



Full length article

Disruption of drug-resistant biofilms using *de novo* designed short α -helical antimicrobial peptides with idealized facial amphiphilicity



Jasmeet Singh Khara^{a,b,c}, Sybil Obuobi^a, Ying Wang^a, Melissa Shea Hamilton^b, Brian D. Robertson^c, Sandra M. Newton^b, Yi Yan Yang^d, Paul R. Langford^{b,1}, Pui Lai Rachel Ee^{a,*,1}

^a Department of Pharmacy, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore

^b Section of Paediatrics, Department of Medicine, St Mary's Campus, Imperial College London, London W2 1PG, United Kingdom

^c MRC Centre for Molecular Bacteriology and Infection, Department of Medicine, Imperial College London, London SW7 2AZ, United Kingdom

^d Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, Singapore 138669, Singapore

ARTICLE INFO

Article history:

Received 23 December 2016

Received in revised form 17 April 2017

Accepted 19 April 2017

Available online 29 April 2017

Keywords:

Antimicrobial peptides (AMPs)

Drug-resistant biofilms

Anti-endotoxic

Ideal facial amphiphilicity

Alpha-helix

ABSTRACT

The escalating threat of antimicrobial resistance has increased pressure to develop novel therapeutic strategies to tackle drug-resistant infections. Antimicrobial peptides have emerged as a promising class of therapeutics for various systemic and topical clinical applications. In this study, the *de novo* design of α -helical peptides with idealized facial amphiphilicities, based on an understanding of the pertinent features of protein secondary structures, is presented. Synthetic amphiphiles composed of the backbone sequence $(X_1Y_1Y_2X_2)_n$, where X_1 and X_2 are hydrophobic residues (Leu or Ile or Trp), Y_1 and Y_2 are cationic residues (Lys), and n is the number repeat units (2 or 2.5 or 3), demonstrated potent broad-spectrum antimicrobial activities against clinical isolates of drug-susceptible and multi-drug resistant bacteria. Live-cell imaging revealed that the most selective peptide, $(LKKL)_3$, promoted rapid permeabilization of bacterial membranes. Importantly, $(LKKL)_3$ not only suppressed biofilm growth, but effectively disrupted mature biofilms after only 2 h of treatment. The peptides $(LKKL)_3$ and $(WKKW)_3$ suppressed the production of LPS-induced pro-inflammatory mediators to levels of unstimulated controls at low micromolar concentrations. Thus, the rational design strategies proposed herein can be implemented to develop potent, selective and multifunctional α -helical peptides to eradicate drug-resistant biofilm-associated infections.

Statement of Significance

Antimicrobial peptides (AMPs) are increasingly explored as therapeutics for drug-resistant and biofilm-related infections to help expand the size and quality of the current antibiotic pipeline in the face of mounting antimicrobial resistance. Here, synthetic peptides rationally designed based upon principles governing the folding of natural α -helical AMPs, comprising the backbone sequence $(X_1Y_1Y_2X_2)_n$, and which assemble into α -helical structures with idealized facial amphiphilicity, is presented. These multifunctional peptide amphiphiles demonstrate high bacterial selectivity, promote the disruption of pre-formed drug-resistant biofilms, and effectively neutralize endotoxins at low micromolar concentrations. Overall, the design strategies presented here could provide a useful tool for developing therapeutic peptides with broad-ranging clinical applications from the treatment and prevention of drug-resistant biofilms to the neutralization of bacterial endotoxins.

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1. Introduction

Recently, the discovery of bacterial resistance to what was reported as “the last line antibiotic”, colistin, has fuelled speculation that the world could be on the brink of the post-antibiotic era [1]. This problem is compounded by biofilms, which are structured aggregates of microbial cells enclosed within an extracellular

* Corresponding author.

E-mail address: phaeplr@nus.edu.sg (P.L.R. Ee).

¹ These authors contributed equally to this work.

polymeric substance (EPS) matrix that adhere to biological or non-biological surfaces [2]. Associated with over 65% of all infections, biofilms are involved in various device-related infections and chronic infections, including cystic fibrosis pneumonia, endocarditis, necrotizing fasciitis and musculoskeletal infections [3]. Despite the immense challenges of eradicating biofilm-related infections, antimicrobials being developed are rarely evaluated for their anti-biofilm activities.

Antimicrobial peptides (AMPs) have gathered considerable interest as a new source of antibiotics due to their broad-spectrum and rapid bactericidal activities, in addition to their ability to synergize with conventional antibiotics against drug-resistant pathogens [4]. AMPs possess immensely diverse sequences, yet fundamentally are comprised of a high proportion of hydrophobic residues and bear a net positive charge, enabling them to selectively target the 'microbial Achilles heel': the negatively charged phospholipid bilayer [5]. Upon contacting microbial membranes, AMPs assume ordered amphipathic structures and disrupt membrane integrity via barrel-stave or toroidal-pore mechanisms, or induce 'micellization' by carpet-like mechanisms [6]. Some peptides possess non-membrane permeabilizing mechanisms, and instead translocate the cytoplasmic membrane to act on intracellular targets [5,6]. Besides their direct antimicrobial activity, AMPs effectively inhibit biofilm formation and disrupt mature biofilms [3]. Moreover, these multifunctional molecules demonstrate diverse immunomodulatory properties including the modulation of chemokine expression, promotion of wound healing and angiogenesis, and reduction of pro-inflammatory mediators in response to bacterial endotoxins such as lipopolysaccharides (LPS) and lipoteichoic acid [7,8].

Many AMPs transition from disordered structures in solution to assume organized, facially amphiphilic conformations, with the clustering of hydrophobic and cationic amino acids into spatially distinct regions [5]. Investigations aimed at optimizing the amphipathic α -helix as a means of enhancing antimicrobial potency uncovered that peptides with idealized facial amphiphilicities, comprising of uninterrupted hydrophobic and cationic segments (Fig. 1A), have demonstrated superior membrane-targeting activities and in turn enhanced selectivities [9,10]. This improvement in antimicrobial activity has been attributed to the enhanced penetration and depolarization of bacterial membranes demonstrated by peptides with idealized facial amphiphilicities [11]. One study found that amongst a series of peptides presenting perfectly amphipathic helices, the peptide demonstrating optimal antibacterial selectivity, LBU2, also had the highest mean hydrophobic moment – a measure of amphiphilicity of an α -helix [12]. The otherwise continuous hydrophobic face of the LL-23 fragment of human cathelicidin is interrupted by a single hydrophilic serine residue at position 9, creating two amphipathic domains [11]. Substitution of serine9 with either of the hydrophobic residues valine or alanine creating the uninterrupted hydrophobic surface found in primate cathelicidins not only resulted in higher antimicrobial activity, but also increased the suppression of LPS-induced pro-inflammatory mediators [11]. These findings highlight the importance of optimizing the facial amphiphilicity of synthetic peptides in order to generate more selective lead compounds to be brought forward for clinical testing.

However, the strategies employed by the majority of these studies have remained largely empirical, often utilizing natural AMPs as templates, or helical wheel projections to perform modifications by replacing, deleting or scrambling amino acid sequences [13]. Additionally, optimized sequences derived from natural AMPs, with high sequence homology to host defense peptides, could inadvertently compromise innate immunity should antimicrobial resistance develop [14]. As such, implementing a rational approach in the *de novo* design of peptides which fold into

predetermined α -helical structures presenting idealized facial amphiphilicity, and with distinct sequences from natural AMPs [15], could allay these concerns. Recently, the repeating sequence (XXYY)_n, devised on the basis of mimicking the hydrophobic periodicity of natural α -helical AMPs, was successfully applied in the development of short synthetic amphipathic α -helical peptides [16–18]. However, in order to maintain a balance between hydrophobic and charged residues, the number of repeat units is restricted to positive integers ($n = 1, 2, 3$, and so forth). Another inherent limitation of this sequence is that it cannot be utilized to generate peptides with idealized facial amphiphilicity for $n > 2$ (Fig 1C1).

