promoter. Methanol (Merck) was subsequently added to induce expression.

2.1.4. DNA purification

Plasmids were purified using EZ-10 kit (Bio Basic). PCR product/DNA fragments were purified using MiniElute kit (Qiagen) and QIAquick kit (Qiagen). In-gel purification was done using Qiaquick gel purification kit (Qiagen). DNA concentration was detected using UV VIS spectrophotometry (NanoDROPoneC, ThermoFisher).

2.1.5. Enzymes

Restriction enzymes XbaI, SalI, XhoI, DpnI, BglI,BstBI, AvrII, the T4 DNA ligase and Polymerase enzymes TAQ, HiFi and Q5 (New England Biolabs) were used.

2.1.6. Synthesis and sequencing of primers and protein fragments

Antimicrobial peptides (AMP) Temporin-PT-a (FFGSVLKLIPKIL) and Bactrocerin-1 (VGKTWIKVIRGIGKSKIKWQ) were chosen for expression. Bactrocerin-1 and Temporin PT-1 were codon optimized, synthesized and cloned in pUC57 by Bio Basic. All the primers were synthesized by IDT Singapore. Sequencing was performed by Bio Basic. Primers for the peptides of interest were designed with XhoI on the 5' primer and Xba1 on the 3' fragment to allow easy ligation to vector backbone. His tag (6x His residues) was added behind each peptide of interest to allow for purification using a His column.

2.2. Methods

2.2.1. Bactericidal assay

Bactericidal potency for synthetic peptides was determined using standard microdilution assays against 10^6 CFU/mL of target microbes. Upon overnight incubation, the microbial suspension was plated on nutrient agar plate and visually observed for colony formation. The lowest peptide concentration that completely kills the inoculated microbes is determined as the minimum bactericidal concentration (MBC). Killing kinetics of the peptides was determined by doing CFU enumeration and optical density at designated time points.

2.2.2. Peptide structural studies

Peptide conformation was determined using two main studies, Circular Dichroism and Two Dimensional Nuclear Magnetic Resonance (2D-NMR). For both studies, candidate peptides were exposed to different solvents, to mimic neutral and microbial membrane environments. Respective spectrums were obtained and deconvoluted for elucidation of peptide structure under the respective conditions.

Biocompatibility studies were conducted with two different cell lines, namely erythrocytes and uroepithelial cells. The respective mammalian cells were exposed to the candidate peptides for 24 h, at physiological temperature (37^{o}C). Viability of the mammalian cells were determined through either hemolytic rate or MTT viability studies.

2.2.3. Anti-biofilm assay

Biofilm dispersal capabilities of the candidate peptide was studied using a modified anti-biofilm assay. A thick layer of biofilm was first established on unmodified PDMS substrate surface. Candidate peptide, at appropriate concentration was exposed to the established biofilm for 24 h, at 37^{o}C. CFU enumeration of the adherent microbial community was conducted to provide an indication of the extent of biofilm disruption.

2.2.4. *In vivo* safety and efficacy studies

In vivo safety and efficacy testing of the candidate peptide was conducted using an established mice wounding model. Briefly, a superficial wound of 6 mm diameter was inflicted on the dorsal of the cleanly shaven mice. 10 μL of microbial suspension (10^4 CFU) was inoculated onto the wound and left to air dry for 5 min. Peptides, encapsulated in appropriate hydrogel systems, was introduced to the wound and secured using Tegaderm™ dressing. Upon being in contact with the wound, peptides will be steadily released from the hydrogel system. Mice were euthanized at 24 h post-infection by carbon dioxide asphyxiation. 1 cm^2 of skin around the wound was excised and homogenized in 1 mL of PBS. CFU enumeration of the bacterial burden was conducted as an indication of the degree of infection manifestation around the wound.

2.2.5. Molecular cloning

PCR products and vector backbone pGAPαZA pAOXαZA were digested with XbaI and XhoI restriction enzymes. The digested products were purified using the Qiaquick column (Qiagen). Ligation was performed using a 3:1 insert to vector molar ratio in a final volume of 20 µl. The reaction tube was incubated at room temperature for 10 min and the reaction arrested by incubating the tube on ice for 5 min. 10 µl of the ligation mix was added to 50 µl of DH5α competent cells (which had been thawed on ice). The reaction tube was incubated on ice for 30 min, and then heat shocked for 45 s at 42°C followed by 2 min on ice. The cells were resuspended in 200 µl of LB media and incubated for 30 min at 37°C to allow for cell recovery. The cells were then spread on a LB plate comprising 50µl/ml zeocin and incubated for 16 h at 37°C.

2.2.6. *P. pastoris* transformation

10 ml of YPD was inoculated with a single colony of *P. pastoris* X33 and grown for 24 h. The cells were diluted to an Optical Density 600nm (OD_{600}) of 0.003 in 40 ml of YPD and grown at 30°C under 200 rpm shaking conditions until an OD_{600} of 1.6 was attained. Cells were then transferred into 50 ml falcon tubes and pelleted by centrifugation at 15,000 rpm for 5 min. The pellet was resuspended in 10 ml of Transformation Buffer (100 mM LiAc, 10 mM DTT, 0.6 M sorbitol, and 10 mM Tris-HCl, pH 7.5. Sigma Aldrich). The cells were incubated for 30 min at room temperature after which the cells were centrifuged and the supernatant discarded. The cells were then transferred into 2 ml tubes and washed 4 times with 1.5 ml of ice-cold 1M sorbitol (Sigma Aldrich). The cells were resuspended in a final volume of 80 µl 1 M ice cold sorbitol.

1 ug of AvrII linearized plasmid in 1-10µl of sterile water were added to the cells which were then transferred to a 0.2 cm Bio-rad Gene Pulser cuvette and incubated on ice for 5 to10 min. The cuvette was pulsed using Bio-Rad Gene Pulser XCell Electroporation System with the settings 2 kV, 25 μF and 200 Ω. 1 ml of ice-cold YPDS (1 % (w/v) yeast extract, 2 % (w/v) Peptone, 2 % (w/v) D-glucose, 1M Sorbitol) was added immediately to the cuvette after the electroporation. The cuvette was incubated at 30°C for 2 h. The cells were transferred into a 1.5 ml tube and pelleted. The supernatant was removed and the remaining 20-30

µl of media was used to resuspend the cells. The cells were then spread on YPD+Zeocin 100ug/ml plate and incubated for 48 h at 30°C. Once electroporated, the linearized AvrII or SacI plasmids will integrate at the GAP or AOX locus by homologous recombination.

2.2.7. Colony PCR for *E. coli* and *P. pastoris*

8 *E. coli* transformants with Zeocin resistance were picked from the transformation plate and resuspended in 10 µl distilled water. A PCR was set up using TAQ DNA polymerase and respective primers which were used for the amplification of the insert. The size of the PCR fragment sufficed to select possible positive clones. 3 positive clones were sent for sequencing using the primer NB23.

8 yeast transformants with Zeocin resistance were picked from the transformation plate and streaked on a YPD+Zeocin plate. After overnight incubation at 30°C, cells were picked and added to 20 µl distilled water and boiled at 95°C for 10 min. 1 µl of this was used as template for colony PCR. NB22 and NB23 were used in the set up of a TAQ DNA polymerase PCR reaction. pGAPZαZA and pAOXαZA vector backbone were used as a positive controls.

