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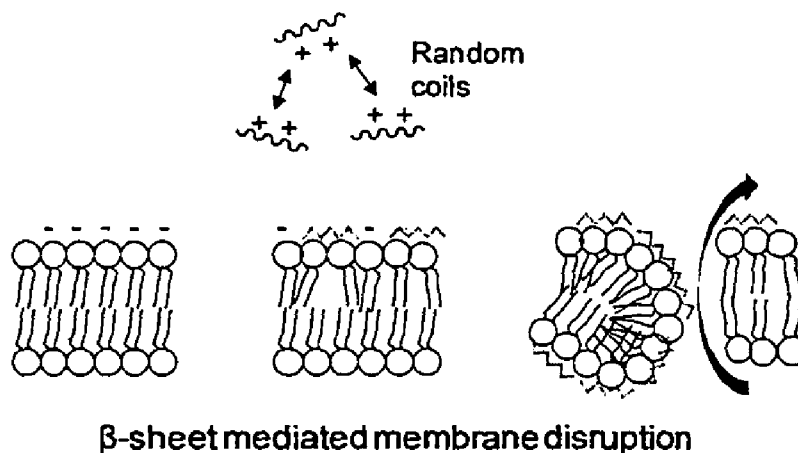
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(57) Abstract: Disclosed are amphiphilic peptides. Also disclosed are methods of treating proliferative disease, bacterial infection, viral infection and fungal infection, endotoxin neutralization and a method of removing biofilm. Also disclosed is the use of the amphiphilic peptides.

PEPTIDES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of priority of Singapore patent application No. 201206407-7, filed August 29, 2012, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[002] The present invention generally relates to antimicrobial peptides and methods for their use.

BACKGROUND OF THE INVENTION

[003] Despite significant improvements in living standards and biomedical technologies over the past century, the global burden of infectious diseases remains exceedingly high and is a major cause of public health, economic and social problems. According to World Health Organization (WHO) statistics, infectious and parasitic diseases such as pneumonia, tuberculosis, meningitis, diarrheal diseases, HIV and malaria are the second leading causes of death worldwide. The widespread and often indiscriminate use of antibiotics in industrialized nations further fuels the problem by contributing to the rapid emergence of drug resistant pathogens, making infectious diseases increasingly difficult to control with the existing classes of antibiotics. The exploding crisis of antibiotic-resistant infections coupled with the on-going dearth in new small-molecule antibiotics development, have spurred considerable efforts toward the discovery and development of membrane active antimicrobial peptides (AMPs) as an alternative class of antimicrobial agents. Naturally occurring antimicrobial peptides, also known as 'host defence peptides', were first discovered as components of the innate immunity, forming the first line of defence against invading pathogens in all living organisms. Unlike conventional antibiotics that inhibit specific biosynthetic pathways such as cell wall or protein synthesis, the majority of the cationic antimicrobial peptides exert their activities via physical disruption of the more negatively charged microbial membrane lipid bilayers to induce leakage of cytoplasmic components leading to cell death. The physical nature of membrane disruption is believed to result in a lower likelihood for drug resistance

development as it becomes metabolically 'costlier' for the microorganism to mutate or to repair its membrane components at the same rate as the damage is being inflicted.

[004] Although more than 1700 naturally occurring antimicrobial peptides from diverse sources including microorganisms, plants and animals have been isolated and characterized in the past 3 decades, only very few AMPs such as polymyxins and gramicidins are being used clinically; and mainly in topical formulations due to their high systemic toxicities. The major challenges identified with the application of antimicrobial peptides as drugs lie in the high cost in synthesizing long peptide sequences, poor stability and unknown toxicity after systemic administration. In efforts to enhance antimicrobial activities and minimize non-specific toxicities, more researchers are increasingly utilizing naturally occurring antimicrobial peptide or protein sequences as templates to perform chemical modifications such as cyclization, sequence truncations, and substitution with D-, β - or fluorinated-amino acids for the generation of new peptide analogs with broader applications for localized or systemic infections within the body. However, current approaches to optimize naturally occurring antimicrobial peptide sequences remain largely empirical at best, making it extremely difficult to delineate general structure-activity relationships especially against the backdrop of massive sequence and structural diversities. Furthermore, many of the new peptide analogs remain long (20 amino acids or more), which might induce significant immunogenicity and ultimately increase the cost for large scale manufacturing. More importantly, it has been suggested that the use of antimicrobial peptides with sequences that are too close to the host defence antimicrobial peptides may trigger the development of resistance towards innate AMPs that could inevitably compromise natural defences against infections, posing significant health and environmental risks.

[005] At the same time, the rapid emergence of antibiotics resistant bacteria and fungi in both the nosocomial and community settings has created a significant strain on healthcare systems around the world. While global incidences of antibiotics resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) and multidrug-resistant *Klebsiella pneumoniae* and *Acinetobacter spp.* have reached epidemic levels, the number of new antibiotics entering the clinical development pipeline has been dismal; with only three new structural classes of antibiotics including the oxazolidinones (linezolid), lipopeptides (daptomycin) and pleuromutilins (retapamulin) entering the market since 2000. This development is especially alarming given that

pathogenic bacteria such as *S. aureus*, *Enterobacter* and *Klebsiella* are developing resistance to vancomycin and carbapenems, which are potent antibiotics traditionally reserved as the last line of defence for vulnerable patients in hospitals. With the on-going dearth in small molecular antibiotics development, the design and identification of alternative classes of antimicrobial agents with new modes of action that can effectively overcome drug resistance mechanisms is more pressing than ever.

[006] As the majority of the antimicrobial peptides exert their antimicrobial activities through a rapid and direct membrane lytic mechanism, they possess an inherent advantage in overcoming conventional mechanisms of antibiotics resistance such as the increased expression of drug efflux pumps on microbial membranes, production of drug degradation enzymes or alteration to drug interaction sites acquired by microbes against small molecular antibiotics targeting specific biosynthetic pathways. Significant barriers limiting the successful clinical translation of antimicrobial peptides, however, include high systemic toxicities as a result of poor microbial membrane selectivities, relatively high manufacturing cost (for long peptide sequences) and susceptibility to degradation by proteases present in biological fluids such as blood serum, wound exudates or lacrimal fluids.

[007] In view of the above, there is a need to provide alternative antimicrobial peptides. There is also a need to provide an alternative method of treating microbial infections.

SUMMARY OF THE INVENTION

[008] In one aspect, there is provided an amphiphilic peptide. The peptide comprises $(X_1Y_1X_2Y_2)_n$ (Formula I), wherein the C-terminal end of the peptide is amidated; X_1 and X_2 is independently of each other a hydrophobic amino acid; Y_1 and Y_2 is independently of each other a cationic amino acid and wherein n is at least 1.

[009] In another aspect, there is provided the peptide as described herein for use as a medicament.

[0010] In another aspect, there is provided a method of treating a bacterial infection or removing bacteria. The method comprises the administration of a pharmaceutically effective amount of a peptide as described herein.

[0011] In another aspect, there is provided a method of neutralizing endotoxins. The method comprising administration of a pharmaceutically effective amount of a peptide as described herein.

[0012] In another aspect, there is provided a method of treating viral based infectious diseases. The method comprising administration of a pharmaceutically effective amount of the peptide as described herein.

[0013] In another aspect, there is provided a method of treating fungal infection or infestation
5 or removing fungus. The method comprising administration of a pharmaceutically effective amount of the peptide as described herein.

[0014] In another aspect, there is provided a method of treating proliferative diseases. The method comprising administration of a pharmaceutically effective amount of the peptide as described herein.

10 [0015] In another aspect, there is provided a use of a peptide as described herein in the manufacture of a medicament for treating a bacterial infection, or removing bacteria, or neutralizing endotoxins, or treating viral based infectious diseases, or treating a fungal infection or infestation, or removing fungus.

15 BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0017] **Figure 1** shows design features of synthetic β -sheet forming peptides. A) shows
20 exemplary peptide of the present disclosure. B) shows an example of the peptide of the present disclosure presented as a linear molecule. C) shows a schematic diagram of possible membrane disruption caused by the peptide of the present disclosure. In brief, in aqueous solutions, the peptides exist as monomeric random coils due to electrostatic repulsion between the protonated Arg and/or Lys residues. However, in the presence of microbial cell
25 membranes, the peptides readily assemble into β -sheet secondary structures stabilized by electrostatic interactions between the positively charged residues and the negatively charged phospholipids, followed by the insertion of their hydrophobic residues into the lipid bilayer to mediate membrane disruption.

[0018] **Figure 2** shows circular dichroism spectra of (a) (VRVK)₂-NH₂ (SEQ ID NO: 11), (b)
30 (IRIR)₂-NH₂ (SEQ ID NO: 12), (c) (IKIK)₂-NH₂ (SEQ ID NO: 13), (d) (IRIK)₂-NH₂ (SEQ ID NO: 17), (e) (IRVK)₂-NH₂ (SEQ ID NO: 14), (f) (FRFK)₂-NH₂ (SEQ ID NO: 15), (g) (VRVK)₃-NH₂ (SEQ ID NO: 20), (h) (IRIK)₃-NH₂ (SEQ ID NO: 21), (i) (IRVK)₃-NH₂ (SEQ

ID NO: 22) in deionized water and 25 mM SDS micelles solution. Figure 2 demonstrates how the peptides of the present disclosure readily self-assembled into β -sheet secondary structures in a microbial membrane mimicking condition (25 mM SDS solution).

[0019] Figure 3 shows haemolytic activities of the synthetic antimicrobial peptides. Figure 3 shows that the peptides of the present disclosure induced minimal or no haemolysis against rat red blood cells at various minimum inhibitory concentration (MIC) values.

[0020] Figure 4 shows plot of viable microbe colony-forming units (CFU) after treatment with representative peptide (IRIK)₂-NH₂ (SEQ ID NO: 17) at various concentrations (i.e. 0, 0.5 $\times$ minimum inhibitory concentration (MIC), MIC and 2 $\times$ MIC). Figure 4 shows that the peptides of the present disclosure are bactericidal at MIC values and above.

[0021] Figure 5 shows FE-SEM images of (a) *Escherichia coli* and (b) *Staphylococcus aureus* treated with broth containing 10% (by volume) of water as control and representative peptides (IRIK)₂-NH₂ (SEQ ID NO: 17) and (IRVK)₃-NH₂ (SEQ ID NO: 22) for 2 h. Figure 5 shows that the peptides of the present disclosure can cause membrane lysis in *Escherichia coli* and *Staphylococcus aureus*.

[0022] Figure 6 shows cell viability of *Staphylococcus aureus* in biofilms treated with various concentrations of (IRIK)₂-NH₂ (SEQ ID NO: 17) and (IRVK)₃-NH₂ (SEQ ID NO: 22) for 24 h as determined using the XTT assay. * $P < 0.01$ relative to (IRVK)₃-NH₂. Figure 6 shows that the peptides of the present disclosure demonstrated a dose-dependent killing of *Staphylococcus aureus* residing in biofilms.

[0023] Figure 7 shows changes in biomass of pre-formed *Staphylococcus aureus* biofilms treated with various concentrations of (IRIK)₂-NH₂ (SEQ ID NO: 17) and (IRVK)₃-NH₂ (SEQ ID NO: 22) for 24 h as determined by crystal violet staining. Figure 7 demonstrates that the peptides of the present disclosure can be used to remove biofilms.

[0024] Figure 8 shows the effects the peptides of the present disclosure with (a) two repeat units (n=2) and (b) three repeat units (n=3) on stability of FITC-labelled LPS aggregates. Figure 8 demonstrates that the peptides of the present invention are effective in disrupting lipopolysaccharide (LPS) aggregates.

[0025] Figure 9 shows the inhibitory effects of the peptides of the present disclosure on LPS-stimulated NO production in NR8383 rat macrophage cell line. Figure 9 demonstrates that the peptides of the present disclosure are effective in neutralizing the effect of LPS.

[0026] Figure 10 shows cell viability of NR8383 rat macrophage cell line after 18 h incubation with the peptides of the present disclosure at various concentrations. Figure 10 shows that the anti-inflammatory property of the peptides of the present disclosure was independent of their effects on cell viability and that the peptides were not cytotoxic at concentrations required for antimicrobial and anti-inflammatory activities.

[0027] Figure 11 shows circular dichroism spectra of (a) IK8 enantiomers, (b) IK12 enantiomers, (c) IK8 stereoisomers and (d) control peptides in microbial membrane-mimicking conditions (25 mM SDS micelles solution). Figure 11 shows the peptides of the present disclosure readily self-assembled to form β -sheet secondary structure in microbial membrane mimicking conditions.

[0028] Figure 12 shows haemolytic activities of (a) IK8 stereoisomers, (b) IK12 enantiomers and (c) IK8 non- β -sheet forming peptide controls in rabbit red blood cells. Figure 12 shows the D-stereoisomers of the peptides of the present disclosure exhibit minimal or no haemolysis at MIC values, with high selectivity for microbial membranes.

[0029] Figure 13 shows antimicrobial activities of IK8-all L and IK8-all D against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* after 6 h treatment with proteases (a) trypsin and (b) proteinase K. Figure 13 shows all the D enantiomers of the peptides of the present disclosure are protease resistant.

[0030] Figure 14 shows plot of viable colony-forming units (CFU) of (a) *Staphylococcus epidermidis*, (b) *Staphylococcus aureus*, (c) *Escherichia coli*, (d) *Pseudomonas aeruginosa*, and (e) *Candida albicans* after 18 h treatment with IK8-all D at various concentrations (i.e. 0, $0.5 \times$ minimum inhibitory concentration (MIC), MIC and $2 \times$ MIC). The designed peptide achieved more than 3 log reductions in colony counts ($> 99.9\%$ kill) for each of the microorganisms at MIC and $2 \times$ MIC values, indicating a bactericidal mechanism of action.