In this study, α -helical amphiphiles with perfectly segregated hydrophobic and cationic faces were rationally designed based upon principles governing the folding of natural α -helical AMPs including: 1) stabilization of the helical coil by repetitive hydrogen bonding between the carbonyl oxygen of each amino acid and the amide hydrogen four residues ahead [19], 2) maximization of hydrophobic moment with periodic distributions of hydrophobic and cationic amino acids having repeat periods corresponding to that of the α -helix of 3.6 residues [20,21], and 3) the incorporation of amino acids possessing helix-forming rather than helix-breaking tendencies [22]. Therefore, the recurring sequence comprising four amino acids (X₁Y₁Y₂X₂)_n – where X₁ and X₂ are hydrophobic amino acids, Y₁ and Y₂ are cationic amino acids, and n is the number repeat units – was devised to preserve the α -helical periodicity, recognized as a key driver of helical formation [23], while ensuring a balance between hydrophobic and charged residues. The synthetic amphiphiles were initially characterized for their α -helical propensity in a membrane-mimicking environment, before evaluation of their therapeutic efficacy against a range of both drug-susceptible and -resistant bacteria. Live-cell imaging studies provided insights into the membrane-lytic antimicrobial mechanisms. Finally, the anti-endotoxin activity of the designed peptides and their versatility in inhibiting biofilm formation and disrupting pre-formed drug-resistant biofilms was also assessed.

2. Material and methods

2.1. Materials

Synthesis of peptides was performed by GL Biochem (Shanghai, China) and their purity was confirmed to be more than 95% by reversed-phase HPLC. Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (PBS) solution, dimethyl sulfoxide (DMSO), propidium iodide (PI), carbenicillin, crystal violet, HPLC grade water, Triton X-100 and LPS from *E. coli* O111:B4 were from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was from Labtech International (Sussex, UK). Bacto Brain Heart Infusion (BHI) was from BD (Sparks, MD, USA) while agar-agar technical for microbiology was from Merck (Darmstadt, Germany). 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazolium bromide (MTT) was from Duchefa Biochemie (Haarlem, Netherlands). The Griess Reagent System was from Promega (Madison, WI, USA) and the Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantitation Kit was from Pierce Biotechnology (Rockford, IL, USA).

2.2. Bacterial strains and growth conditions

The *P. aeruginosa* reference strain (PAO1), laboratory strains of *E. coli* (K-12 MG1655) and methicillin-sensitive *S. aureus* (MSSA 8325-4), and the clinical isolate of *K. pneumoniae* (MIDG1643) were evaluated in this study. Drug-resistant strains include the hospital-acquired methicillin-resistant *S. aureus* (MRSA 252) and multi-drug

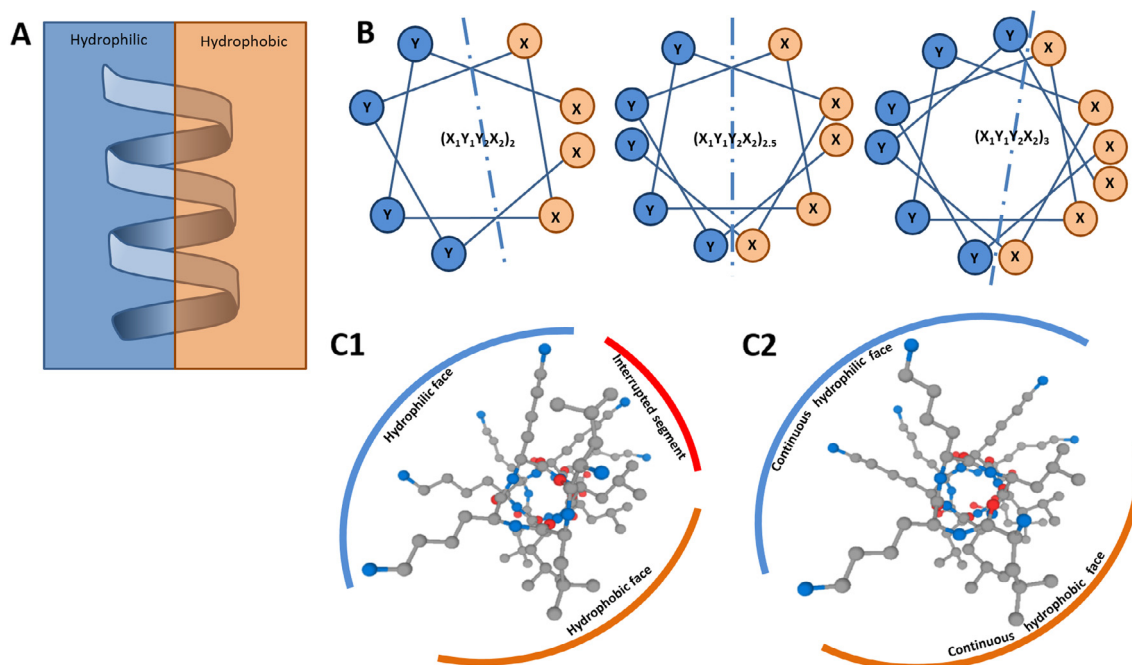


Fig. 1. (A) Schematic illustration and (B) helical wheel projection of α -helical peptides with idealized facial amphiphilicity, possessing the backbone sequence $(X_1Y_1Y_2X_2)_n$, where X_1 and X_2 are hydrophobic amino acids, Y_1 and Y_2 are cationic amino acids, and n is the number repeat units. Ball-and-stick models demonstrating interrupted and continuous hydrophilic and hydrophobic segments in (C1) (LLKK)₃ and (C2) (LKKL)₃, respectively, generated by the Mobyle bioinformatics portal available online at <http://mobyle.rpbs.univ-paris-diderot.fr/> [42].

resistant (MDR) *P. aeruginosa* wound isolates (PA-W1, PA-W14 and PA-W25). All bacterial strains were grown in BHI broth or on BHI agar.

2.3. Peptide characterization

Surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI-TOF MS) spectra were obtained as previously described to verify peptide molecular weights [24]. Briefly, ProteinChip® NP20 arrays (Bio-Rad Laboratories) were primed with 5 μ L HPLC grade water, and 5 μ L of peptide (500 μ M) was applied to the array and allowed to dry for 1 h at room temperature. Each array was washed twice with HPLC grade water and dried for 15 min at room temperature before applying $2 \times 0.7 \mu$ L of 20% α -cyano-4-hydroxycinnamic acid (CHCA) to each spot on the arrays. Spectra were generated using a PCS 4000 SELDI-TOF MS instrument (Bio-Rad Laboratories) at a laser energy of 800 nJ and a focus mass based on the theoretical molecular weight of each individual peptide. The matrix was attenuated to 500 Da and ten shots were obtained per position. Mass accuracy was calibrated externally using All-in-One Peptide standard (Bio-Rad Laboratories).

2.4. Circular dichroism (CD) spectroscopy

CD spectra were acquired using the Chirascan™ CD Spectrometer (Applied Photophysics, Surrey, UK). Peptide solutions were first diluted in SDS surfactant (25 mM) to achieve a final concentration of 0.2 mM. CD spectra were recorded at room temperature from a wavelength of 190 to 240 nm, and at a scanning speed of 60 nm min⁻¹ using a quartz cell with path length of 1.0 mm. Measurements were averaged from 3 runs per peptide and the acquired spectra were converted to mean residue ellipticity using the following equation:

$$\theta_M = \frac{\theta_{\text{obs}}}{10} \cdot \frac{M_{\text{RW}}}{c \cdot l} \quad (1)$$

where θ_M is the mean residue ellipticity (deg cm² dmol⁻¹), θ_{obs} is the observed ellipticity corrected for the blank at a given wavelength (mdeg), M_{RW} is residue molecular weight (M_w /number of amino acids), c is peptide concentration (mg mL⁻¹), and l is the path length (cm).

2.5. MIC measurements

The antibacterial activity of the synthetic α -helical peptides against Gram-positive and Gram-negative bacteria was evaluated using the standard broth microdilution method. The peptides were serially diluted with BHI broth, and 100 μ L was added to each well of the 96-well plate. Bacterial cultures were grown up to mid-log phase and diluted to an initial inoculum of 10^6 CFU mL⁻¹ before seeding an equal volume into each well. The plates were then allowed to incubate at 37 °C in a shaking incubator for 18 h for all strains. The MIC was determined by confirming the absence of bacterial growth both visually and spectrophotometrically by OD₆₀₀ readings taken with the VersaMax Tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Experiments were carried out in triplicate on at least 2 separate occasions.