Agarose (Sigma Aldrich) gel electrophoresis were subsequently ran to ascertain the sizes of the colony PCR fragments. Bio-rad gel tanks and power packs were used to run the gel. Uview (Bio-rad) were added to allow DNA fragments to fluorescnce under UV light. 100 bp molecµlar marker from Bio-rad was used. Amersham Imager was used to visualize the bands on the gel.

2.2.8. Peptide expression

Transformants containing the GAP consitutive promoter were inoculated in 5 ml YPD and grown for 48 h at 30°C and 200 rpm (Thermo Fisher MaxQ 6000 Incubated Shaker). Transformants containing AOX inducible promoter were inoculated in 5 ml BMGY and grown for 48 h at 30°C and 200 rpm to ensure complete glycerol consumption. Subsequently, 1% (v/v) methanol was added twice a day for the next 96 h to induce expression of peptides, at 30°C with shaking at 200 rpm.

2.2.9. Peptide purification and concentration

Cell culture was centrifuged at 10,000 rpm for 5 min and both pellets and supernatant kept separately. The total cell pellet was transferred to a 1.5 ml tube, the excess supernatant removed and 300 µl ice-cold Breaking Buffer (50mM Sodium Phosphate pH 7.4, 5% Glycerol,10 mM PMSF, 10 mM EDTA, 2 mM DTT, protein inhibitor cocktail (Complete Mini, Roche) 1 tablet for 10ml breaking buffer) was added. The cells were resuspended and transferred in an ice-cold screw cap tube containing 300 µl acid-washed glass beads (Sigma Aldrich, 425-600 µm (30-40 U.S. sieve)). The tube was subjected to 2 x 40 s 5.5m/s bead beating (5 min on ice in between) using the MP Fastprep24 (BioMedical). The disrupted cells were transferred to

an ice cold 1.5 ml tube and spun down for 5 min at 15,000 rpm at 4°C. The supernatant (~240 µl) was transferred into a new 1.5 ml tube.

Both supernatant of the harvested cells and whole cell intracellular fractions were purified using HisPur™ Ni-NTA Chromatography Cartridge 90099 (Thermo Scientific), by employing the protocol provided in the instruction manual. Syringe application was used at 1 ml/min. Following elution, samples were concentrated using Amicon® µltra-15 Centrifugal Filter Units with a 3 kDa cut off. Samples were concentrated from 25 ml to 1 ml at 10,000 rpm and 4°C.

2.2.10. SDS-PAGE and Western blot

40 µl of 4x Sample Buffer (Bio-rad) and 1 M DTT (1,4-Dithiothreitol, Sigma Aldrich) were added to 120 µl of His-purified samples, mixed and boiled for 5 min at 95°C. Samples were cooled at room temperature prior to loading onto an SDS-PAGE gel. Both Tris/Glycine and Tris/Tricine gels (Bio-rad) were used to run the samples. Precision Plus Protein™ Dual Xtra Prestained Protein Standards (Bio-rad) was used as a marker. The gels were run at 80 V for 20 min, followed by 150 V for 45 min. Green Fluorescence Protein (GFP) with His tag was used as a positive control. The gel was stained overnight with Instant blue for SDS PAGE and rinsed with water before visualization.

For Western Blot, the proteins were transferred to a 0.2 µm nitrocellulose membrane using the Bio-rad transblot system, where the semi dry method program (Mixed MW 8 min transfer) was employed. The membrane was blocked with 0.05% (v/v) TBS Tween 20, 5% (v/v) fat free milk and incubated for 1 h at room temperature on orbital shaker. The blocking buffer was then removed and 25 ml of anti-His antibody (sc-8036 HRP from Santa Cruz) in TBS Tween 0.05% (v/v), 5% (v/v) fat free milk at 1:200 dilution was added. The membrane was incubated overnight at 4°C on orbital shaker. On the following day, the membrane was washed 3 times with TBS Tween at 5 min each and added with 1.5 ml of Amersham ECL Prime substrate. The membrane was incubated for 5 min and viewed using Amersham chemiluminescent platform.

2.2.11. Determination of minimum inhibitory concentration

Minimum Inhibitory Concentration (MIC) of Bactrocerin-1 and Temporin-PTa against *P. pastoris* was tested by using chemically synthesized peptides. MIC would be the minimum concentration of peptides at which the cells showed no growth. 15.6, 31.3, 62.5, 125, 250, 500, 1000, 2000 and 4000 µg/ml of peptides were added to *P. pastoris* culture (OD_{600} ~0.1) in 96 well plate and incubated at 30°C without shaking. OD_{600} readings of the *P. pastoris* culture were measured at 24 and 48 h. MIC of Bactrocerin-1 and Temporin-PTa against *E. coli*, *S. aureus* and *P. aeruginosa* were also determined as controls. Before the MIC assay was performed, a Colony Forming Unit (CFU) determination assay was conducted. The respective bacteria cells were inoculated in 5ml LB overnight. 50 µl cells in 20% glycerol were aliquoted and stored at -80°C. One

aliquot was used for each assay. 100 µl of thawed cells was inoculated in 5 ml LB and incubated for 1 h at 37°C with shaking. 100 µl of cells were added into the first well of a 96 well plate and 10-fold serial dilutions were performed to achieve 10^7-fold dilution using LB as a diluent. 5 µl was spotted on an LB plate and incubated overnight. The number of colonies for dilutions with countable, single colonies and CFU/ml was subsequently calculated based on this formula:

CFU/ml = no. of colonies x dilution factor x 1000µl / 5µl

With this CFU/ml value, the MIC assay was performed with different strains of bacteria. Synthesized peptides were diluted to 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320 µg/ml. 50 µl of the diluted peptides were mixed with 50 µl of 2 x 10^6 cells/ml of bacteria cells and incubated at 37°C without shaking. OD600 was read every hour to determine the MIC.

2.2.12. Reverse phase high-performance liquid chromatography

Peptides were analyzed by reverse phase high-performance liquid chromatography (RP-HPLC). Agilent Technologies 1200 Infinity II was used together with Poroshell 120, EC-C18 2.7um 4.6 x 150mm (Agilent Technologies). 1.5 ml/min flow rate was used with a gradient run from 10% (v/v) to 80% (v/v) acetonitrile within 10 min. 10 µl of sample was injected per run, and the temperature set at 26.3°C. Wavelength for detection was employed at 220 and 280nm. 20 µg/ml pure synthetic peptides were first analyzed as controls.

3. Results and Discussion

3.1. Peptide engineering towards broad spectrum antimicrobial performance

Our earlier studies previously reported the development of a promising synthetic antimicrobial peptide (AMP) P116 (KKLIKKILKIL)[1], possessing good antimicrobial potency against a broad spectrum of microbes. With the goal to develop AMP-functionalised materials, P116 was further engineered with the addition of a cysteine residue at the N-terminal and amidation of the C-terminal to enhance peptide stability, respectively. The modifications did not adversely affect the antimicrobial performance of the derived peptide, CP116A (CKKLIKKILKIL-NH_2), demonstrating favorable bactericidal potency (MBCs of 11 µM) against target pathogen. However, the lack of an easily detectable amino acid residue (e.g. tryptophan) within the peptide sequence affects ease of peptide assay and has limited the application of CP116A for other studies. The relatively high hemolytic toxicity, which accompanies the potent bactericidal properties, also renders CP116A unsuitable for general clinical antimicrobial application.