Figure 14 demonstrates that similar to the L-enantiomers, the D-enantiomers of the peptide of the present disclosure have bactericidal mechanisms.

[0031] Figure 15 shows FE-SEM images of (a) *Staphylococcus aureus* and (b) *Pseudomonas aeruginosa* treated for 2 h with 125 mg L^{-1} of IK8-all D and MHBII containing 10% (by volume) HPLC water. Figure 15 demonstrates the D-enantiomers of the peptides of the present disclosure can cause membrane lysis in *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

[0032] **Figure 16** shows drug resistance development profiles of (a) *Escherichia coli* and (b) *Staphylococcus aureus* exposed to sub-minimum inhibitory concentrations (MIC) of IK8-all D and various clinically used antibiotics. Figure 16 suggests that the peptide of the present disclosure does not induce any development of drug resistance within the timeframe tested.

5 [0033] **Figure 17** shows intracellular killing of *Staphylococcus aureus* mediated by IK8-all D in infected mouse macrophage cell line RAW264.7 (MOI 10). Figure 17 demonstrates that the peptides of the present disclosure have potent antimicrobial properties against bacteria present within infected cells.

[0034] **Figure 18** shows the circular dichroism spectra of (a) IK8 enantiomers, (b) IK8
10 stereoisomers, (c) IK12 enantiomers and (d) control peptides in deionized water. Figure 18 demonstrates that both the enantiomers and stereoisomers of the peptides of the present disclosure remained as random coils and did not adopt any secondary structures in deionized water.

[0035] **Figure 19** shows MALDI-TOF mass spectra demonstrating proteolytic activity of
15 trypsin and proteinase K on (a) (IRIK)₂-NH₂ (SEQ ID NO: 17) and (b) (irik)₂-NH₂ (SEQ ID NO: 18). Arrow indicates predominant enzyme cleavage site. Figure 19 shows that in contrast to L-enantiomers, the treatment of proteases on D-enantiomer peptides of the present disclosure does not lead to degradation of the D-enantiomer peptides.

[0036] **Figure 20** shows minimum inhibitory concentration (MIC) determination of (a)
20 ciprofloxacin- and (b) gentamicin-resistant *Escherichia coli* after 18 h treatment with the synthetic antimicrobial peptide IK8- all D. Figure 20 demonstrates that D-enantiomers of the peptides of the present disclosure is able to inhibit gentamicin- and ciprofloxacin-resistant *Escherichia coli* at the same concentration as that of wild-type (non-drug resistant) *Escherichia coli*.

25 [0037] **Figure 21** shows cytotoxicity study of IK8-all D against mouse alveolar macrophage RAW264.7 and human foetal lung fibroblast WI-38 cell lines. Figure 21 demonstrates that the D-enantiomers of the peptides of the present disclosure are only cytotoxic at concentrations that are well above the antimicrobial concentrations.

[0038] **Figure 22** shows that the peptides of the present disclosure selectively kills cancer
30 cells (HeLa) and are less cytotoxic towards non-cancerous rat alveolar macrophages (NR8383) and human dermal fibroblast cell lines.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] Table 1 shows the characterization of the peptides of the present disclosure.

[0040] Table 2 shows the minimum inhibitory concentrations (MICs) and selectivity indices of the peptides of the present disclosure.

5 [0041] Table 3 shows the design and characterization of the peptides of the present disclosure.

[0042] Table 4 shows the minimum inhibitory concentrations (MICs) and selectivity indices of the peptides of the present disclosure.

10 [0043] Table 5 shows the minimum microbicidal concentrations (MBCs) of the peptides of the present disclosure against clinically isolated drug resistant microorganisms.

[0044] Table 6 shows the minimum inhibitory concentrations (MICs) of the peptides of the present disclosure against clinically isolated *Mycobacterium tuberculosis*.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

15 [0045] The design and optimization of short synthetic peptides with minimal resemblance to naturally occurring peptide sequences is a useful strategy for the development of safe and efficacious antimicrobial peptides for clinical use. Besides having a cationic character with net charges between +2 to +9 and comprising of ~ 30-50% of hydrophobic amino acid residues, one commonality of various naturally occurring and synthetic antimicrobial
20 peptides lies in the folding of the amphipathic peptides to form secondary structures, often upon contact with microbial membranes. Between the two predominant forms of folded structures including α -helical peptides (e.g. cathelicidins, cecropins, magainins) and β -sheet peptides (e.g. defensins and protegrins), amphipathic β -sheet peptides have been found to be less haemolytic while possessing comparable antimicrobial activities to their α -helical
25 counterparts of equal charge and hydrophobicity. Thus, it is the object of the present disclosure to provide a short synthetic peptide having β -sheet folding structures that have antimicrobial activities and are less haemolytic.

[0046] Thus, in one aspect, there is provided an amphiphilic peptide. In one example, the peptide comprises $(X_1Y_1X_2Y_2)_n$ (Formula I), wherein X_1 and X_2 may be individually selected
30 from the group consisting of hydrophobic amino acids; Y_1 and Y_2 may be individually selected from the group consisting of cationic amino acids; and n may be at least 1.5. In one example, the inventors of the present disclosure designed short synthetic β -sheet folding

peptides consisting of short recurring $(X_1Y_1X_2Y_2)_n$ -NH₂ sequences based upon several basic design principles from naturally occurring β -sheet folding AMPs including, but not limited to: 1) the common occurrence of amphipathic dyad repeats in membrane-spanning β -sheets, 2) the requirement for hydrophobic residues (such as, but not limited to Val, Ile, Phe and Trp) and cationic (such as, but not limited to Arg and Lys) and to interact and perturb microbial cell walls and membranes, and 3) the strong β -sheet folding propensities of Val, Ile, Phe and Trp (Figure 1).

[0047] As used herein, the term "amphiphilic peptide" refers to a peptide that possesses both hydrophilic and lipophilic properties, which is conferred by the peptide having cationic amino acids attached to hydrophobic amino acids. As used herein, the term "hydrophobic amino acid" refers to amino acid residues that are not soluble in water. In one example, the term "hydrophobic amino acid" may include, but is not limited to alanine (A), valine (V), leucine (L), isoleucine (I), phenylalanine (F), methionine (M), tryptophan (W), cysteine (C), tyrosine (Y), histidine (H), threonine (T), serine (S), proline (P) and glycine (G). In one example, the hydrophobic amino acid may be alanine (A), valine (V), leucine (L), isoleucine (I), phenylalanine (F), methionine (M), tryptophan (W) and cysteine (C). In one example, the hydrophobic amino acid may be isoleucine (I) or valine (V).

[0048] As used herein, the term "cationic amino acid" refers to amino acid residues which are soluble in water. In one example, the cationic amino acid may include, but is not limited to arginine (R), lysine (K) and histidine (H).

[0049] In one example, the amphiphilic peptide is an isolated peptide. As used herein, the term "isolated" refers to a peptide free of or substantially free of proteins, lipids, nucleic acids, for example, with which it is naturally associated.

[0050] In one example, the amphiphilic peptide may consist of $(X_1Y_1X_2Y_2)_n$ (Formula I), wherein X_1 and X_2 may be independently of each other a hydrophobic amino acid; Y_1 and Y_2 may be independently of each other a cationic amino acid. Thus, X_1 and X_2 may be the same or different amino acids and Y_1 and Y_2 may be the same or different amino acids.

[0051] In the present disclosure, n may not be 1 because peptides with four amino acids as described herein (for example $X_1Y_1X_2Y_2$ (SEQ ID NO: 1)) do not have antimicrobial activities and do not form β -sheets in microbial membrane environments. Accordingly, n may be between about 1.5 to 5. In one example, n may be 1.5, 2, 2.5, 3, 3.5, 4, 4.5 or 5.

- [0052] In one example, the amphiphilic peptide may include, but is not limited to $X_1Y_1X_2Y_2X_3Y_3$ (SEQ ID NO: 2), $X_1Y_1X_2Y_2X_3Y_3X_4Y_4$ (SEQ ID NO: 3), $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$ (SEQ ID NO: 4), $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5X_6Y_6$ (SEQ ID NO: 5), $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5X_6Y_6X_7Y_7$ (SEQ ID NO: 6),
- 5 $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5X_6Y_6X_7Y_7X_8Y_8$ (SEQ ID NO: 7), $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5X_6Y_6X_7Y_7X_8Y_8X_9Y_9$ (SEQ ID NO: 8) and $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5X_6Y_6X_7Y_7X_8Y_8X_9Y_9X_{10}Y_{10}$ (SEQ ID NO: 9). In one example, X_1 , X_2 , X_3 , X_4 , X_5 , X_6 , X_7 and X_8 , may be the same or independently different amino acids and Y_1 , Y_2 , Y_3 , Y_4 , Y_5 , Y_6 , Y_7 and Y_8 , may be the same or independently different amino acids.
- 10 [0053] In one example, the peptides of the present disclosure may have C-terminal end that is amidated. The term "C-terminal end" is used herein in accordance to its definition as commonly known in the art, that is, can be used interchangeably with any of the following terminologies such as the carboxyl-terminus, carboxy-terminus, C-terminal tail, C-terminus or COOH-terminus, which refer to the end of an amino acid chain, terminated by a free
- 15 carboxyl group (-COOH). As written herein, the peptides as described herein are presented as C-terminal end on the right and N-terminal end on the left. Thus, as used herein, the phrase "C-terminal end is amidated" refers to the substitution of the hydroxyl group of C-terminal end of the peptide of the present disclosure with a primary or secondary amine group. In one example, the peptides as described herein may be $(X_1Y_1X_2Y_2)_n$, which may be $(X_1Y_1X_2Y_2)_n$ -
- 20 NH_2 . In one example, the peptides of the present disclosure may be amidated by process commonly known in the art. Without wishing to be bound by theory, it is believed that C-terminal amidation enhances antimicrobial activities presumably due to the reinforcement of cationic character; the peptides designed for this study were amidated at the C-terminal to confer a high net positive charge.
- 25 [0054] The peptides of the present disclosure does not require secondary structure stabilizer. The phrase "secondary structure stabilizer" refers to molecules that are capable of holding local spatial arrangement of a polypeptide's backbone atoms without regard to the conformations of its side chains. The secondary structure stabilizer as used herein may include, but is not limited to hydrophobic effect, electrostatic interactions and chemical cross-
- 30 links such as disulphide bonds within and between polypeptide chains or metal ions internal cross-linking. In one example, the peptides of the present disclosure are distinct from existing β -sheet peptides known in the art in that they do not require disulphide bridges or other

covalent bond constraints to stabilize the secondary structure. In aqueous solutions, the peptides of the present disclosure may be expected to exist as monomers due to electrostatic repulsion between the protonated Arg and/or Lys residues. In the presence of microbial cell membranes, the peptides of the present disclosure readily fold into secondary β -sheet structures stabilized by electrostatic interactions between the positively charged residues and the negatively charged phospholipids, followed by the insertion of its hydrophobic residues into the lipid bilayer. Thus, in one example, the peptide may be capable of self-assembly into β -sheet structure. As used herein, the term "self-assembly" refers to a type of process wherein a disordered system of pre-existing components forms an organized structure or pattern as a consequence of specific, local interactions among the components themselves, without external direction. In the present disclosure, the peptides may spontaneously form β -sheet structures in microbial membrane environments. As used herein, the phrase "microbial membrane environments" refers to microenvironment in the membrane of target microbes. In one example, this environment may be simulated by the presence of the anionic surfactant SDS. In one example, the peptides may not spontaneously form β -sheet structures in deionised water or aqueous solution.

[0055] As used herein, " β -sheet" or " β -pleated sheet structure" refers to a regular secondary structure of peptides that may comprise at least one β -strand, which is a stretch of polypeptide chain typically 3 to 10 amino acid long with backbone in an almost fully extended formation. In one example, the β -sheet structure of the present invention consists of at least one β -strand.

[0056] Furthermore, in one example, the arrangement of the cationic and hydrophobic amino acids within the beta-sheet forming sequence may not be changed. As shown in examples provided in Table 1 and Figure 11d, rearrangement of cationic and hydrophobic amino acids within the beta-sheet forming sequence can lead to a loss of secondary structure.

[0057] In one example, the peptides of the present disclosure may include: 1) choice of cationic amino acid residue (i.e. Arg vs. Lys vs. combination of both), 2) degree of polarity and bulkiness of the hydrophobic side chain, and 3) a specific length of peptide sequence. In the present disclosure, the various possible configurations of peptides of the present disclosure were systematically investigated for effects on antimicrobial and haemolytic activities. In some examples, peptides containing Arg have been found to have stronger antimicrobial properties compared to Lys-containing. Without wishing to be bound to theory,

it was believed that the stronger antimicrobial properties of Arg rich peptides of the present disclosure may be due to the greater charge density of the guanidinium side chain. However, it is commonly believed in the art that the presence of numerous arginine residues within a peptide sequence is associated with a higher degree of haemolysis and cytotoxicity. Thus, both Arg and Lys were incorporated in the peptide designs to capitalize on the high charge density of Arg for improving antimicrobial effects while tuning the toxicity of the synthetic peptides using the less toxic Lys residue.