2.6. Hemolytic activity test

Evaluation of peptide toxicity against mammalian cells was carried out on freshly drawn RBCs from healthy adult volunteers. Ethics approval for this study was granted by Imperial College London NHS Healthcare Tissue Biobank (sub-collection reference number: Pae-SN-15-001) and written informed consent was obtained from all donors prior to enrolment in the study. RBCs were diluted 25 $\times$ with PBS to obtain a 4% (v/v) suspension for testing of hemolytic activity. Twofold serial dilutions of peptides were performed with PBS to give solutions with final concentrations ranging from 0 to 2000 μ M. 300 μ L of peptide solution was mixed with an equal volume of RBC suspension before incubation at 37 °C for 2 h. Subsequently, the mixtures were centrifuged at 1700g for 5 min and

100 μ L of the supernatant was transferred to each well of the 96-well plate. The extent of hemoglobin release was assessed by spectrophotometric measurements at 576 nm by the VersaMax Tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Untreated RBCs and those treated with 1% Triton X-100 served as negative and positive controls respectively. Data are expressed as mean $\pm$ standard deviation for two independent experiments performed in triplicate. The degree of hemolysis was calculated using the following equation:

Hemolysis(%)

$$= \frac{(OD_{576nm} \text{ of treated sample} - OD_{576nm} \text{ of negative control})}{(OD_{576nm} \text{ of positive control} - OD_{576nm} \text{ of negative control})} \times 100\% \quad (2)$$

2.7. Killing efficiency test

The antimicrobial peptide L12 displayed the highest selectivity for bacterial over mammalian cells and hence, was tested for its ability to kill Gram-positive and Gram-negative bacteria. Peptides were serially diluted with BHI broth to give solutions with final concentrations corresponding to 0.5 $\times$, 1 $\times$, 2 $\times$ and 4 $\times$ MIC. Bacterial cultures were diluted to a cell density of 10⁶ CFU mL⁻¹ and 100 μ L was added to an equal volume of peptide solution. After incubation at 37 °C for 18 h in a shaking incubator, ten-fold serial dilutions of samples was performed with PBS and 10 μ L was plated out in duplicates onto BHI agar. Bacterial colonies were counted following an overnight incubation at 37 °C. Results are expressed as mean log (CFU mL⁻¹) $\pm$ standard deviation of two experiments performed in duplicate.

2.8. Microfluidic live-cell imaging with time-lapse fluorescence microscopy

Live-cell imaging studies were performed at the Facility for Imaging by Light Microscopy (FILM) at Imperial College London with the automated CellASIC ONIX Microfluidic Platform and CellASIC ONIX B04A-03 Microfluidic Bacteria Plates (EMD Millipore Corporation, Hayward, CA, USA) according to previously established protocols [24]. Briefly, *E. coli* (K-12 MG1655) and *S. aureus* (MSSA 8325-4) were grown up to mid-log phase before dilution with BHI broth to achieve a cell density of 10⁷ CFU mL⁻¹. The most selective peptide L12 was prepared at final concentrations equivalent to 4 $\times$ and 8 $\times$ MIC in the presence of PI (10 μ g mL⁻¹). 100 μ L of the diluted bacterial cultures and 350 μ L of the drug solutions were added to the appropriate inlet wells, following which, the microfluidic plate was vacuum-sealed to the F84 manifold. After the CellASIC ONIX FG Software was initiated, loading and follow-on washing of untrapped bacterial cells was performed in accordance with the manufacturer's protocols. Peptide solutions were perfused into culture chambers at the recommended pressure of 2 psi for up to 2.5 h, with the temperature maintained at 37 °C. Negative controls consisted of bacterial cells exposed to medium containing PI only. A $\times$ 63 oil-immersion objective lens was utilized to capture phase contrast and fluorescent images every 10 min with the Zeiss Axiovert 200 M inverted microscope (Carl Zeiss Inc.).

2.9. Inhibition of biofilm formation

The ability of L12 to prevent biofilm formation was studied in 96-well plates using the crystal violet staining assay. Crystal violet stains live and dead cells by binding to negatively charged molecules in bacterial surface membranes, peptidoglycan, and the exopolysaccharide of biofilm matrices. The assay used measures total biofilm biomass and was performed as described previously

[25]. Briefly, bacterial cultures of *S. aureus* (MSSA 8325-4 and MRSA 252) and *P. aeruginosa* (PAO1 and PA-W25) were grown to mid-log phase before diluting to give an inoculum of 10⁶ CFU mL⁻¹. 50 μ L of the bacterial suspension was seeded into each well together with an equal volume of peptide solution. The anti-biofilm activity of the L12 was assessed at concentrations corresponding to 0.06 $\times$ 0.125 $\times$, 0.25 $\times$, 0.5 $\times$, 1 $\times$ and 2 $\times$ MIC. Following overnight incubation at 37 °C to facilitate biofilm formation, the culture media was aspirated and wells washed with deionized water to remove planktonic cells. Adherent biofilms were stained with 0.1% (w/v) crystal violet solution (125 μ L) for 10 min at room temperature and subsequently washed with deionized water to remove the excess dye. 200 μ L of 70% ethanol was transferred to each well to solubilize the dye and the biomass of biofilms was quantified by absorbance measurements at 595 nm taken with the VersaMax Tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data are expressed as mean $\pm$ standard error of the mean (S.E.M.) for three independent experiments performed in triplicate.

2.10. Disruption of pre-formed biofilms

The viability of pre-formed biofilms following peptide treatment was assessed according to previously established protocols [26]. Mid-log phase cultures of PA-W25 and MRSA 252 were diluted to 10⁶ CFU mL⁻¹ and 100 μ L was added to each well. After incubation for 18 h at 37 °C to allow biofilm formation, wells were washed with deionized water and 100 μ L of peptide solution was added at concentrations equivalent to 2 $\times$, 4 $\times$, 8 $\times$ and 16 $\times$ MIC. After 2 h exposure to L12, wells were washed with deionized water before determination of biofilm viability using the MTT assay as described above. Data are expressed as mean $\pm$ standard error of the mean (S.E.M.) for three independent experiments performed in triplicate.

2.11. Bioluminescence imaging of biofilms

To complement the microbiological biofilm assays, bioluminescence imaging studies were performed to visualize the extent of peptide-mediated biofilm eradication and growth suppression. Bioluminescent *P. aeruginosa* (ATCC 9027), transformed using a recombinant plasmid containing the *luxCDABE* gene cassette of *Photobacterium luminescens* excised from pAKlux1.1 and cloned into the PUCP19 vector backbone by electroporation, was employed in the screening of anti-biofilm activity of the synthetic peptides [27]. Transformed *P. aeruginosa* was maintained in LB broth containing carbenicillin (300 μ g mL⁻¹). Preparation of bacterial inocula and treatment of biofilms were conducted according to protocols reported in the previous sections. Inhibition of biofilm growth was assessed after 18 h treatment with L12 up to 4 $\times$ MIC while pre-formed biofilms were evaluated following 2 h exposure to L12 up to 64 $\times$ MIC. The IVIS imaging system was utilized for imaging of bioluminescent biofilms and acquired with Living Image software. Bioluminescence signals are expressed as photons per second per centimeter squared per steradian (p/s/cm²/sr) and presented as an intensity map.

2.12. Cell culture and cytotoxicity testing

The mouse macrophage cell line RAW 264.7 was utilized to assess the cytotoxicity of synthetic peptides by the MTT assay in accordance with previously established protocols [17,24]. Macrophage cells were maintained in DMEM supplemented with 10% FBS and cultured in humidified atmosphere at 37 °C and 5% CO₂. Cells were seeded at a density of 1 $\times$ 10⁴ cells per well in 96-well plates and incubated for 24 h, before exposure to peptide solutions

ranging from 3.90 to 31.3 $\mu\text{g mL}^{-1}$ for an additional 24 h. After treatment, the medium was replaced with 200 μL of fresh DMEM and 40 μL MTT solution (5 mg mL^{-1}) and the cells were incubated for a further 4 h at 37 °C. 150 μL of DMSO was then added to dissolve the resultant formazan crystals and absorbance readings were taken at 595 nm in a VersaMax Tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was determined relative to untreated controls and expressed as $(A_{595\text{nm}} \text{ of treated sample}) / (A_{595\text{nm}} \text{ of control}) \times 100\%$. Data are expressed as mean $\pm$ standard error of the mean (S.E.M.) for three independent experiments performed in triplicate.