In this study, rational engineering using CP116A as a template was conducted with the aim to develop short AMPs with superior bactericidal potency and enhanced biocompatibility was performed (Table 1). The main

engineering strategies include sequential removal of hydrophobic amino acids, rearrangement of amino acids to maximize amphipathicity and reinforcing cationicity through replacement of lysine with arginine. After each round of modification, the performance of the analogue peptides was verified through minimum bactericidal assay and hemolytic assay. A candidate peptide, with the most optimal antimicrobial potency and biocompatibility, was selected for the next round of engineering.

Table 1 shows the bactericidal acitivities and hemocompatibility (at 500 µg/mL) of the parent and analogue peptides. Compared to the CP116A, two of the analogue peptides (23 and 23R) exhibited comparable bactericidal potency and desirable biocompatibility with mammalian erythrocyte cells. The two analogues are selected as candidate AMPs for subsequent studies. 23R, in particular, holds great potential. Despite having similar microbial killing capability (MBC of 11 µM) as CP116A, 23R proved non-cytotoxic towards mammalian cells, with a low hemolytic rate of 9% at a high concentration of 500 µg/mL (350 µM).

Table 1. Modifications to parent peptide CP116A with sequential amino acid(s) replacement for enhanced bactericidal properties and improved biocompatibility. Peptide sequences, which are bolded, represent candidature peptide chosen for the next round of modification. CP116WAV23 (denoted hereafter as 23) and CP116WAV234R (denoted hereafter as 23R) are chosen as the candidature peptides for subsequent studies.

Modification	Peptides	Sequence	Minimum bactericidal concentration (µM)	Hemolytic rate (%)	Peptide modification
0	**CP116A**	**CKKLIKKILKIL-NH2**	11	66	Parent peptide
1	**CP116WA1**	**CKKWIKKILKIL-NH2**	21	70	Replacement of one hydrophobic amino acid with tryptophan (underlined), for easier detection and enhance bactericidal potency
	CP116WA2	CKKLWKKILKIL-NH2	21	76	
	CP116WA3	CKKLIKKILKWL-NH2	21	80	
	CP116WA4	CKKLIKKILKIW-NH2	42	76	
2	CP116WAV1	CKKWIKILKKL-NH2	23	53	Reducing the number of hydrophobic amino acid(s) while maintaining amphipathicity, to improve biocompatibility and augment antibacterial capability
	CP116WAV2	**CKIWKKILKKL-NH2**	11	45	
3	CP116WAV21	CKWKKILKKL-NH2	25	29	
	CP116WAV22	CKIWKKLKKL-NH2	25	31	
	CP116WAV23 (23)	**CKIWKKIKKL-NH2**	25	7	
4	CP116WAV231R1	CKIWKRIKKL-NH2	24	2	Replacing lysine(s) with arginine(s) to improve electrostatic interaction between peptide and bacterial membrane
	CP116WAV231R2	CKIWKKIKRL-NH2	24	3	
	CP116WAV232R	CKIWKRIKRL-NH2	24	3	
	CP116WAV234R (23R)	**CRIWRRIRRL-NH2**	11	9	

3.2. Bactericidal performance of candidate peptides

The two candidate AMPs (23 and 23R) were screened against a variety of gram negative and gram positive microbes. Table 2 shows the minimum bactericidal concentration (MBC) of the respective candidate AMPs against ten chosen pathogens. In general, both 23 and 23R demonstrated broad-spectrum bactericidal properties, targeting both gram negative and gram positive microbes. Compared to 23, 23R fared better in terms of coverage, demonstrating a good degree of potency against 80% of the tested microbes, and bactericidal potency, with a lower MBC values. While both candidate peptides are inferior to gold standard

antibiotics (kanamycin) for some strains, both 23 and 23R are more effective against specific strains of bacteria, such as *P. aeruginosa*, *L. monocytogenes* and *B. subtilis*.

Table 2. MBCs of candidature AMPs 23 and 23R against a wide spectrum of microbes. Antibiotic (kanamycin) served as a positive control for comparison of bactericidal potency.

	23	23R	Kanamycin
	Minimum bactericidal concentration (μM)		
E. coli (UTI89)	26.7 ± 9.2	13.3 ± 4.6	3.4 ± 0.0
P. aeruginosa (PAO1)	63.0 ± 0.0	24.0 ± 13.9	208.3 ± 72.2
S. enterica (ATCC13311)	5.3 ± 2.3	6.7 ± 2.3	7.8 ± 0.0
L. monocytogenes (ATCC4644)	32.0 ± 0.0	6.7 ± 2.3	52.1 ± 18.0
B. subtilis (ATCC6633)	9.3 ± 6.1	4.0 ± 0.0	62.5 ± 0.0
S. aureus (USA300)	42.3 ± 17.9	18.7 ± 12.2	7.8 ± 0.0
E. faecalis (ATCC29212)	>500	166.7 ± 72.2	62.5 ± 0.0

3.3. Structural deconvolution for candidate peptides

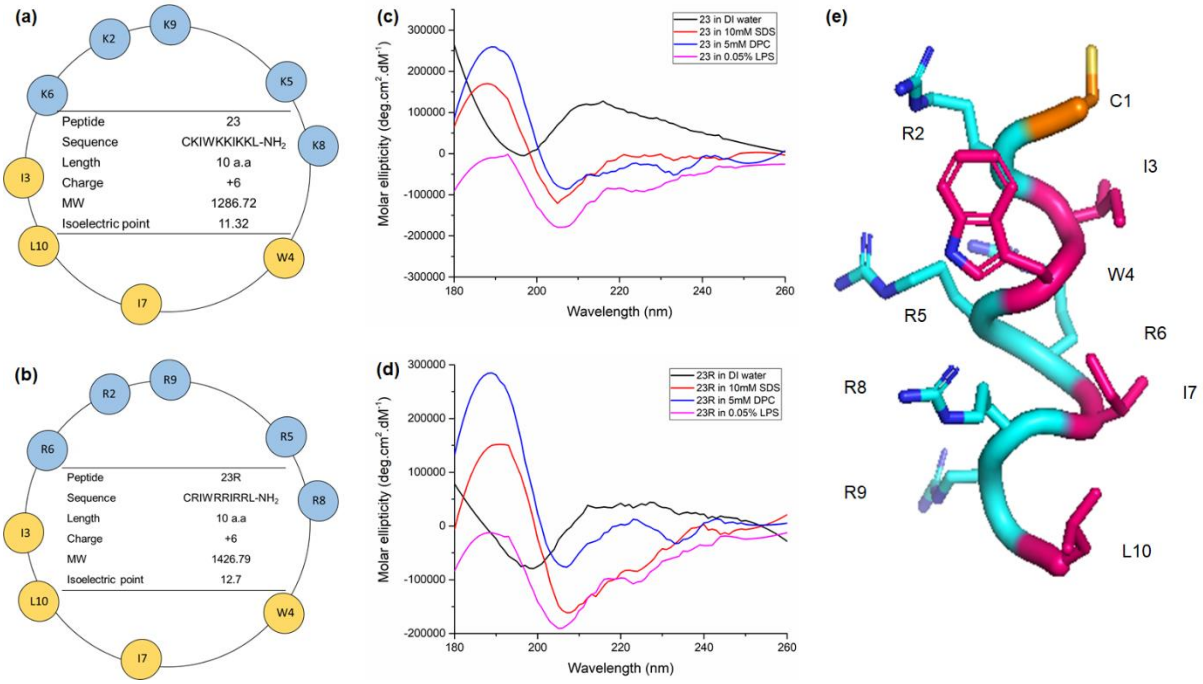

Figure 2. Structural design and deconvolution of candidate peptides, 23 and 23R. Schiffer-Edmundson helical wheel diagram for 23 (a) and 23R (a). Both peptide were designed to maximize amphipathicity, with hydrophobic amino acids on one face and hydrophilic amino acid on the opposite face. Circular dichroism deconvolution of 23 (c) and 23R (d) in different solvent environment. Both candidate AMPs adopted a highly helical conformation upon exposure to 10 mM SDS micelles and microbial lipopolysaccharides. High resolution 2D-NMR analysis and deconvolution of 23R (f) with SDS micelles showed the peptide adopting a highly helical conformation upon interaction with SDS micelles.