[0058] At the same time, in efforts to enhance the stability of the peptides of the present disclosure in biological fluids, the inventors of the present disclosure investigated the effect of stereochemistry on the antimicrobial activities of the synthetic peptides. Thus, in one example, there is provided peptides of the present disclosure which may have each repeating unit n of Formula I comprises independently of each other 1, or 2, or 3 or 4 D-amino acids with the remaining amino acids being L-amino acids. In one example, the repeating unit n of Formula I may comprise independently of each other 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 D-amino acids with the remaining amino acids being L-amino acids. In one example, the repeating unit n of Formula I may comprise independently of each other 2 or 4 or 8 D-amino acids with the remaining amino acids being L-amino acids. As used herein, "L-amino acids" and "D-amino acids" refer to the two isomers that can occur in every amino acid. As written herein, small underlined residues represent D-amino acids while capital non-underlined represents L-amino acid. "L-amino acids" refer to the amino acid isomer which are manufactured in cells and incorporated into proteins. "D-amino acids" refers to isomeric modification to amino acids of the peptides of the present disclosure. Advantageously, "D-amino acids" may not be recognized by human and microbial proteases. Thus, as illustrated in Figures 13 and 19, the peptides of the present disclosure may be prevented from premature proteolytic degradation *in vivo*. However, as found by the inventors of the present disclosure, selective D-amino acid substitution at four positions led to a complete loss of secondary structure. Accordingly, in one example, the each repeating unit n of Formula I may comprise independently of each other 1 or 2 D-amino acids with the remaining amino acids being L-amino acids. In one example, the distribution of D-amino acids in each repeating unit n of Formula I may be identical or different from each other. In one example, the peptide as described herein, wherein n may be 2 and amino acids in position 4 and 6 may be D-amino acids while the remaining amino acids are L-amino acids. As

illustrated in the Experimental Section, selective D-amino acid substitution at positions 4, 6, enabled the retention of secondary structure forming propensity (see Figure 11c).

[0059] In one example, the peptides as described herein may comprise or consists of the sequence $(IY_1IY_2)_n-NH_2$. In one example, the peptides as described herein may comprise or
5 consists of the sequence $(IRX_2K)_n-NH_2$.

[0060] In one example, the peptide as described herein when represented by general formula $X_1Y_1X_2Y_2X_3Y_3$ (SEQ ID NO: 2) may include, but is not limited to irikir-NH₂ (SEQ ID NO: 10), wherein small underlined residues represent D-amino acids. In one example, the peptide as described herein when represented by general formula $X_1Y_1X_2Y_2X_3Y_3X_4Y_4$ (SEQ ID NO:
10 3), may include, but is not limited to VRVKVRVK-NH₂ (SEQ ID NO: 11), IRIRIRIR-NH₂ (SEQ ID NO: 12), IKIKIKIK-NH₂ (SEQ ID NO: 13), IRVKIRVK-NH₂ (SEQ ID NO: 14), FRFKFRFK-NH₂ (SEQ ID NO: 15), WRWKWRWK-NH₂ (SEQ ID NO: 16), IRIKIRIK-NH₂ (SEQ ID NO: 17), irikirik-NH₂ (SEQ ID NO: 18) and IRIkIrIK-NH₂ (SEQ ID NO: 19), wherein small underlined residues represent D-amino acids while capital non-underlined
15 represent L-amino acid. In one example, the peptide as described herein when represented by general formula $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5X_6Y_6$ (SEQ ID NO: 5), may include, but is not limited to VRVKVRVKVRVK-NH₂ (SEQ ID NO: 20), IRIKIRIKIRIK-NH₂ (SEQ ID NO: 21), IRVKIRVKIRVK-NH₂ (SEQ ID NO: 22), and irvkvirkvirk-NH₂ (SEQ ID NO: 23), wherein small underlined residues represent D-amino acids while capital non-underlined
20 represent L-amino acid. In one example, the peptides as described herein may be selected from the group consisting of VRVKVRVK-NH₂ (SEQ ID NO: 11), VRVKVRVKVRVK-NH₂ (SEQ ID NO: 20), IRIRIRIR-NH₂ (SEQ ID NO: 12), IKIKIKIK-NH₂ (SEQ ID NO: 13), IRVKIRVK-NH₂ (SEQ ID NO: 14), FRFKFRFK-NH₂ (SEQ ID NO: 15), WRWKWRWK-NH₂ (SEQ ID NO: 16), IRIKIRIK-NH₂ (SEQ ID NO: 17), IRIKIRIKIRIK-NH₂ (SEQ ID NO:
25 21), irikir-NH₂ (SEQ ID NO: 10), irikirik-NH₂ (SEQ ID NO: 18), IRIkIrIK-NH₂ (SEQ ID NO: 19), IRVKIRVKIRVK-NH₂ (SEQ ID NO: 22), and irvkvirkvirk-NH₂ (SEQ ID NO: 23), wherein small underlined residues represent D-amino acids while capital non-underlined represent L-amino acid.

[0061] Also provided are peptides of the present disclosure for use as a medicament.

30 [0062] In another aspect, there is provided a method of treating a microbial infection or removing microorganism comprising administration of a pharmaceutically effective amount of a peptide of the present disclosure. The terms "treat," "treatment," and grammatical

variants thereof, refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological condition, disorder or disease or obtain beneficial or desired clinical results. Such beneficial or desired clinical results include, but are not limited to, alleviation of symptoms; 5 diminishment of extent of condition, disorder or disease; stabilized (i.e. not worsening) state of condition, disorder or disease; delay or slowing of condition, disorder or disease progression; amelioration of the condition, disorder or disease state, remission (whether partial or total), whether detectable or undetectable; or enhancement or improvement of condition, disorder or disease. Treatment includes eliciting a cellular response that is 10 clinically significant, without excessive levels of side effects. Treatment also includes prolonging survival as compared to expected survival if not receiving treatment.

[0063] As used herein, the term "microbes" or "microorganism" is used in its broadest sense and is therefore not limited in scope to prokaryotic organisms. Rather, the term "microorganism" includes within its scope bacteria, archaea, virus, yeast, fungi, protozoa and 15 algae. In one example, there is provided a method of treating a bacterial infection or removing bacteria comprising administration of a pharmaceutically effective amount of a peptide of the present disclosure.

[0064] In one example, the bacteria may be gram positive or gram negative bacteria. Thus, bacterial infections that may be treated include, but not limited to, those caused by bacteria 20 from the genus of *Acetobacter*, *Acinetobacter*, *Actinomyces*, *Agrobacterium* spp., *Azorhizobium*, *Azotobacter*, *Anaplasma* spp., *Bacillus* spp., *Bacteroides* spp., *Bartonella* spp., *Bordetella* spp., *Borrelia*, *Brucella* spp., *Burkholderia* spp., *Calymmatobacterium*, *Campylobacter*, *Chlamydia* spp., *Chlamydophila* spp., *Clostridium* spp., *Corynebacterium* spp., *Coxiella*, *Ehrlichia*, *Enterobacter*, *Enterococcus* spp., *Escherichia*, *Francisella*, 25 *Fusobacterium*, *Gardnerella*, *Haemophilus* spp., *Helicobacter*, *Klebsiella*, *Lactobacillus* spp., *Lactococcus*, *Legionella*, *Listeria*, *Methanobacterium* extroquens, *Microbacterium* multiforme, *Micrococcus luteus*, *Moraxella catarrhalis*, *Mycobacterium* spp., *Mycoplasma* spp., *Neisseria* spp., *Pasteurella* spp., *Peptostreptococcus*, *Porphyromonas*, *Pseudomonas*, *Rhizobium*, *Rickettsia* spp., *Rochalimaea* spp., *Rothia*, *Salmonella* spp., *Serratia*, *Shigella*, 30 *Staphylococcus* spp., *Stenotrophomonas*, *Streptococcus* spp., *Treponema* spp., *Vibrio* spp., *Wolbachia*, and *Yersinia* spp. In one example, the bacteria may include, but are not limited to *Acetobacter aurantius*, *Acinetobacter baumannii*, *Actinomyces Israelii*, *Agrobacterium*

radiobacter, *Agrobacterium tumefaciens*, *Azorhizobium caulinodans*, *Azotobacter vinelandii*,
Anaplasma phagocytophilum, *Anaplasma marginale*, *Bacillus anthracis*, *Bacillus brevis*,
Bacillus cereus, *Bacillus fusiformis*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus*
mycoides, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacteroides fragilis*, *Bacteroides*
5 *gingivalis*, *Bacteroides melaminogenicus* (*Prevotella melaminogenica*), *Bartonella henselae*,
Bartonella quintana, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Borrelia burgdorferi*,
Brucella abortus, *Brucella melitensis*, *Brucella suis*, *Burkholderia mallei*, *Burkholderia*
pseudomallei, *Burkholderia cepacia* complex, *Burkholderia cenocepacia*,
Calymmatobacterium granulomatis, *Campylobacter coli*, *Campylobacter fetus*,
10 *Campylobacter jejuni*, *Campylobacter pylori*, *Chlamydia trachomatis*, *Chlamydophila*. (such
as *C. pneumoniae*, *Chlamydophila psittaci*, *Clostridium botulinum*, *Clostridium difficile*,
Clostridium perfringens, *Clostridium tetani*), *Corynebacterium diphtheriae*,
Corynebacterium fusiforme, *Coxiella burnetii*, *Ehrlichia chaffeensis*, *Enterobacter cloacae*,
Enterococcus avium, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*,
15 *Enterococcus gallinarum*, *Enterococcus maloratus*, *Escherichia coli*, *Francisella tularensis*,
Fusobacterium nucleatum, *Gardnerella vaginalis*, *Haemophilus ducreyi*, *Haemophilus*
influenzae, *Haemophilus parainfluenzae*, *Haemophilus pertussis*, *Haemophilus vaginalis*,
Helicobacter pylori, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Lactobacillus casei*,
Lactococcus lactis, *Legionella pneumophila*, *Listeria monocytogenes*, *Methanobacterium*
20 *extroquens*, *Microbacterium multiforme*, *Micrococcus luteus*, *Moraxella catarrhalis*,
Mycobacterium avium, *Mycobacterium bovis*, *Mycobacterium diphtheriae*, *Mycobacterium*
intracellulare, *Mycobacterium leprae*, *Mycobacterium lepraemurium*, *Mycobacterium phlei*,
Mycobacterium smegmatis, *Mycobacterium tuberculosis*, *Mycoplasma fermentans*,
Mycoplasma genitalium, *Mycoplasma hominis*, *Mycoplasma penetrans*, *Mycoplasma*
25 *pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pasteurella multocida*,
Pasteurella tularensis, *Peptostreptococcus*, *Porphyromonas gingivalis*, *Pseudomonas*
aeruginosa, *Rhizobium Radiobacter*, *Rickettsia prowazekii*, *Rickettsia psittaci*, *Rickettsia*
quintana, *Rickettsia rickettsii*, *Rickettsia trachomae*, *Rochalimaea henselae*, *Rochalimaea*
quintana, *Rothia dentocariosa*, *Salmonella enteritidis*, *Salmonella typhi*, *Salmonella*
30 *typhimurium*, *Serratia marcescens*, *Shigella dysenteriae*, *Staphylococcus aureus*,
Staphylococcus epidermidis, *Stenotrophomonas maltophilia*, *Streptococcus agalactiae*,
Streptococcus avium, *Streptococcus bovis*, *Streptococcus cricetus*, *Streptococcus faecium*,

- Streptococcus faecalis*, *Streptococcus ferus*, *Streptococcus gallinarum*, *Streptococcus lactis*, *Streptococcus mitior*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus rattus*, *Streptococcus salivarius*, *Streptococcus sanguis*, *Streptococcus sobrinus*, *Treponema pallidum*, *Treponema denticola*, *Vibrio cholerae*, *Vibrio comma*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Wolbachia*, *Yersinia enterocolitica*, *Yersinia pestis* and *Yersinia pseudotuberculosis*. In one example, the bacteria may include, but is not limited to *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Enterococcus spp.*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*.
- 10 [0065] In one example, the bacterial infection may cause conditions such as, but are not limited to pneumonia, tuberculosis, meningitis, diarrhoeal diseases, formation of biofilm, sepsis, listeriosis, gastroenteritis, toxic shock syndrome, hemorrhagic colitis; hemolytic uremic syndrome, Lyme Disease, gastric and duodenal ulcers, human ehrlichiosis, pseudomembranous colitis, cholera, salmonellosis, cat scratch fever, necrotizing fasciitis
- 15 (GAS), streptococcal toxic shock syndrome, nosocomial and community associated infections, atherosclerosis, sudden infant death syndrome (SIDS), ear infections, respiratory tract infections, urinary tract infections, skin and soft tissue infections, nail bed infections, wound infection, septicemia, gastrointestinal disease, hospital-acquired endocarditis and blood stream infections.. In one example, the bacteria may be drug resistant bacteria.
- 20 [0066] Sepsis refers to the major cause of mortality in intensive care units worldwide, which is triggered by the release of lipopolysaccharide molecules from the outer wall of gram-negative bacteria. Currently, besides the intravenous administration of broad spectrum antibiotics, only supportive therapies are given to prevent the aggravation of septic shock syndrome, with no effective therapies against the microbial endotoxin, which is a noxious
- 25 mediator of immune responses. Polymyxin B is the 'gold' standard for endotoxin binding and neutralization, however, its high cytotoxicity negates its practical applicability for systemic applications. As such, there is a need to provide an alternative antimicrobial agent that can simultaneously annihilate causative microorganisms and neutralize endotoxins without having cytotoxic activity towards the mammalian cells. Thus, in one example, the peptide of
- 30 the present disclosure may be used for preventing sepsis.
- [0067] The lipopolysaccharide (LPS) endotoxin, which has potent immunostimulating properties, is an integral structural component in the outer membrane leaflet of Gram-

negative bacteria. LPS is shed continuously during microbial cell growth and division and is liberated in large amounts during cell death, often as a result of antibiotics therapies against bacterial infections. Upon release into the bloodstream, LPS aggregates are dissociated by LPS-binding plasma proteins (LBPs) to form LPS-LBP complexes which stimulate the host
5 monocytes and macrophages to secrete various cytokines (e.g. TNF- α , IL-6, IL-8) and pro-inflammatory mediators (e.g. NO and reactive oxygen species). This activation of the innate immune system triggers a cascade of exaggerated immune responses resulting in a serious clinical syndrome known as septic shock, which could rapidly precipitate in multiple organ failure or death if left untreated. The anionic amphiphilic lipid A domain, which is
10 structurally conserved across most Gram-negative genera, is well-regarded as the active moiety of LPS. The inventors of the present disclosure demonstrates that cationic antimicrobial peptide amphiphiles as described herein present a particularly useful candidate for binding and neutralizing of LPS via electrostatic interactions with the anionic head group by cationic lysine or arginine residues, and dissociation of LPS aggregates via hydrophobic
15 interactions between the alkyl chains of LPS and non-polar amino acid side chains. In one example, there is provided a method of neutralizing endotoxins comprising administration of a pharmaceutically effective amount of a peptide of the present disclosure. In one example, the endotoxins may be bacterial endotoxins or fungal endotoxins. In one example, the endotoxins may be polysaccharides, lipoteichoic acid, lipopolysaccharide or
20 lipooligosaccharide. Figure 9 demonstrates that the peptides of the present disclosure effectively reduce the effects of LPS on macrophages.