2.13. Nitric oxide (NO) production by LPS-stimulated macrophages

The ability of peptides to neutralize LPS activity was studied using protocols described previously [28]. RAW 264.7 cells were seeded at a density of 4×10^4 cells per well in 96-well plates and incubated for 24 h at 37 °C and 5% CO_2 . Following medium removal, cells were stimulated with LPS (100 ng mL^{-1}), either in the presence or absence of peptides for an additional 24 h. NO formation was determined by measuring nitrite concentrations in supernatants using the Griess reagent (0.1% N-1-naphthylethylenediamine dihydrochloride, 1% sulfanilamide and 5% phosphoric acid) according to the manufacturer's protocols. Absorbance readings were taken at 540 nm by the VersaMax Tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA) and standard curves generated with NaNO_2 solutions were used to quantify nitrite levels. Untreated cells and those stimulated with LPS alone served as negative and positive controls respectively. Data are expressed as mean $\pm$ standard error of the mean (S.E.M.) for three independent experiments performed in triplicate.

2.14. Neutralization of endotoxins

The *de novo* designed peptides were evaluated for their LPS-inhibiting activities using the LAL chromogenic assay in accordance with the manufacturer's protocols. Peptides were serially diluted in endotoxin-free water and added to an equal volume of LPS (0.5 EU mL^{-1}) in 96-well plates in a final volume of 50 μL . The plates were incubated for 30 min at 37 °C to facilitate interactions between the peptides and LPS, following which, 50 μL of LAL was added to each well. After additional 10 min incubation at 37 °C, 100 μL of the chromogenic substrate solution (Ac-Ile-Glu-Ala-Arg-p-nitroalanine) was added to each well. Next, the plates were incubated for a further 6 min at 37 °C before adding 50 μL of 25% acetic acid to terminate the reaction. The extent of LPS inhibition was quantified relative to untreated controls by absorbance measurements at 405 nm taken with the VersaMax Tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data are expressed as mean $\pm$ standard deviation for three independent experiments.

2.15. Image and statistical analysis

Processing and compiling of phase and fluorescent images into movies at 2 frames per second were carried out using the ImageJ software. All statistical analysis in this study was performed with GraphPad Prism 6 software (GraphPad Software Inc., CA, USA) using either one-way analysis of variance (ANOVA), with Bonferroni's *post hoc* test for multiple comparisons or a nonparametric Kruskal Wallis multiple comparisons test with Dunn's *post-test*.

3. Results and discussion

3.1. Peptide design and characterization

In this study, short cationic helical peptides with idealized facial amphiphilicities were designed based on the recurring sequence $(X_1Y_1Y_2X_2)_n$, where the hydrophobic residues $X_1 = X_2 = \text{Leu}$ or Ile or Trp , and the cationic residues $Y_1 = Y_2 = \text{Lys}$. This proposed sequence allows for amphipathic peptides of intermediate lengths ($n = 1.5, 2.5, 3.5$, and so forth) while enabling the *de novo* design of peptides with perfectly amphipathic structures for $n \leq 3$ (Fig. 1). The influence of size of the cationic and hydrophobic faces on the biological and hemolytic activities was assessed by comparing 8-mer, 10-mer and 12-mer peptides with varying number of repeat units ($n = 2$ or 2.5 or 3). Besides the inclusion of residues which were helix formers rather than breakers, another key consideration was the selection of amino acids that could enhance peptide selectivity for microbial over mammalian cells. Hence, Lys was selected over Arg as the polar residue due to its high helical propensity [22], and lower cytotoxicity [18]. The hydrophobic residue Leu was also included due to its high helical propensity [22], while Trp was chosen because of its strong interaction with the lipid bilayer interface [29]. Finally, the selection of Ile was supported by evidence that Leu to Ile substitutions produced peptides with identical bactericidal activity, but markedly reduced toxicity [30]. The synthetic peptides were amidated at the C-terminus to improve antimicrobial activity by increasing net positive charge [31].

3.2. Peptide characterization

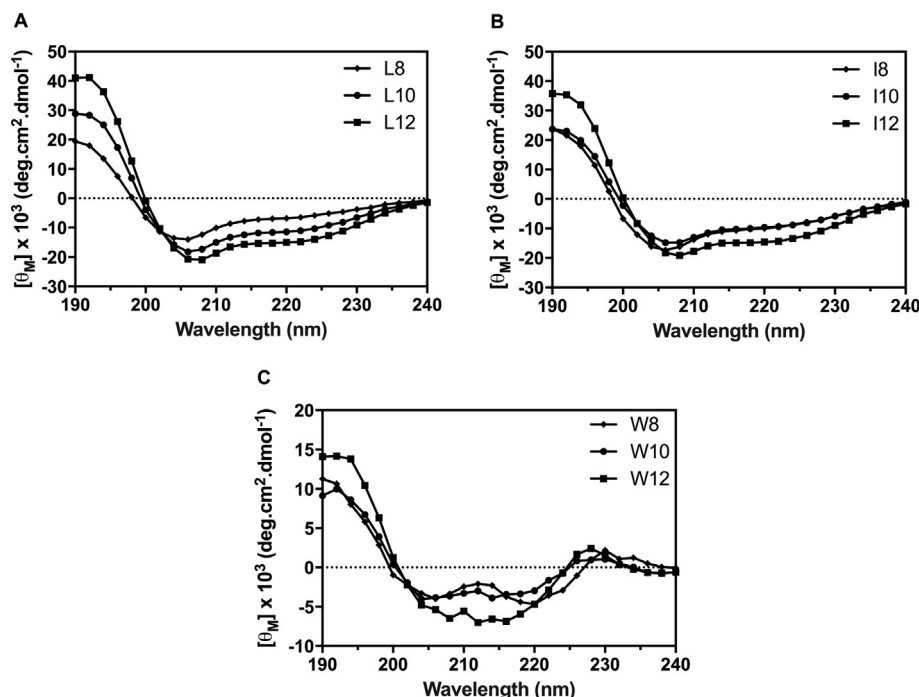
The sequences of the *de novo* peptides, their denotations and molecular weights are provided in Table 1. The molecular weights of the synthetic peptides were verified using SELDI-TOF MS, and agreement between the theoretical and measured molecular weights confirmed that the peptides had been synthesized to the desired specifications. The secondary structures of the synthetic peptides were evaluated in the presence of a 25 mM sodium dodecyl sulphate (SDS) micelle solution to simulate a microbial membrane environment. Under these conditions, the synthetic peptides readily adopted α -helical conformations, as supported by the characteristic double minima at ~ 208 and 222 nm (Fig. 2). In general, increasing the number of repeat units enhanced the helical propensity of synthetic peptides comprising of Leu and Ile residues, evident from the lower mean residue ellipticity values at 222 nm (θ_{222}). While the shorter Trp-containing peptides W8 and W10 displayed strong helical signatures, the longer W12 exhibited a broader minima between 208 and 222 nm (Fig. 2C), possibly due to self-aggregation of the more hydrophobic peptide [32].

3.3. Antimicrobial activity

The antimicrobial activity of the synthetic peptides was studied against a panel of clinically relevant Gram-positive and Gram-negative bacteria and the results are summarized in Table 2. The designed peptides effectively inhibited bacterial growth over a range of concentrations, with L12 and W12 emerging as the most potent antimicrobials exhibiting minimum inhibitory concentrations (MICs) ranging from 7.81 to 31.3 μM . The three least effective peptides, L8, I8 and I10 also exhibited the lowest α -helical conformation (Fig. 2), suggesting that folding of peptides into amphipathic structures was important for antimicrobial activity. This observation is supported by evidence that helix formation is a major driver of peptide insertion into the lipid bilayer [33]. In general, an increase in size of the continuous cationic and hydrophobic

Table 1Design of α -helical peptides with idealized facial amphiphilicity and their molecular weights.