The Schiffer-Edmundson helical wheel diagram is adopted for the design and engineering of the respective peptide analogues. Figures 2(a) and (b) show the Schiffer-Emundson helical wheel diagrams for 23 and 23R respectively. As illustrated in the diagrams, both peptides are engineered to optimize amphipathicity, with the hydrophobic and cationic amino acid residues organized on opposite faces. These distinct faces each affect the antimicrobial action of the peptides and should be optimized to enhance initial electrostatic interaction and facilitate penetration of AMPs into microbial membrane.

In order for the aforementioned peptide design to be valid, the ability for the peptide to adopt a helical conformation is an important pre-requisite. Circular dichroism spectroscopy was used to investigate the secondary structures of the candidate peptides in different environments. Both 23 (Figure 2c) and 23R (Figure 2d) possess similar circular dichroism spectroscopic fingerprints in different solvent environments. In the presence of neutral deionized water, the AMPs exhibited disordered conformations with low molar ellipticity above 210 nm and negative band near 195 nm[2,3]. In the presence of bacteria membrane-mimicking sodium dodecyl sulfate (SDS) micelles, lipopolysaccharides (LPS) and dodecylphosphocholine (DPC) micelles, 23 and 23R adopted alpha helical conformations, characterized by negative troughs at 206 nm and 225 nm coupled with a positive peak at 190 nm[4], with 23R possessing a higher degree of alpha helicity, with peaks and troughs of greater magnitudes.

The detailed 3D conformation of 23R was further studied using two dimensional nuclear magnetic resonance (2D-NMR) spectroscopy (Figure 2e). Results of the 3D deconvolution of 23R is in agreement with circular dichroism spectroscopy results. Arrangement of the amino acid residues corresponds with the Schiffer-Edmundson alpha helical representation, where hydrophobic and cationic resides are organized on opposite faces, maximizing amphipathicity.

3.4. Biocompatibility of candidate peptides

Biocompatibility of candidate AMPs was assessed using (i) erythrocyte hemolysis, and (ii) human epithelial cell viability assays. Figures 3a and 3b show that even at high peptide concentration (175 μM), no severe cytotoxic effect was observed for both AMPs, with hemolytic rate (<10% hemolysis) and cell viability (>90% cell viability) maintained within acceptable clinical limits. Given 23R's superior antimicrobial performance for clinical translation and further application studies, 23R was selected as candidate AMP for further analysis.

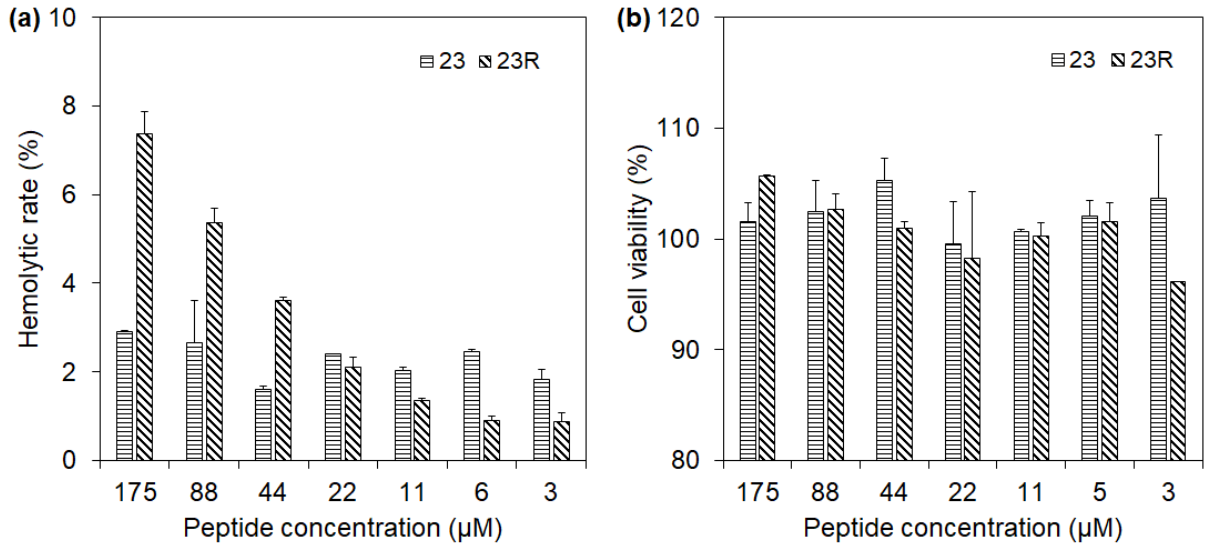

Figure 3. Biocompatibility of candidate peptides, 23 and 23R. Both peptides demonstrated excellent biocompatibility, with (a) low erythrocyte hemolysis and (b) high mammalian cell viability.