[0068] Another problem that bacteria may pose towards human is the formation of biofilms. The formation of biofilms is a significant problem that is implicated in a variety of both the medical field and the non-medical field. Biofilm formation occurs when microbial cells
25 adhere to each other and are embedded in a matrix of extracellular polymeric substance (EPS) on a surface. The growth of microbes in such a protected environment that is enriched with biomacromolecules (e.g. polysaccharides, nucleic acids and proteins) and nutrients allow for enhanced microbial cross-talk and increased virulence. As a result, physiological and phenotypic changes particularly in the growth rate and gene transcription patterns of the
30 microbes entrapped within the biofilm have been widely described. The inability of antibiotics to traverse the extracellular polymeric substance and/or inactivation of antibiotics by components of the extracellular polymeric substance is believed to be responsible for the

ineffectiveness of antibiotics to inhibit or kill biofilm microbes at conventional doses. This phenomenon, coupled with the upregulation of drug resistance genes, often leads to the rapid development of antibiotic resistance in biofilms, making their successful eradication an immense challenge in the medical setting. Thus, there is a need to provide a method of removing biofilm.

[0069] In view of the above, in another aspect, there is provided a method of removing a biofilm comprising administration of a pharmaceutically effective amount of the peptide of the present disclosure. In one example, the biofilm may occur on surfaces. The term "surface" used herein, refers to any surface whether medical or industrial, that provides an interface between a fluid, such as a liquid or air, and a solid. The interface between fluid and solid can be intermittent, and can be caused by flowing or stagnant fluid, aerosols, or other means for air-borne fluid exposure. A surface refers, in some examples, to a plane whose mechanical structure is compatible with the adherence of bacteria or fungi. In the context of the peptides and methods described herein, the terminology "surface" encompasses the inner and outer aspects of various instruments and devices, both disposable and non-disposable, medical and non-medical. Examples of non-medical uses include the hull of a ship, dockyard, food processors, mixers, machines, containers, water tanks, water filtrations, purification systems, preservatives in food industries, personal care products such as shampoo, cream, moisturizer, hand sanitizer, soaps and the like. Examples of medical uses include the entire spectrum of medical devices. Such "surfaces" may include the inner and outer aspects of various instruments and devices, whether disposable or intended for repeated uses. Examples include the entire spectrum of articles adapted for medical use, including scalpels, needles, scissors and other devices used in invasive surgical, therapeutic or diagnostic procedures; implantable medical devices, including artificial blood vessels, catheters and other devices for the removal or delivery of fluids to patients, artificial hearts, artificial kidneys, orthopaedic pins, plates and implants; catheters and other tubes (including urological and biliary tubes, endotracheal tubes, peripherally insertable central venous catheters, dialysis catheters, long term tunnelled central venous catheters, peripheral venous catheters, short term central venous catheters, arterial catheters, pulmonary catheters, Swan-Ganz catheters, urinary catheters, peritoneal catheters), urinary devices (including long term urinary devices, tissue bonding urinary devices, artificial urinary sphincters, urinary dilators), shunts (including ventricular or arterio-venous shunts); prostheses (including breast implants, penile prostheses,

vascular grafting prostheses, heart valves, artificial joints, artificial larynxes, otological implants), vascular catheter ports, wound drain tubes, hydrocephalus shunts, pacemakers and implantable defibrillators, dental implants, fillings, dentures and the like. Other examples will be readily apparent to practitioners in these arts. Surfaces found in the medical environment also include the inner and outer aspects of pieces of medical equipment, medical gear worn or carried by personnel in the health care setting. Such surfaces can include counter tops and fixtures in areas used for medical procedures or for preparing medical apparatus, tubes and canisters used in respiratory treatments, including the administration of oxygen, of solubilised drugs in nebulisers and of aesthetic agents. Also included are those surfaces intended as biological barriers to infectious organisms in medical settings, such as gloves, aprons and face-shields. Commonly used materials for biological barriers may be latex-based or non-latex based. An example for a non-latex based biological barrier material may include vinyl. Other such surfaces can include handles and cables for medical or dental equipment not intended to be sterile. Additionally, such surfaces can include those non-sterile external surfaces of tubes and other apparatus found in areas where blood or body fluids or other hazardous biomaterials are commonly encountered. In one example, the biofilm may be comprised on catheters and medical implants.

[0070] In another aspect, there is provided a method of treating viral infectious diseases comprising the administration of a pharmaceutically effective amount of a peptide of the present disclosure. In one example, the viral infectious diseases may be caused by virus including, but not limited to infections or infectious diseases caused by adenoviruses, herpes viruses, poxviruses, parvoviruses, reoviruses, picornaviruses, togaviruses, orthomyxoviruses, rhabdoviruses, paramyxoviruses, papillomaviruses, retroviruses (such as Human Immunodeficiency Virus) and hepadnaviruses. In one example, the viral infectious disease may include, but is not limited to common cold, influenza, chickenpox, cold sores, Ebola, AIDS, avian influenza, SARS, dengue, herpes, shingles, measles, mumps, rubella, rabies, human papillomavirus, viral hepatitis, coxsackie virus, Epstein Barr virus and the like.

[0071] In another aspect, there is provided a method of treating fungal infections or infestations, or removing fungus comprising administration of a pharmaceutically effective amount of a peptide of the present disclosure. As used herein, the term "fungi" (and derivatives thereof, such as "fungal infection") includes, but is not limited to, references to organisms (or infections due to organisms) of the following genus: *Absidia*, *Ajellomyces*,

Arthroderma, *Aspergillus*, *Blastomyces*, *Candida*, *Cladophialophora*, *Coccidioides*, *Cryptococcus*, *Cunninghamella*, *Epidermophyton*, *Exophiala*, *Filobasidiella*, *Fonsecaea*, *Fusarium*, *Geotrichum*, *Histoplasma*, *Hortaea*, *Issatschenkia*, *Madurella*, *Malassezia*, *Microsporum*, *Microsporidia*, *Mucor*, *Nectria*, *Paecilomyces*, *Paracoccidioides*, *Penicillium*,
5 *Pichia*, *Pneumocystis*, *Pseudallescheria*, *Rhizopus*, *Rhodotorula*, *Scedosporium*, *Schizophyllum*, *Sporothrix*, *Trichophyton*, and *Trichosporon*. For example, fungal infections caused by species such as, but is not limited to *Absidia corymbifera*, *Ajellomyces capsulatus*, *Ajellomyces dermatitidis*, *Arthroderma benhamiae*, *Arthroderma fulvum*, *Arthroderma gypseum*, *Arthroderma incurvatum*, *Arthroderma otae* and *Arthroderma vanbreuseghemii*,
10 *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus niger*, *Blastomyces dermatitidis*, *Candida albicans*, *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis* and *Candida pelliculosa*, *Cladophialophora carrionii*, *Coccidioides immitis* and *Coccidioides posadasii*, *Cryptococcus neoformans*, *Cunninghamella* Sp, *Epidermophyton floccosum*, *Exophiala dermatitidis*, *Filobasidiella*
15 *neoformans*, *Fonsecaea pedrosoi*, *Fusarium solani*, *Geotrichum candidum*, *Histoplasma capsulatum*, *Hortaea werneckii*, *Issatschenkia orientalis*, *Madurella grisea*, *Malassezia furfur*, *Malassezia globosa*, *Malassezia obtusa*, *Malassezia pachydermatis*, *Malassezia restricta*, *Malassezia slooffiae*, *Malassezia sympodialis*, *Microsporum canis*, *Microsporum fulvum*, *Microsporum gypseum*, *Microsporidia*, *Mucor circinelloides*, *Nectria haematococca*,
20 *Paecilomyces variotii*, *Paracoccidioides brasiliensis*, *Penicillium marneffeii*, *Pichia anomala*, *Pichia guilliermondii*, *Pneumocystis jiroveci*, *Pneumocystis carinii*, *Pseudallescheria boydii*, *Rhizopus oryzae*, *Rhodotorula rubra*, *Scedosporium apiospermum*, *Schizophyllum commune*, *Sporothrix schenckii*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton verrucosum* and *Trichophyton violaceum*, and *Trichosporon asahii*, *Trichosporon cutaneum*,
25 *Trichosporon inkin* and *Trichosporon mucoides*. In one example, the fungal infection as described herein may cause by *C. albicans*. In one example, the fungal infection may be caused by a drug resistant fungus. Exemplary use of the peptides as described herein for inhibiting, treating or removing fungus is provided in Tables 2 and 4.

[0072] The terms "decrease", "reduced", "reduction", "decrease", "removal" or "inhibit" are
30 all used herein generally to mean a decrease by a statistically significant amount. However, for avoidance of doubt, "reduced", "reduction" or "decrease", "removal", or "inhibit" means a decrease by at least 10% as compared to a reference level, for example a decrease by at

least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% decrease (e.g. absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level (e.g., in the absence of a peptide as described herein).

[0073] The inventors of the present disclosure also found that the peptides as described herein may induce cell death in cancerous cell lines. Thus, in another aspect, there is provided a method of treating proliferative diseases may comprise administration of a pharmaceutically effective amount of a peptide as described herein. In one example, the proliferative diseases may include, but are not limited to tumour, cancer, malignancy or combinations thereof. In one example, the proliferative diseases may include, but are not limited to of colorectal cancer, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangio-endotheliosarcoma, synovioma, mesothelioma, Ewing's tumour, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, gastric cancer, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma of the head and neck, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, liver metastases, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, thyroid carcinoma such as anaplastic thyroid cancer, Wilms' tumour, cervical cancer, testicular tumour, lung cancer, small cell cancer of the lung, non-small cell cancer of the lung, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma. Exemplary use of the peptides as described herein for inducing cancerous cell death is provided in Figure 22.

[0074] In another aspect, there is provided the use of a peptide of the present disclosure in the manufacture of a medicament for treating bacterial infection, or removing bacteria, or neutralising endotoxins, or treating viral based infectious diseases or treating a fungal infection or infestations, or removing fungus or treating proliferative diseases. In one example, the use may further comprise providing the peptide of the present disclosure for

administration into a subject in need thereof. In one example, wherein the medicament is to be administered into a subject in need thereof.

[0075] In one example, the subject or patient may be an animal, mammal, human, including, without limitation, animals classed as bovine, porcine, equine, canine, lupine, feline, murine, ovine, avian, piscine, caprine, corvine, acrine, or delphine. In one example, the patient may be a human.

[0076] In one example, the peptide as described herein may be provided as a composition or a pharmaceutical composition. The compositions as described herein may be administered in a number of ways depending upon whether local or systemic treatment is desired.

10 Administration may be topical, pulmonary (e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal) or systemic such as oral, and/or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. In one example, the route of
15 administration may be selected from the group consisting of systemic administration, oral administration, intravenous administration and parenteral administration

[0077] Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be
20 desirable.

[0078] Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

25 [0079] Compositions as described herein include, but are not limited to, solutions, pastes, ointment, creams, hydrogels, emulsions, liposome-containing formulations, and coatings. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

[0080] The formulations as described herein, which may conveniently be presented in unit
30 dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the

formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

[0081] The compositions as described herein may be formulated into any of many possible dosage forms including, but not limited to tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions as described herein may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

[0082] In one example, the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product.

[0083] The compositions as described herein may additionally contain other adjunct components conventionally found in pharmaceutical compositions. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritic, astringents, local anaesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present disclosure. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colourings, flavourings and/or aromatic substances and the like which do not deleteriously interact with the peptide(s) of the formulation.

[0084] The term "pharmaceutically effective amount" as used herein includes within its meaning a sufficient but non-toxic amount of the compound as described herein to provide the desired effect, that is, causing a Log reduction in the number of microorganisms of at least 1.0, which means that less than 1 microorganism in 10 remains. The modified peptides of the present disclosure may provide Log reductions in the number of microorganisms of at

least about 2.0, or at least about 3.0, or at least about 4.0, or at least about 5.0, or at least about 6.0, or at least about 7.0. The exact amount required will vary from subject to subject depending on factors such as the species being treated, the age and general condition of the subject, the severity of the condition being treated, the particular agent being administered, 5 the mode of administration, and so forth. Thus, it is not possible to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation.