Peptide denotation	No. of repeat units [n]	Repeat unit	Peptide sequence	Theoretical M_w	Measured M_w^a	Charge
L8	2	LKKL	LKKLLKKL-NH ₂	982.36	982	+4
L10	2.5	LKKL	LKKLLKKLLK-NH ₂	1223.69	1224	+5
L12	3	LKKL	LKKLLKKLLKKL-NH ₂	1465.02	1465	+6
I8	2	IKKI	IKKI IKKI-NH ₂	982.36	982	+4
I10	2.5	IKKI	IKKI IKKI IKKI-NH ₂	1223.69	1224	+5
I12	3	IKKI	IKKI IKKI IKKI-NH ₂	1465.02	1466	+6
W8	2	WKKW	WKKW WKKW-NH ₂	1274.57	1276	+4
W10	2.5	WKKW	WKKW WKKW WKKW-NH ₂	1588.95	1589	+5
W12	3	WKKW	WKKW WKKW WKKW-NH ₂	1903.33	1904	+6

^a Molecular weights (M_w) determined by SELDI-TOF MS.**Fig. 2.** CD spectra representing α -helical propensity of peptides with the backbone sequence (A) (LKKL)_n (B) (IKKI)_n and (C) (WKKW)_n in 25 mM SDS micelle solution, where n = 2, 2.5 and 3.**Table 2**Minimum inhibitory concentrations (MICs) and HC₅₀ values of peptides with idealized facial amphiphilicity against Gram-positive and Gram-negative bacteria.

Antimicrobial peptide	MIC [μ M]				HC ₅₀ [μ M]
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	
L8	500	>500	>500	>500	>2000
L10	31.3	>500	62.5	>500	>2000
L12	7.81	31.3	7.81	15.6	>2000
I8	>500	>500	>500	>500	>2000
I10	500	>500	>500	>500	>2000
I12	15.6	>500	15.6	250	>2000
W8	31.3	250	>250	62.5	>2000
W10	15.6	62.5	15.6	15.6	>2000
W12	15.6	15.6	15.6	7.81	483

faces in the amphipathic helix corresponded with reduced MIC values. Upon closer examination, the choice of non-polar amino acid was a key determinant of the minimum number of residues required in each uninterrupted segment for peptide activity. Amongst the hydrophobic amino acids studied, the incorporation of Ile proved least effective, as a minimum of six residues in each of the continuous cationic and hydrophobic faces was necessary for activity. Even then, I12 recorded a 16- and 32-fold higher MIC

against the Gram-positive *S. aureus* as compared to L12 and W12 respectively. For Leu-containing peptides, cationic and hydrophobic segments consisting of at least five amino acids were required to inhibit bacterial growth, in line with findings from previous reports [9,34]. However, only four residues per segment were sufficient to render peptides, comprising of bulky Trp residues, active. The superior potency of Trp-containing amphiphiles could be attributed to the strong affinity of Trp for membrane interfaces,

which facilitates peptide penetration and disruption of the lipid bilayer [29,35]. Therefore, the inclusion of bulky Trp residues could prove effective in designing shorter helical antimicrobial peptides, although peptide toxicity should also be given due consideration.

3.4. Hemolytic activity and cell selectivity

The synthetic peptides were evaluated for their hemolytic activity using 4% (v/v) blood from two healthy donors and as shown in Fig. 3, all peptides induced minimal hemolysis at their respective MICs. The Ile-containing peptides (I8, I10 and I12), and those consisting of two repeat units (L8, I8 and W8) displayed the lowest hemolysis of <1.5% even at 2000 μ M. Notably, the most active peptide L12 only induced 16% hemolysis at 2000 μ M, a concentration 150-fold greater than its GM MIC value. Trp incorporation (W8, W10 and W12) produced peptides with stronger hemolytic activity with W10 and W12 inducing hemolysis of 28% and 78% at 2000 μ M respectively. This corresponded to a far lower HC_{50} value, defined as the lowest peptide concentration producing 50% hemolysis of red blood cells (RBCs), of 483 μ M for W12 as compared to all other peptides (Table 2).

To enhance clinical utility of the amphipathic helical peptides, they should preferentially interact with microbial over mammalian cell membranes in order to maximize antimicrobial efficacy while minimizing toxicity. The selectivity index (SI), defined as the ratio of HC_{50} to MIC values, is one measure devised to aid in the selection of the best therapeutic candidates, with higher SI values deemed more desirable. Of the nine peptides, L12 displayed the highest SI against all four strains (Appendix A Table A1). Although both L12 and W12 emerged with relatively similar MICs against the panel of bacteria, the lower hemolytic activity displayed by L12 translated into a twofold superior SI against *K. pneumoniae* and *S. aureus*, and an eightfold superior SI against *E. coli* and *P. aeruginosa*, as compared to W12 (Appendix A Table A1). Among the Trp-containing peptides, W10 possessed the optimal composition with the highest SI against all four strains, despite recording a higher MIC values against *K. pneumoniae* and *S. aureus* as compared to W12. While increasing the number of lipophilic Trp residues increased both antimicrobial and hemolytic activities, the reduction in the HC_{50} value for W12 occurred to a far greater extent than the corresponding decrease in MICs. Thus, limiting the continuous hydrophobic face to ≤ 5 Trp residues may be crucial in designing more selective peptides, as the expansion of this uninterrupted segment beyond that proved detrimental to the SI.

3.5. Antimicrobial mechanisms

The *in vitro* killing efficiency of the synthetic peptides were evaluated against a panel of Gram-positive and Gram-negative bacteria. L12 demonstrated killing efficiencies of >99.9% at 2 $\times$ MICs of all four species (Appendix A Fig. A1). The antimicrobial activity was more significant at 4 $\times$ MIC with L12 achieving >4 log reduction for *P. aeruginosa*, >5 log reduction for *E. coli* and *K. pneumoniae*, and >6 log reduction for *S. aureus*. AMPs adopting amphipathic helical structures are recognized to interact with bacterial membranes in order to exert their antimicrobial effect by creating pores, or inducing widespread collapse of membrane structural integrity [36]. In this study, live-cell imaging of bacterial cells was performed using the CellASIC™ ONIX Microfluidic Platform to elucidate the antimicrobial mechanisms of the most selective peptide L12. The DNA intercalating dye PI was utilized to detect peptide-mediated membrane damaged to *E. coli* and *S. aureus* following treatment with L12 at concentrations up to 8 $\times$ MIC. As shown in Appendix A Fig. A2A and Movie A1, *E. coli* cells doubled continuously without taking up the fluorescent dye in the absence of L12. Under similar conditions, *S. aureus* cells also

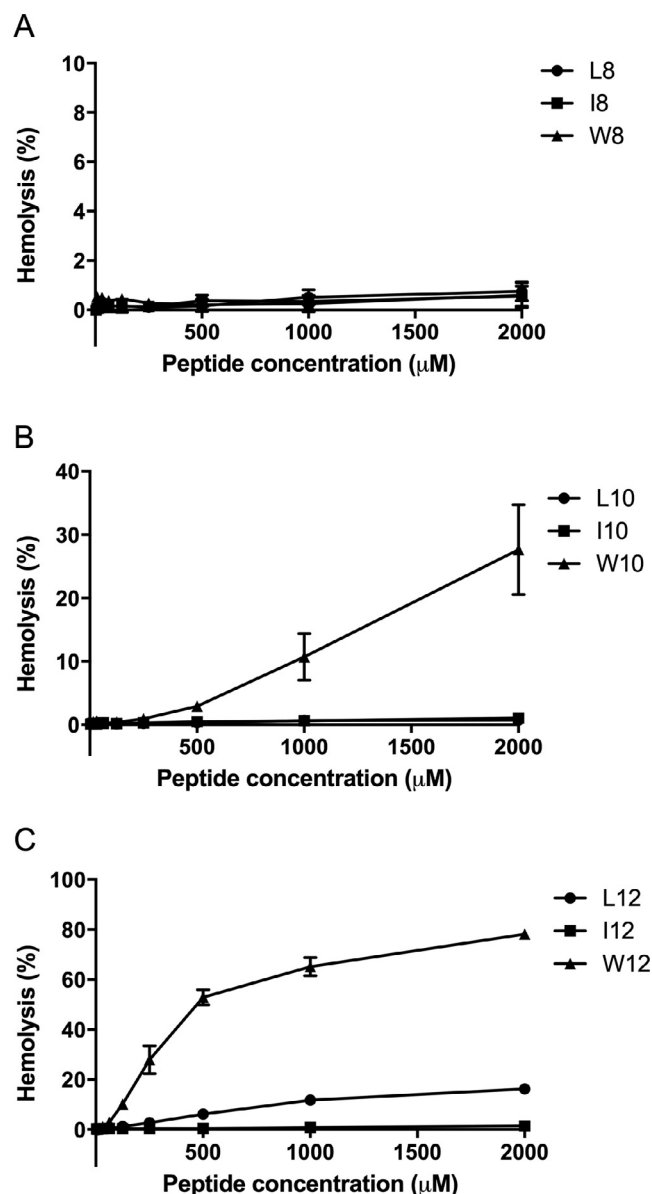


Fig. 3. Hemolytic activity of synthetic α -helical peptides possessing (A) 2 repeat units, (B) 2.5 repeat units and (C) 3 repeat units tested against blood from two healthy donors. Peptides induced minimal hemolysis at concentrations corresponding to the respective MICs.