3.5. Kinetics and mode of bactericidal action of candidate peptides

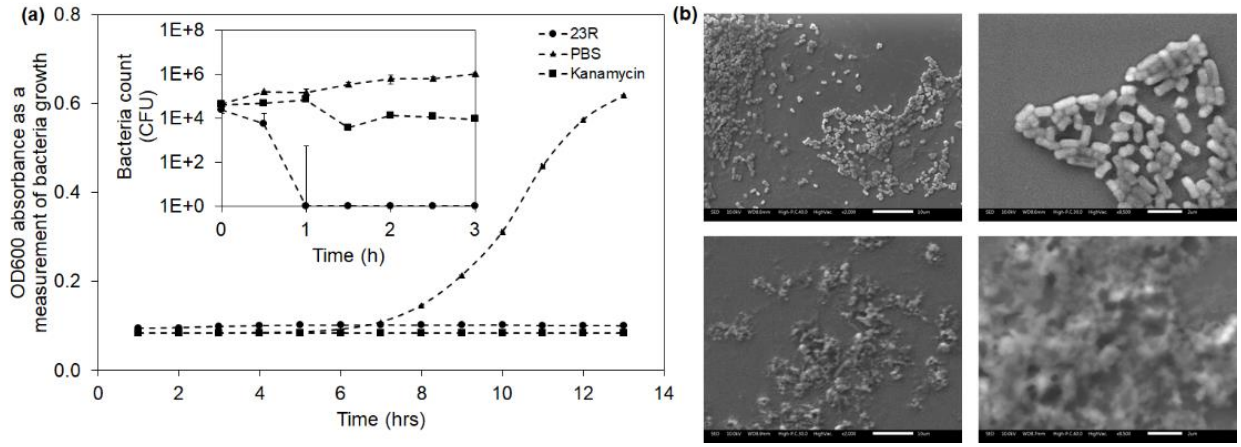

Figure 4. (a) Candidate AMP 23R's antimicrobial efficacy against E. coli (UTI89) over a 13 h time period in comparison to PBS (negative control) and Kanamycin (positive control). Cell growth, as indicated by increase in absorbance at OD_{600}, was measured at 30 min time intervals. To narrow down on the time required for 23R to complete its bactericidal action, CFU enumeration-based kinetic assay was conducted for the first 3 hours (inset). 23R completes its bactericidal action within 1.5 h of incubation with 10^6 CFU/mL of microbes. (b) SEM micrograph of E. coli cells treated with PBS (top) and candidate peptide 23R (bottom). In contrast to the cells treated with PBS, which showed distinct and undisrupted microbial cell membrane, microbes treated with 23R showed disrupted cell membrane with no distinct cell present.

23R kills microbes in a concentration-dependent manner. At a peptide concentration of 13.3 ± 4.6 μM, 23R killed *E. coli* over 18 h of incubation (Table 2). To further understand the bactericidal kinetics of of 23R, a time-kill kinetics assay was conducted for a period of 13 h (Figure 4a). Based on the OD_{600} absorbance measurement, 23R exhibited comparable bacterial killing profile to kanamycin, completely inhibiting

microbial growth within 3 h. Figure 4a inset shows the time kill curves, based on CFU enumeration, for the first 3 h. CFU enumeration revealed the feature of 23R's rapid bactericidal action, where killing of the inoculated microbes was completed within 1.5 h upon initial exposure.

Figure 4b shows a SEM micrograph of the microbes upon exposure to PBS (top) and 23R (bottom). In contrast to microbes treated with PBS, which present themselves as distinct cells with smooth cytoplasmic membrane, the 23R-treated microbes showed pronounce membrane wrinkling and surface blebbing. The cytoplasmic membranes were severely disintegrated with fibrous and cellular materials dispersed outside the cells as a result of leakage and cell lysis. The SEM result suggests that 23R adopts a membrane targeting mode of action, where upon initial contact with the microbial membrane, the AMP realigns and inserts into the phospholipid bilayer, inducing localized defects in the lipid packing. Beyond a certain critical AMP concentration, the membrane eventually disintegrates, causing leakage of cellular content and cell death.

3.6. Biofilm dispersion property of 23R

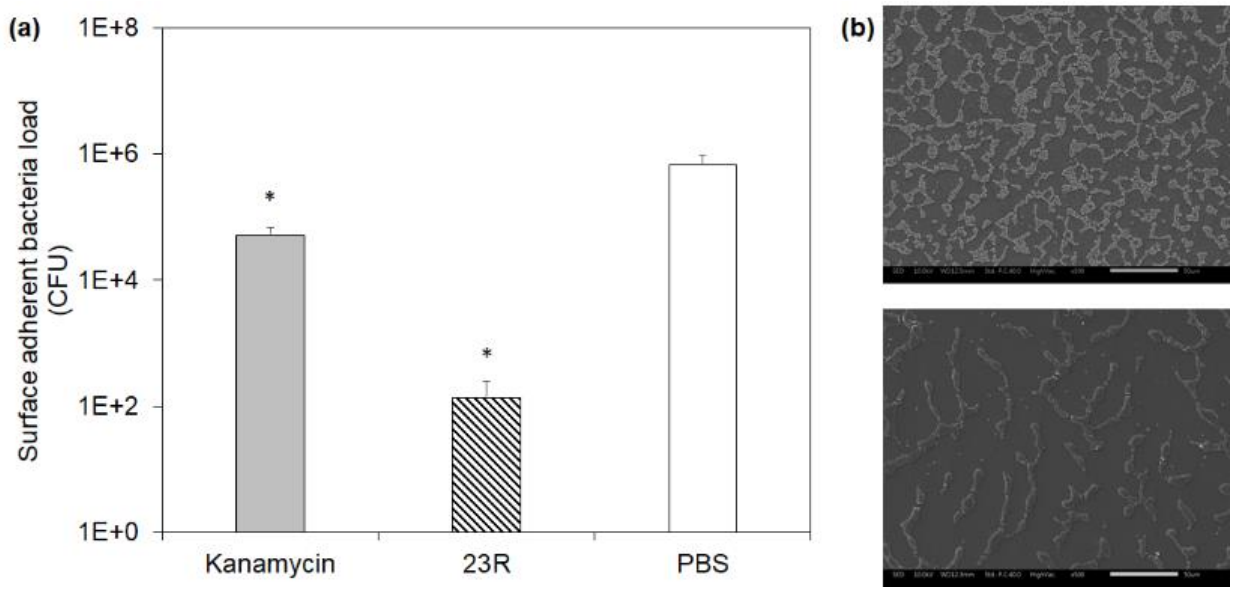

*Figure 5. Biofilm dispersal capability of candidate AMP 23R. (a) CFU enumeration of bacteria concentration on substrate surface provides a quantitative indication of the extent of biofilm remaining on the surface upon treatment with respective antibacterial compounds. 23R demonstrated superior biofilm dispersal properties, effectively reducing established biofilm by 3 log. (b) SEM micrograph of the 23R treated biofilm showed corresponding result. Well-established biofilm was significantly dispersed upon treatment with 23R for 72 h. * indicates $p < 0.05$ in comparison to negative control (PBS) respectively.*

The capability of candidate peptide 23R to effectively target and disperse biofilm was investigated. A thick film of biofilm was firmly established on the surface of PDMS substrate, followed by exposure to respective antimicrobial agents and PBS for 72 h. CFU enumeration (Figure 5a) and SEM micrograph (Figure 5b) of the treated PDMS substrates show that biofilms were significantly dispersed upon treatment with 23R and kanamycin. 23R outperformed kanamycin (1 log reduction) with a massive 4 log reduction and dispersal of

the established biofilm, demonstrating capability of the AMP to effectively target microbes protected within the tough extracellular polymeric substance.