[0085] Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is 10 effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. The administering physician can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of the composition, and can generally be estimated based on EC50s found to be effective in *in vitro* 15 and *in vivo* animal models or based on the examples described herein. In general, dosage is from 0.01 μg to 100 g/kg of body weight, and may be given once or more daily, weekly, monthly or yearly. The treating physician can estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the subject undergo maintenance 20 therapy to prevent the recurrence of the disease state, wherein the composition is administered in maintenance doses, ranging from 0.01 μg to 100 g/kg of body weight, once or more daily, to once every 2 years.

[0086] In one example, the composition may be administered in an amount of between any one of about 0.01 μg , 0.05 μg , 0.1 μg , 0.5 μg , 1 μg , 5 μg , 10 μg , 20 μg , 30 μg , 40 μg , 50 μg , 25 60 μg , 70 μg , 80 μg , 90 μg , 100 μg , 110 μg , 120 μg , 130 μg , 140 μg , 150 μg , 160 μg , 170 μg , 180 μg , 190 μg , 200 μg , 210 μg , 220 μg , 230 μg , 240 μg , 250 μg , 260 μg , 270 μg , 280 μg , 290 μg , 500 μg , 1 mg, 1.5 mg, 2 mg, 2.5 mg, 3 mg, 3.5 mg, 4 mg, 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg, 75 mg, 100 mg, 125 mg, 150 mg, 175 mg, 200 mg, 225 mg, 250 mg to any one of about 0.01 μg , 0.05 μg , 0.1 μg , 0.5 μg , 1 μg , 5 μg , 10 30 μg , 20 μg , 30 μg , 40 μg , 50 μg , 60 μg , 70 μg , 80 μg , 90 μg , 100 μg , 110 μg , 120 μg , 130 μg , 140 μg , 150 μg , 160 μg , 170 μg , 180 μg , 190 μg , 200 μg , 210 μg , 220 μg , 230 μg , 240 μg , 250 μg , 260 μg , 270 μg , 280 μg , 290 μg , 500 μg , 1 mg, 1.5 mg, 2 mg, 2.5 mg, 3 mg, 3.5 mg,

4 mg, 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg, 75 mg, 100 mg, 125 mg, 150 mg, 175 mg, 200 mg, 225 mg, 250 mg, 300 mg/kg of body weight of the patient.

[0087] In one example, the concentration of the administered composition is about 1 to about 100 mg/Kg of body weight of the patient, about 5 to about 100 mg/Kg of body weight of the patient, about 10 to about 100 mg/Kg of body weight of the patient, about 20 to about 100 mg/Kg of body weight of the patient, about 30 to about 100 mg/Kg of body weight of the patient, about 1 to about 50 mg/Kg of body weight of the patient, about 5 to about 50 mg/Kg of body weight of the patient and about 10 to about 50 mg/Kg of body weight of the patient.

[0088] As used herein, the term "about", in the context of amounts or concentrations of components of the formulations, typically means $\pm 5\%$ of the stated value, more typically $\pm 4\%$ of the stated value, more typically $\pm 3\%$ of the stated value, more typically, $\pm 2\%$ of the stated value, even more typically $\pm 1\%$ of the stated value, and even more typically $\pm 0.5\%$ of the stated value.

[0089] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0090] As used herein the term "consisting essentially of" refers to those elements required for a given example. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that example of the invention.

[0091] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that given example.

[0092] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0093] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

EXAMPLE 1 INVESTIGATION INTO THE ANTIMICROBIAL PROPERTIES OF PEPTIDES OF THE PRESENT DISCLOSURE (L-PEPTIDES)

Materials

[0094] Peptides used in this study were synthesized by GL Biochem (Shanghai, China) and purified to more than 95% using analytical reverse phase (RP)-HPLC. The molecular weights of the peptides were further confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS, Model Autoflex II, Bruker Daltonics Inc., U.S.A.) using α -cyano-4-hydroxycinnamic acid (4-HCCA) as matrix. 4-HCCA was purchased from Sigma-Aldrich (Singapore) and used in saturated acetonitrile/water (1:1 volume ratio) after re-crystallization. Phosphate-buffered saline (10 $\times$ PBS) was purchased from 1st Base (Singapore) and diluted to the intended concentration before use. Mueller-Hinton Broth II (MHB II) and yeast mould broth (YMB) powders were purchased from BD Diagnostics (Singapore) and re-constituted according to the manufacturer's instructions. *Staphylococcus epidermidis* (ATCC No. 12228), *Staphylococcus aureus* (ATCC No. 29737), *Escherichia coli* (ATCC No. 25922), *Pseudomonas aeruginosa* (ATCC No. 9027) and yeast

Candida albicans (ATCC No. 10231) were obtained from ATCC (U.S.A) and cultivated according to the recommended protocols. Lipopolysaccharide (LPS) and FITC-conjugated LPS from *E. coli* 0111:B4 were purchased from Sigma-Aldrich. Griess reagent system was obtained from Promega (U.S.A.) and used according to the manufacturer's protocol.

5

Circular Dichroism (CD) Spectroscopy

[0095] Each peptide was first dissolved at 0.5 mg mL⁻¹ in deionized (DI) water alone or DI water containing 25 mM of SDS surfactant. The CD spectra were recorded at room temperature with a CD spectropolarimeter (JASCO Corp. J-810), using a quartz cell with 1.0 mm path length. CD spectra were acquired with solvent subtraction from 190 to 240 nm at 10 nm/min scanning speed and were averaged from 5 runs per peptide sample. The acquired CD spectra were converted to mean residue ellipticity using the following equation:

$$\theta_M = \frac{\theta_{obs}}{10} \cdot \frac{M_{RW}}{c \cdot l}$$

[0096] where θ_M refers to the mean residue ellipticity (deg·cm²·dmol⁻¹), θ_{obs} is the observed ellipticity corrected for DI water at a given wavelength (mdeg), M_{RW} is the residue molecular weight (M_w /number of amino acid residues), c is the peptide concentration (mg·mL⁻¹), and l is the path length (cm).

Haemolysis Testing

[0097] Fresh rat red blood cells were subjected to 25 × dilution with PBS to obtain an approximate 4 % (by volume) suspension for use in this experiment. 300 μL of red blood cell suspension was added to each tube containing equal volume (300 μL) of peptide solutions in PBS. The tubes were incubated at 37 °C for 1 h before subjected to centrifugation at 1000 × g for 5 min. Aliquots (100 μL) of supernatant were transferred to each well of a 96-well plate and analysed for haemoglobin release at 576 nm using a microplate reader (TECAN, Switzerland). Red blood cells suspension incubated with PBS was used as negative control. Absorbance of red blood cells lysed with 0.1 % v/v Triton X-100 was used as the positive control and taken to be 100 % haemolytic. Percentage of haemolysis was calculated using the following formula: Haemolysis (%) = [(O.D._{576 nm} of treated sample – O.D._{576 nm} of negative control)/(O.D._{576 nm} of positive control – O.D._{576 nm} of negative control)] × 100. Data are expressed as mean ± standard deviations of 4 replicates.

Minimal Inhibitory Concentration (MIC) Measurements

[0098] The antimicrobial activities of the polymers were investigated against Gram-positive *S. epidermidis* and *S. aureus*, Gram-negative *E. coli* and *P. aeruginosa*, and yeast *C. albicans* using the broth microdilution method. Bacterial cells were cultivated in MHBII at 37 °C and yeast cells were grown in YMB at room temperature under constant shaking at 300 rpm to reach mid-logarithmic growth phase. The microbial suspensions were diluted with the appropriate broths and adjusted to give an initial optical density (O.D.) reading of approximately 0.07 at a wavelength of 600 nm on a microplate reader (TECAN, Switzerland), which corresponds to McFarland Standard No. 1 (approximately 3×10^8 CFU mL⁻¹). Peptides were dissolved in 0.2 µm filtered HPLC grade water and subjected to a series of two-fold dilutions using the appropriate broths. 100 µL of microorganism suspension with an initial loading level of 3×10^5 CFU mL⁻¹ was added to an equal volume (100 µL) of peptide solution to achieve final concentrations ranging from 3.9 to 500 mg L⁻¹ and with water concentration fixed at 10% (by volume) in each well of a 96-well plate. After 18 h or 42 h incubation with shaking at 37 °C or room temperature for bacteria and yeast, respectively, the MIC was taken as the lowest polymer concentration at which no microbial growth was observed visually and there was no change in O.D. readings from 0 h. Microbial cells in broth containing 10% (by volume) of HPLC grade water as well as pure broth alone were used as the negative controls. To ascertain aseptic handling, wells containing pure broth without microbes were included in each experiment. Each test was performed in 6 replicates on at least 2 independent occasions.

Determination of Killing Efficiency

[0099] After 18 h treatment of microorganisms with various concentrations of peptides at 0.5 × MIC, MIC and 2 × MIC, the respective samples were subjected to a series of ten-fold dilutions and plated onto LB agar plates. The plates were then incubated overnight and counted for colony-forming units. A sample containing microbes treated with 10% by volume water was used as a negative control. Results are expressed as Log(CFU/mL) and as % Kill = [(cell count of control – survivor count of polymer-treated microbes)/cell count of control] × 100.

Field Emission-scanning Electron Microscopy (FE-SEM) Imaging

[00100] *E. coli* suspension at $\sim 3 \times 10^8$ CFU ml⁻¹ (100 μ L) was incubated with an equal volume of broth containing 20% (by volume) of water and a lethal dose (250 mg L⁻¹) of a representative peptide in a 96 well-plate for 2 h. Eight replicates of each condition was
5 pooled into a microfuge tube, pelleted down at $5000 \times g$ for 5 min, and rinsed twice with PBS. The samples were then fixed with 4% formaldehyde at room temperature for 15 min, followed by rinsing with DI water. Dehydration of the cells was performed using a series of graded ethanol solutions (35, 50, 75, 90, 95 and 100%). The samples were allowed to air-dry, mounted on carbon table, and sputter coated with platinum for imaging with a FE-SEM setup
10 (JEOL JSM-7400F, Japan).

Biofilm growth inhibition and biomass assay

[00101] Overnight cultures of *S. aureus* diluted to 3×10^6 CFU ml⁻¹ were added into each well of a 96-well plate at a volume of 100 μ L per well and allowed to adhere overnight
15 at 37 °C with gentle shaking at 100 rpm. The wells were then rinsed once with 100 μ L of PBS to remove planktonic cells and loosely attached cells, and replenished with 100 μ L of fresh broth. Biofilm formation was allowed to proceed up to 6-8 days with daily rinsing and media changes before use in experiments. To determine the effects of peptide treatment on viability of *S. aureus* in biofilms, 100 μ L of (IRIK)₂-NH₂ (SEQ ID NO: 17) and (IRVK)₃-NH₂ (SEQ
20 ID NO: 22) solutions at MIC, 4 $\times$ MIC and 8 $\times$ MIC levels were added into each well and allowed to incubate for 24 h. Subsequently, the solutions were removed and 120 μ L of activated XTT solution was added into each well. After 4 h incubation, 100 μ L aliquots from each well was transferred to a new 96-well plate for the determination of absorbance using a microplate reader (TECAN, Switzerland) at measurement and reference wavelengths of 490
25 nm and 660 nm, respectively. Relative cell viability was expressed as $[(A_{490}-A_{660})_{\text{sample}}/(A_{490}-A_{660})_{\text{control}}] \times 100\%$. Data are expressed as mean $\pm$ standard deviations of four replicates per concentration.

[00102] The biomass of the biofilms was estimated by a crystal violet staining assay. Briefly, the formed biofilms were first treated with the peptides for 24 h as described above.
30 After aspirating the culture medium, the biofilms were washed once with PBS, fixed with methanol for 15 min at room temperature and stained with 100 μ L of 0.1% (weight by volume) crystal violet for 10 min. The excess crystal violet dye was removed by rinsing the

wells with DI water for five times. The dye that is associated with the biofilm was extracted using 100 μL of 33% glacial acetic acid per well and quantified by measuring the absorbance at a wavelength of 570 nm using a microplate reader (TECAN, Switzerland). The relative amount of biomass remaining after peptide treatment was expressed as a percentage of the control treated with broth containing 10% (by volume) of water. Data represent mean $\pm$ standard deviations of four replicates per concentration.

FITC-LPS Binding Assay

[00103] 50 μL of 1 $\mu\text{g mL}^{-1}$ FITC-LPS in PBS was treated with an equal volume of peptide solution (50 μL) in each well of a black 96-well plate with clear bottom. The interactions of the peptides with the FITC-conjugated LPS were studied by exciting the FITC-LPS at 480 nm and monitoring the emission of FITC at 516 nm in the presence of increasing concentrations of peptides (3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 mg L^{-1}) using a fluorescence microplate reader (TECAN, Switzerland) at 0 h and 2 h. 100 μL of PBS containing 10% by volume of water was included as a blank. Fluorescence intensity of 100 μL FITC-LPS (0.5 $\mu\text{g mL}^{-1}$) in PBS was used as a negative control. The percentage change in fluorescence intensity was calculated as follows: $\% \Delta F(\text{AU}) = [(F_{\text{Sample}} - F_{\text{Blank}}) / (F_{\text{Control}} - F_{\text{Blank}})] \times 100] - 100$. Results are expressed as mean $\pm$ standard deviations of 4 replicates.

Cell Culture

[00104] The rat macrophage cell line NR8383 was maintained in FK15 growth medium supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin and cultured at 37 $^{\circ}\text{C}$ under an atmosphere of 5% CO_2 and 95% humidified air.