excluded PI and replicated uninterruptedly (Appendix A Fig. A3A and Movie A4). However, exposure of *E. coli* to L12 at 4 $\times$ MIC rapidly induced fluorescent staining of bacterial cells within 15 min (Appendix A Fig. A2B and Movie A2). The effect was more pronounced at 8 $\times$ MIC with PI uptake into bacterial cells occurring within 5 min of peptide treatment (Appendix A Fig. 4A and Movie A3). Similarly, *S. aureus* exhibited fluorescence within 15 min of peptide introduction at both 4 $\times$ and 8 $\times$ MIC (Movies A5 and A6). Moreover, the replication of L12-treated *E. coli* and *S. aureus* cells was promptly halted after 25 min exposure to 8 $\times$ MIC, suggesting that permeabilization of bacterial membranes likely culminated in cell death. Live-cell imaging studies also provide a useful means of assessing the relative kinetics of the membrane-permeabilizing activity of AMPs when comparing Gram-positive versus Gram-negative bacteria. Treatment of *S. aureus* with L12 at 8 $\times$ MIC resulted in rapid fluorescent staining of >90% and ~100% of cells within 15 and 35 min respectively (Fig. 4B and Movie A6). For

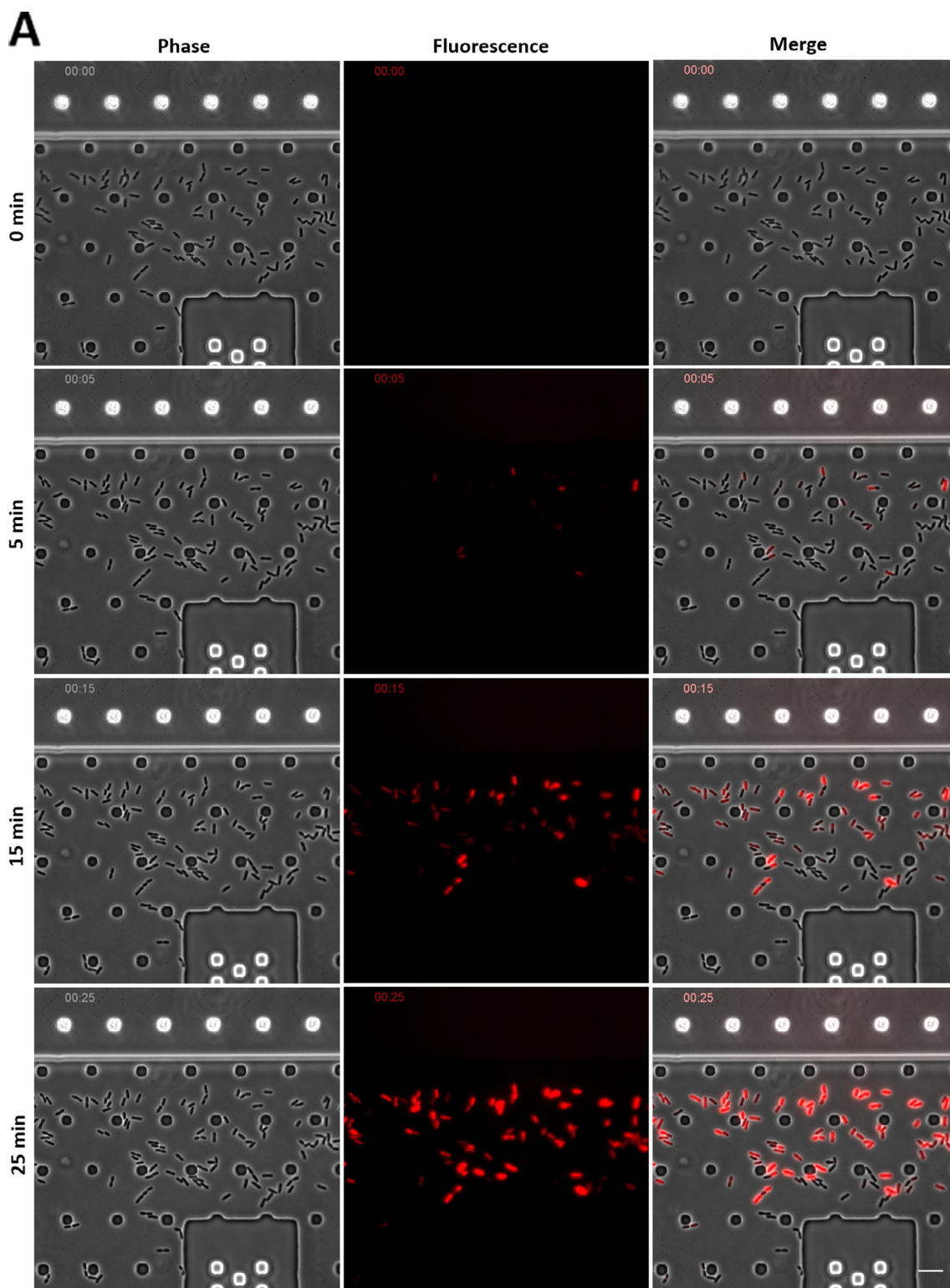


Fig. 4. Time-lapse fluorescence microscopy images of (A) *E. coli* (K-12 MG1655) and (B) *S. aureus* (MSSA 8325-4) following treatment with L12 at 8× MIC in the presence of the membrane impermeable dye, PI. The uptake of PI into bacterial cells within minutes of exposure supports the rapid membrane-lytic antimicrobial mechanisms of the synthetic peptides. Scale bar = 10 μ m.

E. coli however, membrane permeabilization was far more gradual with ~65% of cells displaying fluorescence within 15 min and >90% staining only achieved at 45 min ([Appendix A Fig. 4A and Movie A3](#)). The more rapid fluorescence observed for *S. aureus* is consistent with the fact that Gram-positive bacteria lack an outer mem-

brane, hence binding of PI to intracellular DNA is readily achieved upon compromise of cytoplasmic membrane integrity. The presence of an outer membrane in Gram-negative bacteria necessitates that both outer and inner membranes are sequentially