3.7. Resistance development of 23R

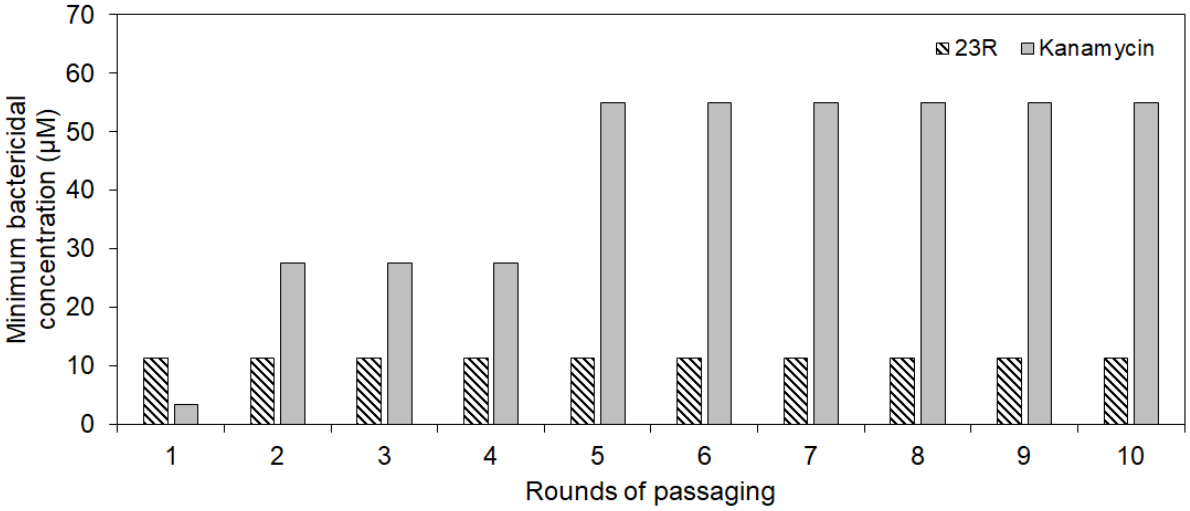

Figure 6. Upon subjecting to repeated exposure to sub-MBC concentration of 23R and multiple rounds of sub-culturing, there were no significant changes in the MBC of the candidate AMP. In comparison to kanamycin, which saw a gradual increase of its MBC, 23R does not induce resistance in microbes after 10 passages.

Excessive use of antibiotics has led to rapid development of resistance in microbes. As a consequence of inappropriate antibiotic therapy, such as suboptimal dosing, poor pharmacokinetics, usage of low-quality drugs and poor patient compliance, microbes are exposed often exposed to sub-MBC doses of antibiotics[5]. At such concentration, the microbes can continue to proliferate, despite at a reduced growth rate, increasing the rate of adaptive evolution and encouraging resistance development[6]. Due to human and external factors, more often than not, exposure to sub-MBC concentration of antimicrobial compounds cannot be avoided. In view of this, the next generation antimicrobial compound should ideally have minimum tendency for resistance development.

A simple study was conducted to investigate the likelihood of resistance development against 23R. Briefly, bacteria exposed to sub-MBC concentration of respective antimicrobial agents (23R and kanamycin) were sub-cultured in a fresh media. The microbial suspension was then subjected to the standard antimicrobial assay to ascertain the MBC. Microbial suspension exposed to sub-MBC concentration of respective tested antimicrobial compounds was withdrawn and sub-cultured in fresh media for the next round of assay. The process was repeated for 10 passages.

Figure 6 shows the variation of MBCs over a period of 10 passages. MBC for kanamycin climbs steadily with increasing passages, from 3 µM to 55 µM, indicating the development of resistance in the microbes. On the contrary, no resistance development was observed for microbes exposed to sub-lethal concentration of 23R, as the MBCs remained constant at 11 µM over the 10 passages. This could be attributed to the 23R's membrane targeting mode of bactericidal action, reducing the plausibility of microbial developing alternative pathways and/or defensive mechanisms to circumvent the antimicrobial effect.

3.8. *In vivo* antimicrobial efficacy

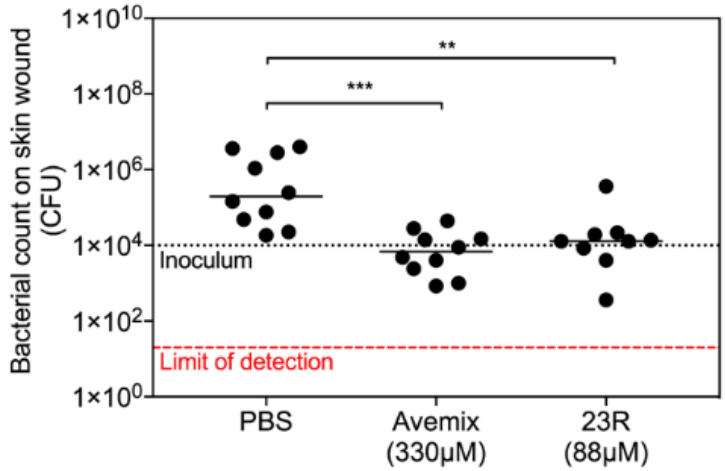

*Figure 7. In vivo antimicrobial efficacy testing of candidate AMP. 23R effectively inhibited microbial proliferation on the skin wound (>10^4 CFU/wound). Antimicrobial performance of 23R under in vivo condition is comparable to well-established veterinary antibiotics (Avemix®). ** and *** indicates $p < 0.005$ and $p < 0.0005$ in comparison to negative control (PBS) respectively.*

In vivo bactericidal performance of the candidate peptide 23R was evaluated using an adapted mouse wounding model[7,8]. Higher titer microbial count was established on majority of the mice (80%) treated with PBS, with a median burden of 1.94 x 10^5 CFU and maximum burden of 4 x 10^6 CFU at 24 h post infection. Both Avemix® and 23R demonstrated significant antibacterial effect, inhibiting bacterial manifestation and keeping wound microbial count relatively constant (~10^3 to 10^4 CFU). Avemix® (median: 6.8 x 10^3 CFU) performed slightly better with an overall lower microbial burden as compared to 23R (1.28 x 10^4 CFU).

The *in vivo* result highlighted the capability of the candidate peptide to maintain its potent bactericidal activity under dynamic, physiological environment.

3.9. Peptide expression studies in *P. pastoris*

The second aim of this project is to study the possibility of efficient production of the peptides in a host organism to allow scalable use of promising peptides. *P. pastoris* is generally regarded as safe, able to grow to high cell densities and secretes little endogenous proteins, thus enabling easy purification of the target peptide. Peptide candidates like 23R would make an interesting model peptide given the presence of RR in its sequence. KK, RR or KR residues are recognized by the KEX2 proteases in the trans-Golgi network, often resulting in AMP cleavage leading to the permanent loss or reduction of antimicrobial effect. Small ubiquitin-related modifier (SUMO) proteins serve as molecular chaperones in eukaryotes to regulate key cellular processes. We hypothesized that the fusion of such peptides to the human isoform of SUMO3, which is unaffected by endogenous yeast proteases, may sterically mask the peptides from proteolytic activity of KEX2. SUMO3 as a fusion partner may also restrict toxicity to *P. pastoris*, hence allowing stable expression of the cationic AMP to high titers[9]. Subsequent purification and treatment of SUMO3-HHC36 fusion with SUMO3-specific protease will release AMP as a free molecule and restore its antimicrobial activity. The feasibility and validity of this platform to express wild type AMP candidates, i.e. Bactrocerin-1 and Temporin-PTa, was first studied.