Endotoxin Neutralization Assay

[00105] NR8383 cells were plated at a density of 4×10^4 and stimulated with LPS (100 ng mL^{-1}) from *E. coli* 0111:B4 in the presence (3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 mg L^{-1}) or absence of peptides in each well of a 96-well plate for 18 h at 37 $^{\circ}\text{C}$. Untreated cells and cells that were stimulated with LPS alone served as the positive and negative controls, respectively. The amount of NO produced was estimated by quantifying the concentration of the stable NO metabolite nitrite in the isolated supernatant fractions using the Griess reagent (1% sulphanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride, 5% phosphoric

acid) according to the manufacturer's protocol. The absorbance was measured at 540 nm and nitrite concentrations were determined using a standard reference curve prepared from known concentrations of NaNO₂ solutions.

5 Cytotoxicity Testing

[00106] The rat macrophage cell line NR8383 was seeded at a density of 4×10^4 cells per well of a 96-well plate and treated with increasing concentrations of peptides (3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 mg L⁻¹) for 18 h at 37 °C. Subsequently, 20 µL of CellTiter-Blue[®] reagent was added into each well and the plate was incubated for a further 4 h. The fluorescence intensity readings of the wells were determined at excitation wavelength of 560 nm and emission wavelength of 590 nm using a microplate reader. Control wells containing peptide solutions in the absence of cells were included to determine background fluorescence. % Cell viability = $[(F_{\text{treated sample}} - F_{\text{corresponding background}})/(F_{10\% \text{ water control}} - F_{10\% \text{ water control background}})] \times 100$. Data are expressed as mean $\pm$ standard deviations of 4 replicates.

Results

[00107] In this study, short amphiphilic peptides containing 8 or 12 amino acid residues were designed by segregating the cationic lysine and/or arginine amino acids in the polar face and various hydrophobic amino acids in the opposite non-polar face. The molecular weights of the synthesized peptides were verified by MALDI-TOF mass spectrometry and the observed molecular weights are listed in Table 1. It can be seen that the experimentally determined molecular weights were in close agreement with the calculated masses, indicating that the products correspond to the designed sequences.

[00108] **Table 1.** Synthetic β -sheet forming peptide designs and molecular weight characterizations

No. of repeat units (n)	Repeat unit	Peptide sequence	SEQ ID NO:	Theoretical M _w	Observed M _w ^a
2	VRVK	VRVKVRVK-NH ₂	11	982.28	984.22
	IRIR	IRIRIRIR-NH ₂	12	1094.42	1096.77
	IKIK	IKIKIKIK-NH ₂	13	982.37	984.00
	IRIK	IRIKIRIK-NH ₂	17	1038.39	1040.16

3	IRVK	IRVKIRVK-NH ₂	14	1010.34	1012.06
	FRFK	FRFKFRFK-NH ₂	15	1174.46	1176.46
	WRWK	WRWKWRWK-NH ₂	16	1330.61	1333.59
	VRVK	VRVKVRVKVRVK-NH ₂	20	1464.91	1467.16
	IRIK	IRIKIRIKIRIK-NH ₂	21	1549.07	1551.43
	IRVK	IRVKIRVKIRVK-NH ₂	22	1506.99	1510.43

[a] Measured by MALDI-TOF MS, apparent $M_w = [M_w + H]^+$

- [00109] In deionized water, each peptide adopted a random coil structure that is characterized by a minimum at ~195 nm in aqueous solutions due to intermolecular electrostatic repulsion between the protonated lysine and/or arginine residues. Under microbial membrane-mimicking hydrophobic environments (using 25 mM SDS micelles solution), however, the synthetic peptides readily self-assembled into β -sheet secondary structures with characteristic CD spectra showing a maximum at ~200 nm and minimum at ~218 nm (Figure 2).
- [00110] The antimicrobial activities of the synthetic peptides were tested against a representative set of clinically relevant microorganisms including Gram-positive *S. epidermidis* and *S. aureus*, Gram-negative *E. coli* and *P. aeruginosa*, and yeast *C. albicans*. As shown in Table 2, the designed peptides displayed broad spectrum antimicrobial activities against the panel of microorganisms tested with geometric mean (GM) minimum inhibitory concentrations (MICs) ranging from 13.3 to 162.7 mg L⁻¹. Overall, the peptide (IRIR)₂-NH₂ (SEQ ID NO: 12) for which all the cationic residues were Arg exhibited the best antimicrobial activities, with the lowest GM MIC value of 13.3 mg L⁻¹. This was followed closely by (IRIK)₂-NH₂ (SEQ ID NO: 17) and (IKIK)₂-NH₂ (SEQ ID NO: 13), with GM MIC values of 23.4 and 38.3 mg L⁻¹, respectively. As can be seen from these results, the peptide with 2 Arg and 2 Lys residues gave rise to antimicrobial effects that were in between its 4 Arg or 4 Lys counterparts. Non-polar amino acids, which are reported to possess high β -sheet forming propensities, were systematically varied based on increasing degree of hydrophobicity and bulkiness while retaining the combination of 2 Arg and 2 Lys cationic residues (Table 1). Among the peptides incorporating Val, Ile, Phe and Trp, (IRIK)₂-NH₂ (SEQ ID NO: 17) demonstrated the most effective antimicrobial effect against the panel of five microorganisms tested. The replacement of the second hydrophobic Ile residue in the

peptide repeat unit with Val to give (IRVK)₂-NH₂ (SEQ ID NO: 14) resulted in a slight decrease in antimicrobial activities. This result strongly suggests that the Ile residues were essential for the strong antimicrobial effects observed. The antimicrobial effects of the peptides with n = 3 repeat units were investigated with sequences containing Val and Ile.

5 From Table 2, (VRVK)₃-NH₂ (SEQ ID NO: 20) demonstrated the best overall antimicrobial effects, followed by (IRVK)₃-NH₂ (SEQ ID NO: 22) and (IRIK)₃-NH₂ (SEQ ID NO: 21). In comparison to the clinically used lipopeptide polymyxin B, several of the β -sheet forming peptides, including (IRIR)₂-NH₂ (SEQ ID NO: 12), (IKIK)₂-NH₂ (SEQ ID NO: 13), (IRIK)₂-NH₂ (SEQ ID NO: 17) and (VRVK)₃-NH₂ (SEQ ID NO: 20) were found to have a wider

10 spectrum of antimicrobial activities as seen from their lower GM MIC values (13.3 - 34.6 vs. 41.4).

[00111] **Table 2.** Minimum inhibitory concentrations (MICs) and therapeutic indices of synthetic antimicrobial peptides

SEQ ID NO:	Antimicrobial peptide	MIC (mg L ⁻¹)					GM ^a (mg L ⁻¹)	HC ₁₀ ^b (mg L ⁻¹)	SI ^c
		<i>S. epidermidis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>			
11	(VRVK) ₂ -NH ₂	62.5	> 500	250	> 500	15.6	—	> 2500	> 10 ^d
12	(IRIR) ₂ -NH ₂	3.9	31.3	7.8	7.8	15.6	13.3	150	11.3
13	(IKIK) ₂ -NH ₂	2.0	125	31.3	31.3	2.0	38.3	700	18.3
17	(IRIK) ₂ -NH ₂	3.9	62.5	15.6	31.3	3.9	23.4	1050	44.8
14	(IRVK) ₂ -NH ₂	7.8	250	62.5	62.5	7.8	78.1	> 2500	> 32.0
15	(FRFK) ₂ -NH ₂	7.8	250	15.6	62.5	62.5	79.7	1600	20.1
16	(WRWK) ₂ -NH ₂	7.8	62.5	15.6	125	250	92.2	1000	10.8
20	(VRVK) ₃ -NH ₂	1.0	62.5	15.6	62.5	31.3	34.6	> 2500	> 72.3
21	(IRIK) ₃ -NH ₂	1.0	62.5	125	500	125	162.7	500	3.1
22	(IRVK) ₃ -NH ₂	2.0	31.3	15.6	62.5	125	47.3	1250	26.4
	Polymyxin B	15.6	125	2.0	2.0	62.5	41.4	> 2500	> 60.4

15 [a] Geometric mean (GM) of MIC values for the 5 microorganisms tested.

[b] Hemolysis concentration 10% (HC₁₀) is defined as the lowest peptide concentration that induces $\geq 10\%$ hemolysis.

[c] Selectivity index (SI) is calculated as $\frac{HC_{10}}{GM}$.

[d] Calculated as 2500 divided by 250 mg L⁻¹.

[00112] For the β -sheet forming peptides to qualify as suitable therapeutic candidates, their antimicrobial activities should be considered in the light of their selectivity for microbial cell membranes so as to minimize toxicities towards mammalian cells. As shown in Figure 3, the synthetic peptides induced minimal or no haemolysis against rat red blood cells at various MIC values. The selectivity indices (SIs) of the peptides were further evaluated as a comparison of the safety and efficacies of the different peptide sequences (Table 2). SIs of the various peptides were calculated as the ratio of the HC₁₀ value (defined as the lowest peptide concentration that induces 10% or more haemolysis) to the GM (geometric mean MICs of the 5 microbial strains tested). With the exception of (IRIK)₃-NH₂ (SEQ ID NO: 21), all of the peptides tested were found to have high SIs of greater than 10, indicating that they are highly attractive candidates suitable for both external and systemic applications in the body. In particular, the present disclosure showed the combination of both Arg and Lys residues in (IRIK)₂-NH₂ (SEQ ID NO: 17) led to marked improvements in the SI from 11.3 and 18.3 of the single cationic amino acid peptide sequences (IRIR)₂-NH₂ (SEQ ID NO: 12) and (IKIK)₂-NH₂ (SEQ ID NO: 13), respectively to a value of 44.8. The acute *in vivo* toxicity testing was performed via intravenous tail vein injection of solutions containing representative peptides of n = 2 [(IRIK)₂-NH₂ (SEQ ID NO: 17)] and n = 3 [(IRVK)₃-NH₂ (SEQ ID NO: 22)] repeat units in mice. The former was selected due to its superior SI among peptides with n = 2 repeat units, while the latter was chosen due to its better antimicrobial activities, selectivities and endotoxin neutralization properties (to be discussed later) among peptides with n = 3 repeat units. (IRIK)₂-NH₂ (SEQ ID NO: 17) and (IRVK)₃-NH₂ (SEQ ID NO: 22) were respectively found to have lethal dose, 50% (LD₅₀; dose required to kill 50% of mice after a specific test period) values of 35.2 mg/kg. The LD₅₀ values of the designed peptides contrast favorably with that reported for polymyxin B (5.4 mg/kg) and gramicidin (1.5 mg/kg).

[00113] To elucidate the antimicrobial mechanism, colony counting experiments were performed after treating the panel of microorganisms with different concentrations of (IRIK)₂-NH₂ (SEQ ID NO: 17). For each of the five microorganisms tested, the peptide achieved killing efficiencies of close to 100% at the respective MIC values, hence supporting a bactericidal mechanism (Figure 4). The surface morphology of *E. coli* after treatment with (IRIK)₂-NH₂ (SEQ ID NO: 17) and (IRVK)₃-NH₂ (SEQ ID NO: 22) was investigated under field-emission scanning electron microscopy (FE-SEM). As shown in Figure 5, the surface of *E. coli* and *S. aureus* treated with the peptide appeared rough and uneven as compared to the

relatively smooth surface of the control sample treated with broth containing 10% (by volume) of water. This observation is consistent with the membrane lytic mechanism of the various naturally occurring and synthetic AMPs reported in the literature. Compared to conventional antibiotics that inhibit various targets within the biosynthetic pathways of microorganisms, the physical disruption of microbial cell membranes by the peptides is expected to provide an inherent advantage in the clinical setting due to the reduced likelihood for the development of mutations that confer multidrug resistance.

Anti-biofilm ability

[00114] The anti-biofilm ability of the synthetic antimicrobial peptides on pre-formed biofilms, which is intrinsically more challenging than preventing biofilm formation, was investigated next. As shown in Figure 6, peptides (IRIK)₂-NH₂ (SEQ ID NO: 17) and (IRVK)₃-NH₂ (SEQ ID NO: 22) demonstrated a dose-dependent killing of the *S. aureus* residing in biofilms. (IRIK)₂-NH₂ was found to induce significantly higher levels of killing across the three concentrations tested ($P < 0.01$), with cell viability drastically reduced to less than 10% at $4 \times \text{MIC}$ level within 24 h. To determine the relative amounts of biomass remaining after treatment with the peptides, crystal violet staining and solubilisation of the biofilms were performed. Consistent with the decrease in *S. aureus* cell viability, the amount of biomass in the treated biofilms was observed to be reduced in a dose-dependent manner (Figure 7). These results, taken together, provide direct evidence that the 8 and 12 amino acids long peptides are highly effective at killing microbes within biofilms and could efficiently mediate the dispersion of biofilm matrices.

LPS and endotoxin neutralisation

[00115] In order to evaluate the ability of the synthetic peptides to bind and dissociate LPS aggregates, FITC-conjugated LPS was conjugated with the various peptides and monitored the changes in fluorescent intensities over 2 h. In aqueous solutions, FITC sequestered within LPS aggregates exhibit self-quenching, resulting in low fluorescent intensities. Conversely, when FITC-LPS aggregates dissociate, the fluorescence increases due to a dequenching effect. As seen in Figure 8a, beta-sheet forming peptides with $n = 2$ did not seem to induce discernible disruption of FITC-LPS aggregates as evident from the lack of changes in fluorescent intensity up to peptide concentrations of 500 mg L^{-1} . The

corresponding peptides with 3 repeat units, however, produced a strong dose-dependent enhancement of the fluorescent intensities of FITC-LPS (Figure 8b). Among the three peptide sequences, (IRIK)₃-NH₂ (SEQ ID NO: 21) and (IRVK)₃-NH₂ (SEQ ID NO: 22) induced a greater percentage change in fluorescent intensities at lower concentrations, which was followed lastly by (VRVK)₃-NH₂ (SEQ ID NO: 20).