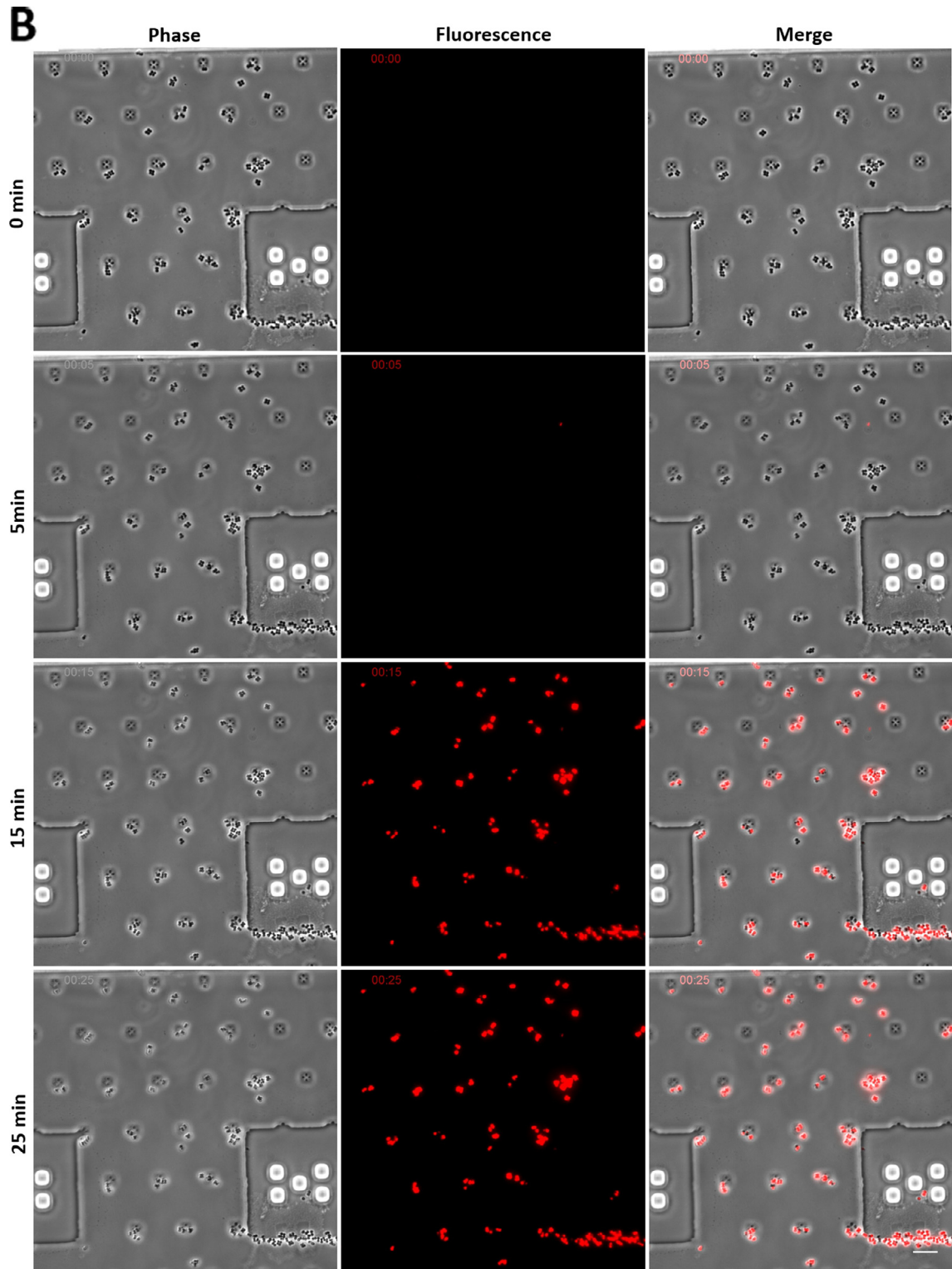


Fig. 4 (continued)

permeabilized before PI can gain access to DNA, thus slowing down the rate at which PI can diffuse into the bacterial cell.

3.6. Inhibition of MDR bacteria and biofilms

The antimicrobial activity of the three most selective peptides was further evaluated against drug-resistant bacteria including

MRSA 252 and MDR *P. aeruginosa* wound isolates (PA-W1, PA-W14 and PA-W25). As shown in Table 3, the synthetic peptides exhibited MICs ranging from 7.81 to 125 μ M against the drug-resistant pathogens. The observed broad-spectrum inhibitory activity of W10, W12 and L12 against drug-resistant bacteria likely stems from the non-specific membrane-lytic mode of action displayed by most AMPs [37]. While both L12 and W12 demonstrated

Table 3Minimum inhibitory concentrations (MICs) of synthetic peptides against clinical isolates of MRSA 252 and MDR *P. aeruginosa* (PA-W1, PA-W14 and PA-W25).

Antimicrobial peptide	MIC [μ M]			
	MRSA 252	PA-W1	PA-W14	PA-W25
L12	62.5	15.6	15.6	15.6
W10	15.6	31.3	125	125
W12	7.81	15.6	31.3	15.6

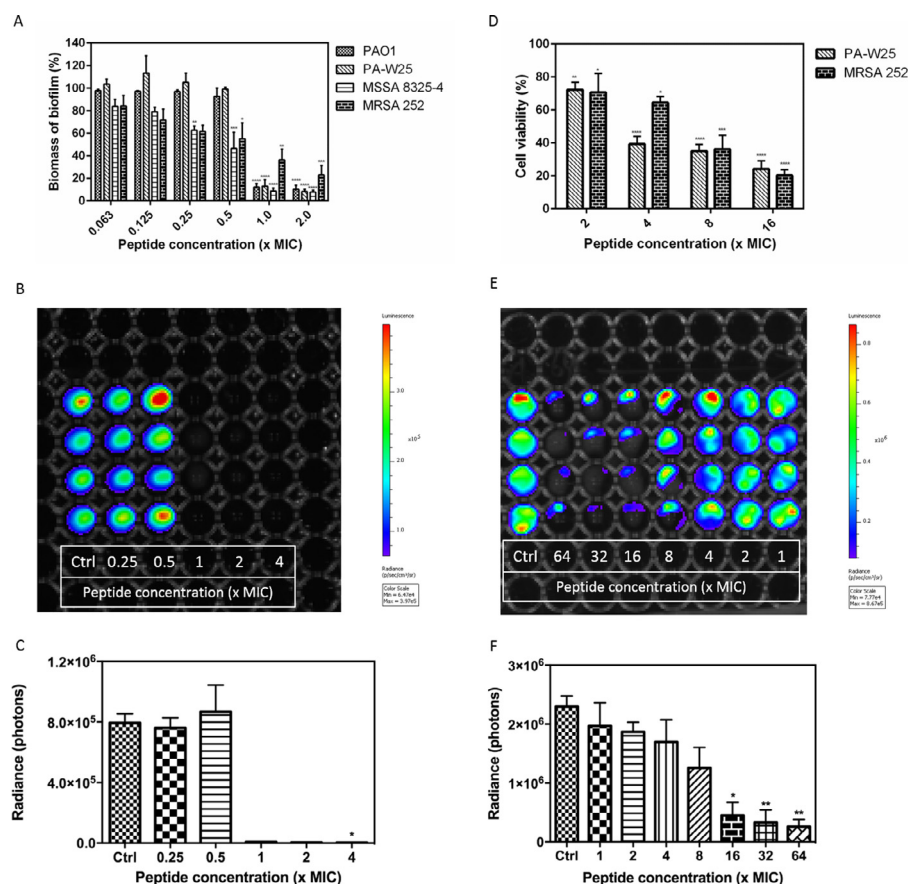


Fig. 5. (A) Inhibition of drug-susceptible and -resistant *P. aeruginosa* and *S. aureus* biofilms formation following overnight exposure to L12. (B) IVIS imaging and (C) radiance quantification of biofilm growth inhibition of bioluminescent *P. aeruginosa* treated with L12 overnight. (D) Cell viabilities of pre-formed PA-W25 and MRSA 252 biofilms after treatment with L12 for 2 h. (E) IVIS imaging and (F) radiance quantification of pre-formed biofilm disruption of bioluminescent *P. aeruginosa* exposed to L12 for 2 h. L12 inhibits biofilm formation at 1 $\times$ and 2 $\times$ MIC, and effectively disrupts pre-established biofilms at supra-MIC levels. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

relatively similar activity against MDR *P. aeruginosa*, W12 recorded an 8-fold lower MIC than L12 against MRSA. This observation is in line with previous findings by Deslouches et al. who demonstrated that incorporating Trp residues can render peptides more potent against *S. aureus*, possibly due to its stronger preference for the lipid bilayer [12].