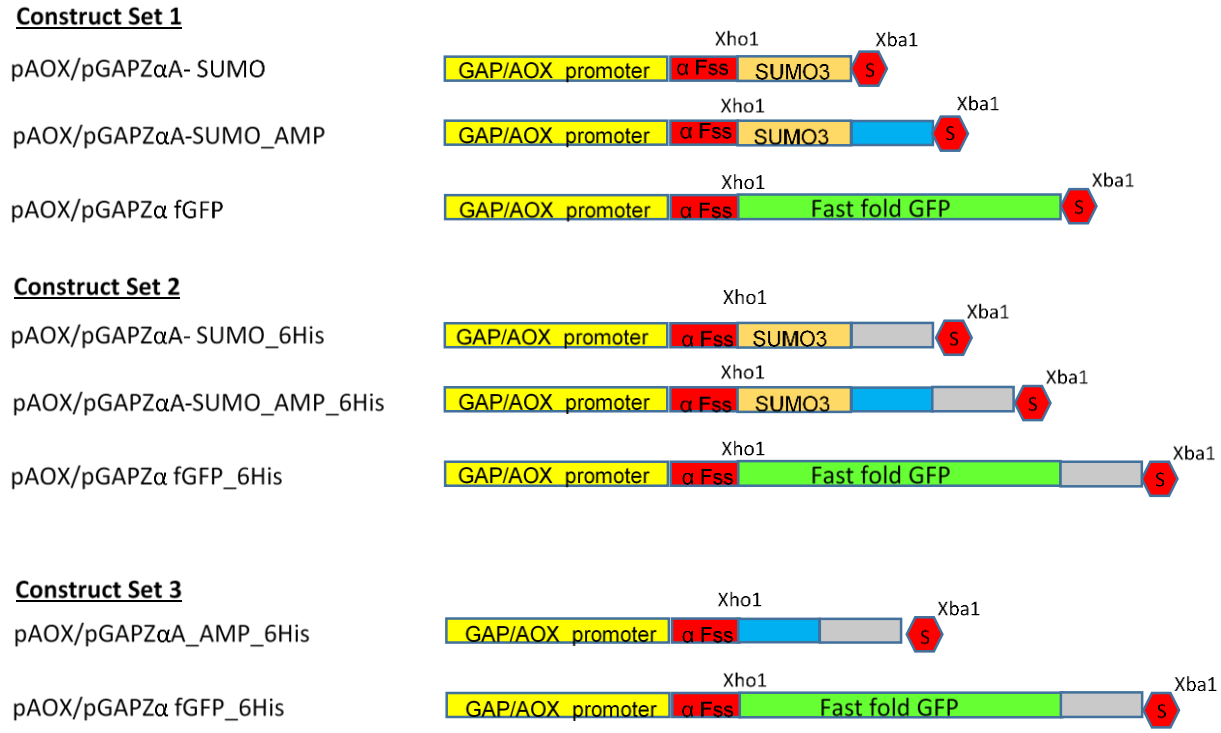

Figure 8. Schematic representation of constructs designed to study AMP expression. Construct Set 1 incorporates AOX/GAP promoter followed by alpha factor signal sequence then SUMO3, followed by AMPs. All constructs have stop codons at the end and are flanked with XhoI and XbaI restriction sites. Construct Set 2 comprises of the same eliminants as Set 1, except for the addition of 6x His tag (grey) before the stop codon. Construct Set 3 is without SUMO 3.

SUMO3 was employed as a carrier protein to study if increasing the protein construct size could improve the overall product yield (Figure 8). Following expression, cleavage of the peptide from SUMO3 would be performed by SUMO peptidase. However, analysis of both the total cell lysate and supernatant post-expression by SDS-PAGE stained by Commassie Blue did not reveal any observable bands that corresponded to the correct size of Temporin-PTa (2.5 kDa) and Bactrocerin-1 (2.3 kDa). 6x-His residues were incorporated as protein tag to aid peptide detection. Both the total cell lysate and extracellular supernatant were purified by immobilized metal affinity chromatography (IMAC) using commercial Ni-NTA column, concentrated and subjected to Western Blot analysis using anti-His antibodies. A 6x-His tagged Green Fluorescence Protein (GFP) was employed as a control for peptide expression. While the GFP positive control showed a clear band corresponding to 37 kDa, no band was detected for IMAC-purified total cell lysate and extracellular supernatant of both Temporin-PTa (2.5 kDa) and Bactrocerin-1 (2.3 kDa) (data not shown). The absence of bands for both secreted and intracellular fractions suggests that the peptides were either present in negligibly low amounts or they were not expressed. The purified total cell lysate and supernatant samples were further analysed using RP-HPLC. The absence of peaks corresponding to Temporin-PTa and Bactrocerin-1 suggests that both peptides were not expressed to sufficient amount for detection. The lower detection limit for the chemically synthesized Temporin-PTa and Bactrocerin-1 was determined to be ~ 20 µg/ml.

3.10. Minimum inhibitory concentrations (MIC) of wild type AMP candidates

To ascertain if host cell viability contributed to sub-optimal expression levels of the peptides, MIC of Temporin-PTa and Bactrocerin-1 against *P. pastoris* was performed.

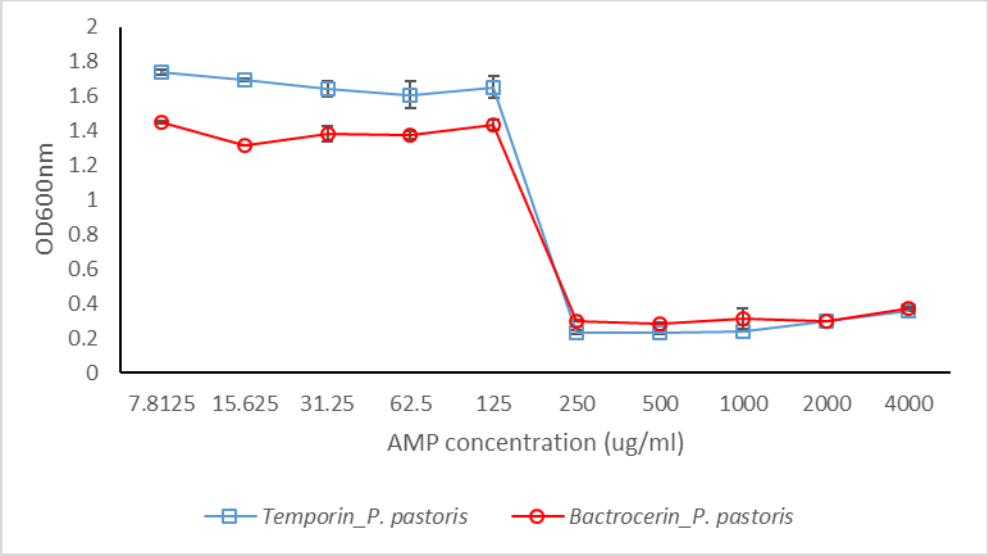

Figure 9. MIC of Bactrocerin-1 and Temporin-PTa against P. pastoris*.*

The MIC for both peptides against *P. pastoris* was found to be approximately 125 μg/ml (Figure 9), which opens the way for the use of AOX promoter to allow cells to attain maximum cell density before induction to produce AMPs. Compared to other host organisms such as *E. coli*, *S. aureus* and *P. aeruginosa* (Figure 10), the use of *P. pastoris* seemed adequate for high expression studies of the model peptides chosen.