[00116] The endotoxin neutralizing capabilities of the synthetic peptides was established by estimating the amount of pro-inflammatory nitrogen oxide released via the quantification of the stable NO metabolite nitrite concentration present in the cell culture media after co-incubation of the rat macrophage cell line NR8383 with 100 ng mL⁻¹ LPS and the various peptides. The peptides were found to effectively inhibit LPS-stimulated NO production, with significantly decreased nitrite concentrations compared to the non-peptide treated control even at low peptide concentrations of 15.6 mg L⁻¹ (Figure 9). The degree of LPS neutralization mediated by the various peptides was in the order of (IRIK)₃-NH₂ (SEQ ID NO: 21) > (IRVK)₃-NH₂ (SEQ ID NO: 22) > (VRVK)₃-NH₂ (SEQ ID NO: 20), which was in close agreement with the trend observed earlier in the FITC-LPS interaction assay. Importantly, at 125 mg L⁻¹ of (IRIK)₃-NH₂ (SEQ ID NO: 21) and (IRVK)₃-NH₂ (SEQ ID NO: 22), the nitrite concentration was reduced to the control level. The cytotoxicities of the peptides was further evaluated against the NR8383 cell line and it was found that the cell viabilities was in excess of 80% up to concentrations of 62.5, 125 and 125 mg L⁻¹ for (VRVK)₃-NH₂ (SEQ ID NO: 20), (IRVK)₃-NH₂ (SEQ ID NO: 22) and (IRIK)₃-NH₂ (SEQ ID NO: 21), respectively (Figure 10). These results confirmed that the good anti-inflammatory properties of the synthetic peptides were independent of their effects on cell viability and that the peptides were not cytotoxic at antimicrobial and anti-inflammatory doses, indicating their suitability for systemic administration. Although (IRIK)₃-NH₂ (SEQ ID NO: 21) possessed the most potent anti-inflammatory activity among the three peptides, its relatively weak antimicrobial activities (GM MIC value = 162.7 mg L⁻¹) and lower selectivity index (SI = 3.1) suggests that the second best anti-inflammatory peptide (IRVK)₃-NH₂ (SEQ ID NO: 22), which has a much lower geometric mean MIC value of 47.3 and higher selectivity index of 26.4 (Table 2), is a more suitable candidate for the safe and efficacious treatment of bloodstream infections.

In the present disclosure, the inventors designed a series of short synthetic β -sheet folding peptides based upon the common occurrence of amphipathic dyad repeats in natural β -sheet

spanning membrane proteins. The designed β -sheet folding peptides demonstrated broad spectrum antimicrobial activities against Gram-positive *S. epidermidis* and *S. aureus*, Gram-negative *E. coli* and *P. aeruginosa* as well as yeast *C. albicans*. Optimal peptide sequences of $n = 2$ and $n = 3$ repeat units, namely (IRIK)₂-NH₂ (SEQ ID NO: 17) and (IRVK)₃-NH₂ (SEQ ID NO: 22), were found to possess high selectivity indices of 44.8 and 26.4, respectively. Acute *in vivo* toxicity testing in mice revealed higher intravenous LD50 values for the optimal synthetic peptides as compared to the clinically used polymyxin B and gramicidin. Further to the highly efficient killing of planktonic microbes, the synthetic peptides were also demonstrated to be potent inhibitors of bacterial growth in biofilms. Additionally, treatment of the biofilms with the various peptides resulted in a drastic decrease in biomass, indicating that the peptides could effectively translocate through and cause dispersion of the biofilm matrix. Additionally, synthetic peptides with 3 repeat units, including (VRVK)₃-NH₂ (SEQ ID NO: 20), (IRVK)₃-NH₂ (SEQ ID NO: 22) and (IRIK)₃-NH₂ (SEQ ID NO: 21), demonstrated endotoxin binding and neutralizing capabilities with minimal or no cytotoxicities induced at the concentrations required for functional effects. Taken together, our findings clearly demonstrated that the rationally designed synthetic β -sheet folding peptides are highly selective and have potential for use as broad spectrum antimicrobial agents to overcome the prevalent problem of multidrug resistance in a wide range of bacterial- or fungal-based infectious disease related applications including, but not limited to, the prevention and eradication of therapeutically challenging biofilms on open wounds, catheters or implants and the neutralization of microbial endotoxins for improved treatment of bloodstream infections.

EXAMPLE 2 INVESTIGATION INTO THE ANTIMICROBIAL PROPERTIES OF PEPTIDES OF THE PRESENT DISCLOSURE (D-PEPTIDES)

Materials

[00117] Peptides used in this study were synthesized by GL Biochem (Shanghai, China) and purified to more than 95% using analytical reverse phase (RP)-HPLC. The molecular weights of the peptides were further confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS, Model Autoflex II, Bruker Daltonics Inc., U.S.A.) using α -cyano-4-hydroxycinnamic acid (4-HCCA) as matrix. 4-HCCA was purchased from Sigma-Aldrich (Singapore) and used in saturated

acetonitrile/water (1:1 volume ratio) after re-crystallization. Phosphate-buffered saline (10 × PBS) was purchased from 1st Base (Singapore) and diluted to the intended concentration before use. Cation-adjusted Mueller-Hinton broth II (MHB II) and yeast mould broth (YMB) powders were purchased from BD Diagnostics (Singapore) and reconstituted according to the manufacturer's instructions. *Staphylococcus epidermidis* (ATCC No. 12228), *Staphylococcus aureus* (ATCC No. 6538), *Escherichia coli* (ATCC No. 25922), *Pseudomonas aeruginosa* (ATCC No. 9027) and yeast *Candida albicans* (ATCC No. 10231) were obtained from ATCC (U.S.A.) and cultured according to the recommended protocols. Ciprofloxacin, gentamicin sulfate and penicillin G were obtained from Sigma-Aldrich.

Circular dichroism (CD) Spectroscopy

[00118] Each peptide was first dissolved at 0.5 mg mL⁻¹ in deionized (DI) water alone or DI water containing 25 mM of SDS surfactant. The CD spectra were recorded at room temperature with a CD spectropolarimeter (JASCO Corp. J-810), using a quartz cell with 1.0 mm path length. CD spectra were acquired with solvent subtraction from 190 to 240 nm at 10 nm min⁻¹ scanning speed and were averaged from 5 runs per peptide sample. The acquired CD spectra were converted to mean residue ellipticity using the following equation:

$$\theta_M = \frac{\theta_{obs}}{10} \cdot \frac{MRW}{c \cdot l}$$

where θ_M refers to the mean residue ellipticity (deg cm² dmol⁻¹), θ_{obs} is the observed ellipticity corrected for DI water at a given wavelength (mdeg), MRW is the residue molecular weight (Mw · number of amino acid residues⁻¹), c is the peptide concentration (mg mL⁻¹), and l is the path length (cm).

Minimal inhibitory concentration (MIC) measurements

[00119] The antimicrobial activities of the β -sheet forming peptides were investigated against *S. epidermidis* and *S. aureus* (Gram-positive), *E. coli* and *P. aeruginosa* (Gram-negative), and *C. albicans* (yeast) using the broth microdilution method. Prior to the experiment, bacteria cells were cultivated in MHB II at 37 °C and yeast cells were grown in YMB at room temperature under constant shaking at 300 rpm overnight to reach mid-logarithmic growth phase. The microbial suspensions were diluted with the appropriate broths and adjusted to give an initial optical density (O.D.) reading of approximately 0.07 at a

wavelength of 600 nm on a microplate reader (TECAN, Switzerland). The O.D. reading corresponds to McFarland Standard No. 1 (approximately 3×10^8 CFU mL⁻¹). The peptides were dissolved in HPLC grade water and subjected to a series of two-fold dilutions using the appropriate broths. Subsequently, 100 µL of microorganism suspension with an initial loading
5 level of 3×10^5 CFU mL⁻¹ was added to an equal volume (100 µL) of polymer solutions to achieve final polymer concentrations ranging from 3.9-500 mg L⁻¹ and with water content fixed at 10% (by volume) in each well of a 96-well plate. After 18 h incubation with shaking at 37 °C or room temperature, the MIC was taken as the lowest polymer concentration at which no microbial growth was observed visually and with no change in O.D. readings from
10 0 h. Microbial cells in broth containing 10% (by volume) water as well as pure broth alone were used as the negative controls. To ensure aseptic handling, wells containing pure broth without microbes were included in each experiment. Each test was performed in 6 replicates on at least 2 independent occasions.

15 Killing efficiency testing

[00120] After 18 h treatment of microorganisms with various concentrations of peptide ($0.5 \times \text{MIC}$, MIC and $2 \times \text{MIC}$), the respective samples were subjected to a series of ten-fold dilutions and plated onto LB agar plates. The plates were then incubated overnight and counted for colony-forming units. A sample containing microbes treated with 10% (by
20 volume) water was used as a control. Results are expressed as Log(CFU/mL) and as % Kill = [(cell count of control – survivor count of peptide-treated microbes)/cell count of control] × 100.

Field emission-scanning electron (FE-SEM) microscopy analysis

25 [00121] *S. aureus* and *P. aeruginosa* suspensions at $\sim 3 \times 10^8$ CFU mL⁻¹ (100 µL) were separately incubated with an equal volume of broth containing final concentrations of 10% (by volume) of HPLC water or 125 mg L⁻¹ of IK8- all D in a 96 well-plate for 2 h. Eight replicates of each condition was pooled into a microfuge tube, pelleted down at 4000 rpm for 5 min, and rinsed twice with PBS. The samples were then fixed with 4% formaldehyde at
30 room temperature for 20 min, followed by rinsing with deionized water. Dehydration of the cells was performed using a series of graded ethanol solutions (35, 50, 75, 90, 95 and 100%).

The samples were mounted on a copper tape, allowed to air-dry and sputter coated with platinum for imaging using a FE-SEM setup (JEOL JSM-7400F, Japan).

Haemolytic activity testing

- 5 [00122] Fresh rabbit red blood cells were subjected to $25 \times$ dilution with RPMI1640 to obtain an approximate 4 % (by volume) suspension for use in this experiment. 300 μ L of red blood cells suspension was added to each tube containing equal volume (300 μ L) of peptide solutions in RPMI1640. The tubes were incubated at 37 °C for 1 h before subjected to centrifugation at $1000 \times g$ for 5 min. Aliquots (100 μ L) of supernatant were transferred to
- 10 each well of a 96-well plate and analysed for haemoglobin release at 576 nm using a microplate reader (TECAN, Switzerland). Red blood cells suspension incubated with RPMI1640 was used as negative control. Absorbance of red blood cells lyzed with 0.1 % v/v Triton X-100 was used as the positive control and taken to be 100 % haemolytic. Percentage of haemolysis was calculated using the following formula: Haemolysis (%) = [(O.D.576 nm of treated sample - O.D.576 nm of negative control)/(O.D.576 nm of positive control - O.D.576 nm of negative control)] $\times$ 100. Data are expressed as mean $\pm$ standard deviations of 4 replicates.

Drug resistance study and antimicrobial activities against drug resistant bacteria

- 20 [00123] *E. coli* and *S. aureus* (initial loading level of 3×10^5 CFU mL⁻¹) were treated repeatedly for up to 20 passages with various concentrations of the peptide IK8-all D as well as the clinically used ciprofloxacin, gentamicin and penicillin G antibiotics according to the broth microdilution method described earlier. After the end of 18 h incubation at each passage, bacterial cells (at $\frac{1}{4}$ MIC of the particular passage) were sub-cultured and allowed to
- 25 grow to reach mid-logarithmic growth phase before being used in the subsequent MIC testing. By recording the changes in MIC with each passage, i.e. MIC at passage n normalized to that at the initial passage (MIC_n/MIC_0), drug resistance development can be monitored. At the end of this assay, the ciprofloxacin and gentamicin resistant *E. coli* cultures developed were treated with various concentrations of IK8-all D to determine if it could
- 30 effectively overcome antibiotics drug resistance.

Cell culture

[00124] Mouse alveolar macrophage cell line RAW264.7 and human foetal lung fibroblast WI-38 cells were respectively maintained in DMEM and RPMI media, supplemented with 2 mM L-glutamine, 1.5 g L⁻¹ sodium bicarbonate and 10% FBS, and cultured at 37 °C under an atmosphere of 5% CO₂ and 95% humidified air.

5

Intracellular bacteria killing

[00125] RAW264.7 cells were seeded at a density of 4×10^5 per well of a 12-well plate. Following an overnight incubation, the cells were infected for 1 h with 13.3 µL of processed bacteria (3.0×10^8 CFU mL⁻¹) to achieve a 10:1 multiplicity of infection. The cells
10 were then rinsed twice with 1 × PBS and incubated with 1 mL of fresh media containing 50 µg mL⁻¹ gentamicin for 45 min to eliminate extracellular bacteria. The media in each well were removed and the infected cells were incubated with fresh media containing 10% (by volume) water or various concentrations of IK8-all D (2, 3.9, 7.8, 15.6 and 31.3 mg L⁻¹) for up to 4 and 8 h. At the respective time points, the infected cells were trypsinized, rinsed twice
15 with PBS and lysed with 800 µL of sterile water with incubation at room temperature for 10 min, followed by 5 min sonication. The intracellular bacteria count was determined by plating serially diluted cultures onto LB agar and enumerated after 24 h.