Besides their efficacy against planktonic bacteria, AMPs could provide useful alternatives to address challenging biofilm-related infections [3]. Thus, the most selective peptide L12 was evaluated for its ability to inhibit biofilm formation at sub- and supra-MIC levels. As shown in Fig. 5A, L12 prevented the development of both drug-susceptible and resistant biofilms to similar extents. Though ineffective at sub-MIC levels, L12 significantly reduced the biomass of PAO1 and PA-W25 biofilms by $\sim 90\%$ at both 1 $\times$ and 2 $\times$ MIC ($p \leq 0.0001$). These findings were corroborated by bioluminescent imaging of *P. aeruginosa* which revealed that L12 effectively inhibited biofilm formation at 1 $\times$, 2 $\times$ and 4 $\times$ MIC (Fig. 5B) with $>99\%$ reduction in luminescence signal (Fig. 5C). Unlike the inhibition of *P. aeruginosa* biofilms, the reduction in biomass of *S. aureus* bio-

films occurred in a dose-dependent manner (Fig. 5A). L12 inhibited MSSA and MRSA biofilms by $\sim 37\%$ and 39% at 0.25 $\times$ MIC respectively, and $\sim 53\%$ and 45% at 0.5 $\times$ MIC respectively. The anti-biofilm properties of AMPs are partly attributed to direct killing of planktonic bacteria, hence, the prevention of biofilm formation at sub-MIC suggests that alternative mechanisms could potentially be involved. It has been shown that coating AMPs onto surfaces reduces bacterial adherence in turn inhibiting biofilm formation [26] and this may contribute here.

The treatment of mature biofilms also poses a significant clinical problem as these sessile bacterial communities are inherently resistant to antimicrobials due to poor drug penetration through the EPS matrix, and the existence of subpopulations of stationary phase and persister cells with drug tolerant phenotypes [38]. Hence L12 was further evaluated for its potential to disrupt pre-formed biofilms of drug-resistant pathogens. As shown in Fig. 5D, L12 displayed significant concentration-dependent reduction in cell viabilities of pre-established biofilms of both MRSA 252 and MDR PA-W25 at 8 $\times$ and 16 $\times$ MIC within 2 h of treatment

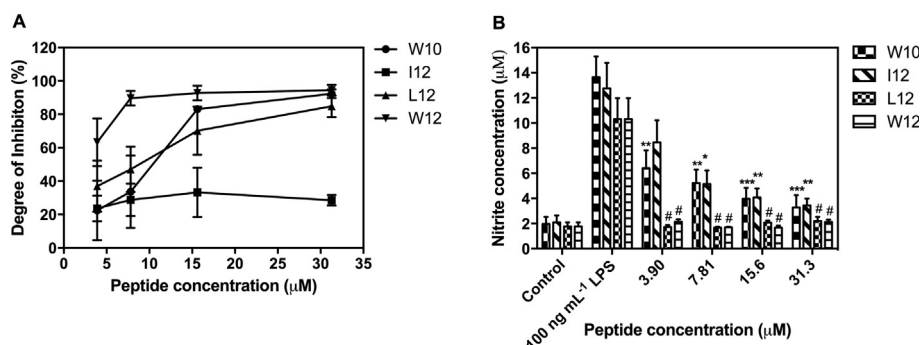


Fig. 6. The ability of *de novo* designed peptides to (A) inhibit LPS within 30 min exposure and (B) restrict LPS-stimulated NO production from RAW 264.7 cells following 24 h treatment with peptides at various concentrations. The synthetic peptides L12 and W12 strongly inhibited LPS and effectively inhibited NO production at sub-MIC concentrations of 3.9 μM . (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, # $p \leq 0.0001$).

($p \leq 0.0001$). Bioluminescent imaging studies of pre-formed *P. aeruginosa* biofilms confirmed that L12 effectively disrupts mature biofilms at supra-MIC levels after 2 h (Fig. 5E), accompanied by significant reductions in luminescence at 16 $\times$ ($p \leq 0.05$), and 32 $\times$ and 64 $\times$ MIC ($p \leq 0.01$) (Fig. 5F). All in all, these findings underscore the potential utility of the *de novo* AMPs presented here in tackling the mounting problem of MDR biofilms and infections.

3.7. Anti-endotoxin activity

Host innate immune responses during sepsis, elicited by exposure to the Gram-negative bacterial cell wall component, LPS, could lead to septic shock and eventually death if uncontrolled. The neutralization of endotoxins by amphipathic peptides could provide an effective means of blocking the overproduction of inflammatory mediators induced by LPS. Thus, the LPS-inhibiting activities of the *de novo* peptides were evaluated from 3.9 to 31.3 μM using the LAL chromogenic assay, as cell viabilities were >70% for all peptides, except L12 which recorded ~30% cell viability at the highest concentration (Appendix A Fig. A4). As shown in Fig. 6A, the most hydrophobic 12-mer, W12, inhibited LPS most effectively ($\geq 90\%$) even at low peptide concentrations of 7.81 μM . In comparison, I12 and L12 only neutralized ~29% and 47% of LPS respectively at similar concentrations. Increasing the number of repeat units by 0.5 contributed to stronger LPS-inhibition for W12 as compared to W10 at all concentrations tested. Taken together, these findings suggest that both peptide length and hydrophobicity are important determinants of the LPS-neutralizing properties of amphipathic peptides.

The overproduction of the pro-inflammatory mediator NO has been implicated in the pathogenesis of septic shock, inducing harmful effects including tissue damage and myocardial depression [39]. Hence, the ability of the peptides to restrict LPS-induced macrophage activation was assessed by quantifying nitrite production from LPS-stimulated RAW 264.7 cells treated with peptides. The most potent inhibitors L12 and W12 limited NO production to levels similar to controls even at the lowest peptide concentration of 3.9 μM ($p \leq 0.0001$) (Fig. 6B). W10 and I12, however, demonstrated a dose-dependent reduction in NO release from macrophages. While NO production was enhanced >6-fold in the absence of peptides, treatment with 7.81 μM of W10 and I12 suppressed NO production to concentrations ~2.7 and 2.5-fold respectively, higher than that of controls. In general, there was close agreement between the LPS-binding and neutralizing activities of the peptides, except for I12 which demonstrated similar LPS-blocking activity as W10 despite exhibiting the lowest LPS-binding affinity. This finding suggests that in addition to the direct binding of LPS by I12, other mechanisms may be involved in the

suppression of pro-inflammatory responses induced by LPS [40,41]. Overall, the short amphiphilic peptides presented in this study could potentially be developed as anti-inflammatory agents in the treatment of endotoxemia by blocking LPS-mediated induction of inflammatory mediators.

4. Conclusions

In summary, the design principles proposed in this study, with peptides comprising of the backbone sequence $(X_1Y_1Y_2X_2)_n$, were successful in producing broad-spectrum α -helical antimicrobial peptides. Optimization of peptide selectivity by varying the number of repeat units and choice of hydrophobic amino acid, found that the sequences $(LKKL)_3$ and $(WKKW)_{2.5}$ possessed the highest selectivity. The antimicrobial activity of L12 was likely due to pore formation, inducing rapid permeabilization of Gram-positive and Gram-negative bacterial membranes within minutes. L12 also effectively inhibited the formation, and promoted the disruption, of drug-resistant biofilms. Finally, both L12 and W12 demonstrated potent suppression of LPS-induced pro-inflammatory mediators even at low peptide concentrations of 3.9 μM . All in all, the design strategies presented in this study could provide a useful tool for developing therapeutic peptides with broad-ranging clinical applications from the treatment and prevention of drug-resistant biofilms to the neutralization of bacterial endotoxins.

Funding

This research is supported by the Singapore Ministry of Health's National Medical Research Council under its Individual Research Grant Scheme (NMRC/1298/2011 and NMRC/OFIRG/0026/2016) awarded to P.L.R.E. and Y.Y.Y., the European Union's Seventh Framework Program (EC-GA 279185), partly funded by the British Society of Antimicrobial Chemotherapy (BSAC) Project Grant (GA2011-01P) awarded to S.M.N. and P.R.L., the Imperial College London Biomedical Research Centre (BRC), the Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology and Research, Singapore), the Imperial College London Elsie Widdowson Fellowship Award to S. M.N., and the National University of Singapore President's Graduate Fellowship to J.S.K. and Y.W.

Acknowledgments

We would like to thank Dr John Tregoning (Imperial College London) for providing the MSSA and MRSA strains, Prof Paul Williams (University of Nottingham) for his kind gift of the *P. aeruginosa* wound isolates, and Dr Martin Goldberg (University of

Birmingham) for the *E. coli* strain. The plasmid pAKlux1.1 (Addgene plasmid #14074) was a gift from Attilia Karsi (Mississippi State University). We would also like to acknowledge Dr Jonathan Taylor and Dr Steve Matthews (Imperial College London) for their assistance with the CD spectroscopy experiments, and the Imperial College London Film Facility, Debora Keller and Stephen Rothery for providing microscopy support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2017.04.032>.

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