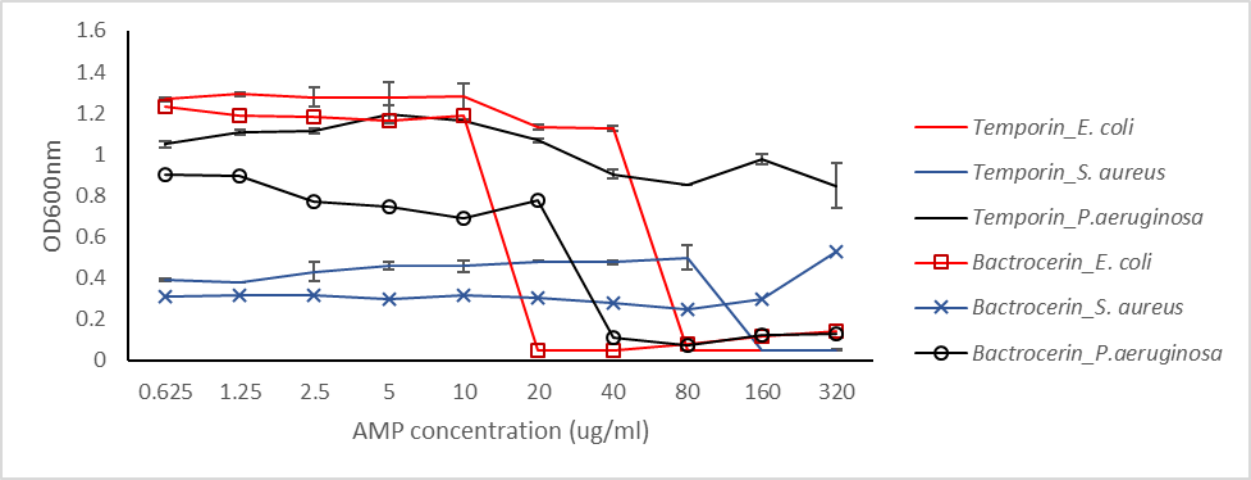

Figure 10. MIC of Bacterocin and Temporin against E. coli, S. aureus *and* P. aeruginosa.

3.11. Comparison with *E. coli* bioprocessing platform

In our earlier efforts to assess AMPs in *E. coli*[15], a fusion protein construct was designed to comprise two fusion tags, i.e. hexa(His) and maltose binding protein (MBP), which were consecutively added to the N-terminal of the target peptide, i.e. human beta defensin 28 (hBD28), followed by a spacer and the TEVp cleavage site, respectively. Recombinant His-MBP-hBD28 was expressed predominantly as soluble proteins, which constituted approximately 30% w/w of the total soluble protein expressed under optimized fermentation conditions (i.e. 23 °C, harvested at 2 h post-induction) in a 2 L culture, as determined by SDS-PAGE analysis. Although the expression of the MBP fusion tag is energetically expensive due to the relatively large molecular size of MBP (i.e. 42 kDa) compared to hBD28, the MBP tag supported the production of soluble His-MBP-hBD28 in this study. Although purification of His-MBP-hBD28 can be performed using both amylose affinity and Ni^{2+}-IMAC columns, the overall higher His-MBP-hBD28 recovery and purification selectivity for the monomeric fusion protein demonstrated by Ni^{2+}-IMAC compared to amylose affinity chromatography justified the use of Ni^{2+}-IMAC chromatography in hBD28 bioprocessing. The higher loss of monomeric fusion protein in amylose affinity purification step could be caused by reduced binding of the MBP fusion protein due to steric constraints caused by MBP. Optimised Ni^{2+}-IMAC purification achieved a His-MBP-hBD28 yield of 57±7% and 71±6% purity. A cleavage yield of 97±2% was achieved upon 14 h of incubation of purified fusion protein with a modified tobacco etch virus protease (TEVp), as determined by densitometry and SDS-PAGE analyzes. Purified HBD28 was finally recovered from an acid precipitation step at >95% purity and 67±12 step yield without compromising peptide structure and activity.

The use of *P. pastoris* was intended to further streamline the bioprocess flowsheet, by minimizing the number of purification steps to recover the purified peptide. Due to extended time involved in construct design, we were unable to scale up the expression of model AMP candidates in *P. pastoris* to comparable scale of that of our previous work in *E. coli*, i.e. 2 L, within the reporting period, and are currently doing so.

4. Concluding remarks and ongoing/future work

Using a parent peptide that showed promising broad spectrum antimicrobial characteristics, rational engineering was performed to enhance cationicity, hydrophobic-to-charge ratio and amphipathicity of the peptide analogues. One of the peptide analogues, 23R, stood out as a promising candidate, demonstrating broad spectrum antimicrobial characteristics, with low minimum bactericidal concentration (MBC) values against most gram-positive and gram-negative bacteria. The adoption of an alpha-helical conformation when exposed to microbial membrane appears to be important to disrupt phospholipid arrangement and eventual disintegration of bacteria membrane. The rapid membrane targeting mode of action also minimizes the possibility of resistance development by the microbes. Safety and efficacy studies of 23R in an animal wounding model demonstrated that the candidate peptide is able to perform under the dynamic physiological condition, significantly inhibiting microbial manifestation at the wound site. This peptide is expected to have broad applicability in antimicrobial protection.

To study the scalability of antimicrobial peptide production in biological platforms, *P. pastoris* was chosen as the host biological organism, where the stable expression and production of AMPs in the range of 3.7 kDa – 7.5 kDa in *P. pastoris* has been reported[10-12], albeit at lower than desirable titers. Expression of lower molecular weight AMPs remains a challenge, in part, due to the higher susceptibility of short peptides to endogenous protease degradation. The use of human serum albumin (HSA) as the C-terminal fusion carrier protein, had recently been shown to enable stable expression of a 2.1 kDa Apidaecin. Despite a reported yield of ~700 mg/l in fed-batch bioreactor mode, the combined Apidaecin-HAS fusion was 70 kDa with 3% w/w of the fusion protein comprising the actual AMP[13]. In this study, the feasibility of producing low MW AMPs, namely Bactrocerin-1 and Temporin-PTa, was evaluated using SUMO3 as the carrier protein and *P. pastoris* as the platform strain. Although we were unable to produce sufficient titer of either recombinant AMP for detection by Western blot and HPLC, the results have contributed to better understanding on AMP bioprocessing requirements. Firstly, conventional Western blotting may not be sufficiently sensitive to detect small peptides, given the propensity of these peptides to detach from blotted membranes. Tomisawa *et al.* demonstrated that low MW peptides (≤ 4 kDa) readily dissociate from PVDF membranes on gentle shaking and more so in the presence of detergents[14]. To resolve this problem, vacuum-assisted Western blotting is currently being studied as an alternative detection platform, which eliminates the need for any form of shaking during the downstream immunodetection steps, and thus could prove more suitable for the detection of low MW AMP. Additionally, we re-evaluated our experimental design and are growing *P. pastoris* culture in larger volumes. It is likely that the amount of AMP produced at small culture volume was

not sufficient to compensate for downstream purification losses, resulting in failure to detect trace production titers. Our earlier studies expressing disulfide-bonded AMPs in 2L *E. coli* cultures achieved a 30% overall yield and >95% peptide purity[15]. A downstream purification strategy involving both cation exchange and RP-HPLC in succession are also currently being studied in place of IMAC. AMPs of 3.7 kDa sizes have been successfully purified at high concentration using the suggested purification strategy whereas the smallest AMP reportedly purified by IMAC was 5.4 kDa[16-17]. Matrix Assisted Laser Desorption / Ionization time-of-flight (MALDI-TOF) mass spectrometry can be used to validate the molecular fingerprint of purified recombinant AMP with that produced by chemical synthesis[12,18]. Quantitative transcriptomic analysis are ongoing to characterize the gene expression of both Bactrocerin-1 and Temporin-PTa in *P. pastoris*, in relation to the concentration of methanol inducer and growth environment factors[19].

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