Cytotoxicity testing

20 [00126] RAW264.7 and WI-38 cells were respectively seeded at a density of 1.5×10^4 and 1×10^4 per well of a 96-well plate. Following overnight incubation, the cells were treated with 1.0 to 125 mg L⁻¹ of IK8-all D for 48 h. Subsequently, the incubation media in each well were replaced with 100 µL of growth media and 10 µL of MTT solution (5 mg·mL⁻¹ in PBS) and the cells were incubated for 4 h at 37°C according to the manufacturer's directions.
25 Resultant formazan crystals formed in each well were solubilized using 150 µL of DMSO upon removal of growth media. A 100 µL aliquot from each well was then transferred to a new 96-well plate for determination of absorbance using a microplate spectrophotometer at wavelengths of 550 nm and 690 nm. Relative cell viability was expressed as $[(A_{550} - A_{690})_{\text{sample}} / (A_{550} - A_{690})_{\text{control}}] \times 100\%$. Data are expressed as mean ± standard deviations from
30 two independent experiments performed in four replicates per concentration.

Results

Peptide Design and Characterization

[00127] Example 1 above described the design of short synthetic β -sheet forming peptides composed of naturally occurring L-amino acids and demonstrated their broad spectrum and highly selective antimicrobial activities against clinically relevant microorganisms. In this study, substitutions with D-amino acids were performed using optimal β -sheet folding peptides (IRIK)₂-NH₂ (SEQ ID NO: 17) (IK8-all L) and (IRVK)₃-NH₂ (SEQ ID NO: 22) (IK12-all L) of n = 1.5, 2 or 3 repeat units respectively, which were previously optimized for antimicrobial activities, selectivities and endotoxin neutralization properties. All the designed peptides were amidated at the C-terminal to confer a high net positive charge for enhanced antimicrobial activities. As shown in Table 3, the observed molecular weights of the synthesized peptide sequences determined using MALDI-TOF mass spectrometry were in close agreement with theoretical values, indicating that the products agree closely with the designed compositions.

15 **Table 3.** Design and characterization of β -sheet forming peptides.

Peptide designation	Sequence ^[a]	SEQ ID NO:	Theoretical M _w	Observed M _w ^[b]
IK8-all L	IRIKIRIK-NH ₂	17	1038.39	1039.84
IK8-all D	<u>irikir</u> ik-NH ₂	18	1038.39	1040.28
IK8-4D	<u>Irk</u> <u>Irk</u> -NH ₂	24	1038.39	1039.81
IK8-2D	IRIK <u>Irk</u> -NH ₂	19	1038.39	1039.74
IK6-all D	<u>irikir</u> -NH ₂	10	797.06	798.23
IK12-all L	IRVKIRVKIRVK-NH ₂	22	1506.99	1510.43
IK12-all D	<u>irvkirvkirvk</u> -NH ₂	23	1506.99	1508.26
Control-all L	IIRKIIRK-NH ₂	25	1038.39	1040.07
Control-all D	<u>iirkiirk</u> -NH ₂	26	1038.39	1040.81
Control-4D	<u>Irk</u> <u>Irk</u> -NH ₂	27	1038.39	1040.90

[a] Small underlined residues represent D-amino acids.

[b] Measured by MALDI-TOF MS, apparent M_w = [M_w + H]⁺

[00128] Secondary structure formation of the designed peptides was investigated in microbial membrane-mimicking environments using CD spectroscopy. IK8-all L and IK12-

all L readily self-assembled to form β -sheet secondary structures in 25 mM SDS micelles solution as evident from a characteristic maxima at ~ 200 nm and minima at ~ 218 nm (Figure 11a and 11b). Corresponding enantiomers IK8-all D and IK12-all D were exact mirror images with ellipticities that were approximately equivalent but opposite in sign as compared to that of their L-counterparts. Interestingly, the selective D-amino acid substitution at positions 2, 4, 6, 8 (IK8-4D) in the optimal IK8-all L sequence led to a complete loss of secondary structure, whereas substitutions only at positions 4, 6 (IK8-2D) enabled the retention of β -sheet forming propensity (Figure 11c). As β -sheet formation is governed by intermolecular hydrogen bonding between the side chain functional groups in a given peptide, extensive D-amino acid incorporation in IK8-4D possibly affected the spatial positioning of functional groups within the peptide structure, leading to the loss of interactions between adjacent peptide molecules to disrupt β -sheet formation. The rearrangement of cationic and hydrophobic amino acids within the β -sheet forming sequence IK8-all L to yield control peptides (IIRK)₂-NH₂ (Control-all L; SEQ ID NO: 25), (iirk)₂-NH₂ (Control-all D; SEQ ID NO: 26) and (IirK)₂-NH₂ (Control-4D; SEQ ID NO: 275) (Table 3) led to a loss of secondary structures; with their existence as random coils under the test conditions even though the overall charge and hydrophobicity of the molecules remained unchanged (Figure 11d). This result thus indicates that the alternate hydrophobic and hydrophilic spatial positioning of the amino acids is essential for the formation of β -sheets in membrane conditions. As shown in Figure 18, the designed peptides remained as random coils in deionized water, hence demonstrating the specificity of the respective β -sheet forming peptides for the amphiphilic microbial membrane-mimics.

Antimicrobial Activities and Selectivities

[00129] The antimicrobial activities of the designed peptides were evaluated against a panel of clinically relevant microorganisms, including *S. epidermidis* and *S. aureus* (Gram-positive), *E. coli* and *P. aeruginosa* (Gram-negative) as well as *C. albicans* (yeast).

Table 4. Minimum inhibitory concentrations (MICs) and selectivity indices of synthetic antimicrobial peptides

Antimicrobial peptide	MIC (mg L ⁻¹)					GM ^{a)} (mg L ⁻¹)	HC ₁₀ ^{b)} (mg L ⁻¹)	SI ^{c)}
	S.	S.	E.	P.	C.			

	<i>epidermidis</i>	<i>aureus</i>	<i>coli</i>	<i>aeruginosa</i>	<i>albicans</i>			
IK8-all L	3.9	62.5	15.6	31.3	3.9	23.4	2000	85.5
IK8-all D	2.0	3.9	3.9	7.8	3.9	4.3	1750	407.0
IK8-4D	> 500	> 500	>500	> 500	> 500	—	> 2000	—
IK8-2D	3.9	31.3	15.6	7.8	3.9	12.5	1600	128.0
IK6-all D	—	250	62.5	125	15.6	113.3	—	—
IK12-all L	2.0	31.3	15.6	62.5	125	47.3	> 125 ^{e)}	>> 2.6
IK12-all D	1.0	7.8	7.8	15.6	31.3	12.7	> 125 ^{e)}	>> 9.8
Control-all L	> 500	> 500	>500	> 500	500	—	> 2000	—
Control-all D	> 500	> 500	>500	> 500	250	—	> 2000	—
Control-4D	125	> 500	31.3	125	62.5	85.9 ^{d)}	> 2000	> 23.3

[a] Geometric mean (GM) of MIC values for the 5 microorganisms tested.

[b] Haemolysis concentration 10% (HC₁₀) is defined as the lowest peptide concentration that induces $\geq 10\%$ haemolysis.

5 [c] Selectivity index (SI) is calculated as $\frac{HC_{10}}{GM}$.

[d] GM of MIC values for the 4 microorganisms which the peptide has activity against.

[e] Precipitation occurred in RPMI1640 media from 125 mg L⁻¹.

10 [00130] From Table 4, it can be seen that peptide sequences which assembled into β -sheets under microbial membrane-mimicking conditions demonstrated substantially stronger and broader spectrum of activities against all the microorganisms tested as compared to the non- β -sheet forming peptides regardless of stereochemistry (Table 4). For instance, the geometric mean (GM) minimum inhibitory concentrations (MICs) of the various β -sheet forming peptides ranges from 4.3-113.3 mg L⁻¹, whereas the non- β -sheet forming control peptides and IK8-4D displayed no or marked reduced antimicrobial activities. This observation thus demonstrates that the self-assembly of the designed AMPs to form β -sheet

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secondary structures is crucial for their strong antimicrobial properties. This finding is consistent with literature reports in which the bioactivity of AMPs is frequently associated with the segregation of cationic and hydrophobic amino acid residues on opposite planes upon formation of secondary structures leading to disruption of membrane lipid bilayers.

5 Interestingly, the selective substitution of D-amino acids in the 2nd and 3rd position of the non- β -sheet forming IIRK motif (Control-all L) to obtain (IirK)₂-NH₂ (Control-4D) partially restored antimicrobial activities against *S. epidermidis*, *E. coli*, *P. aeruginosa* and *C. albicans* (Table 4). In particular, the MIC values against *E. coli* were increased by ~ 16-folds from > 500 to 31.3 mg L⁻¹. The antimicrobial activities of this peptide, however, remained lower than

10 the β -sheet forming peptides of equal length, charge and hydrophobicity (i.e. IK8-all L or IK8-all D).

[00131] Additionally, it was also found that the substitution with D-amino acids considerably improved antimicrobial activities in β -sheet forming peptides regardless of the number of repeat units (n = 2 or 3) or peptide length. This effect was especially pronounced

15 with the shorter IK8-all D peptide with n = 2 repeat units for which the MIC values against the various bacteria were 2-16 fold lower than its L-enantiomer. In comparison, IK12-all D mediated 2-4 fold reduction in MIC values with enhanced antifungal activity against *C. albicans* as compared to its corresponding L-enantiomer. Among the various peptides, IK8-all D demonstrated the most potent antimicrobial activities, with a very low geometric mean

20 (GM) MIC value of 4.3 mg L⁻¹ against the various types of microorganisms, which is superior to that obtained for the clinically used lipopeptide antibiotic polymyxin B (41.4 mg L⁻¹) under identical testing conditions. A further reduction in the peptide length to 6 D-amino acids (n = 1.5) led to a reduction in antimicrobial activities with a higher GM MIC value of 113.3 mg L⁻¹, indicating that β -sheet forming peptides with n = 2 repeat units provided the most optimal

25 structure for antimicrobial activities.

[00132] The haemolytic activities of the synthetic peptides were evaluated using 4% (by volume) rabbit blood. As reported previously, the all L-amino acids containing β -sheet forming peptides displayed minimal or no haemolysis at MIC values, with high selectivities for microbial membranes (also shown in Figure 2 and Table 2). In this present study, it was

30 found that the difference in the haemolytic activities between enantiomeric forms of β -sheet forming peptides of n = 2 repeat units was small, with high HC₁₀ values of 2000 and 1750 mg L⁻¹ for IK8-all L and IK8-all D, respectively (Table 4). With the strong improvements in

antimicrobial activities observed earlier, these results clearly demonstrated the superior selectivities of the D-enantiomers toward the anionic microbial membranes over zwitterionic mammalian cell membranes, giving rise to very high SIs of 407.0 and $\gg 9.8 \text{ mg L}^{-1}$ for IK8-all D and IK12-all D, respectively (Table 4). Taken together, these results suggest that the D-enantiomer of the $n = 2$ synthetic β -sheet forming peptide, IK8-all D, with its broad spectrum and highly selective antimicrobial activities may be a strong therapeutic candidate for the treatment of drug resistant microbial infections.

[00133] Consistent with their poor antimicrobial activities, the non- β -sheet forming peptides, Control-all L and Control-all D, induced minimal or no haemolysis up to 2500 mg L^{-1} (Figure 12c), indicating similarly weak interactions with red blood cell membranes. Likewise, the D-amino acid substituted non- β -sheet forming peptide Control-4D, which demonstrated some degree of antimicrobial activities against several microorganisms, also induced minimal haemolysis, hence giving rise to a high selectivity index (SI) of > 23.3 (Table 4). These results suggest that the altered peptide conformation obtained upon substitution with D-amino acids could still permit a moderate degree of interaction with certain microbial membrane compositions without affecting eukaryotic membranes in the absence of secondary structure formation (Figure 11d).

Resistance to Protease Degradation

[00134] One of the major factors limiting the clinical utility of AMPs lies in their instability to rapid degradation by proteases which are present abundantly in biological fluids and/or secreted by microorganisms. In order to evaluate the proteolytic stability of the designed β -sheet forming peptides, the D- and L-isoforms of the $n = 2$ peptide sequence (IK8-all D or IK8-all L, respectively) were treated with the broad spectrum serine proteases, trypsin and proteinase K, for 6 h and evaluated their antimicrobial activities against *S. aureus*, *E. coli* and *P. aeruginosa*. As seen in Figure 13, untreated IK8-all D and IK8-all L effectively inhibited the growth of all three microorganisms after 18 h incubation. Treatment of IK8-all L with the proteases, however, led to a complete loss in bacterial inhibitory activities, suggesting that it has been degraded by the proteases present. IK8-all D, on the other hand, retained its antibacterial activities against the various microorganisms. The structural integrity of synthetic peptides following treatment with both proteases was also evaluated using MALDI-TOF mass spectroscopy. As seen from the MALDI-TOF mass spectra, the treatment

of IK8-all L with both proteases led to the degradation of the intact peptide resulting in multiple lower molecular weight peaks (Figure 19a), hence accounting for the loss of antibacterial activities observed earlier. The cleavage sites of both enzymes for the IK8-all L peptide are illustrated in Figure 19a with the mass spectra peaks assigned to the major fragments. Conversely, IK8-all D remained intact after protease treatment, with no lower molecular weight products observed (Figure 19b). This reduced susceptibility to enzymatic degradation, coupled with the superior antimicrobial activities and selectivities observed earlier strongly suggests that IK8-all D is a promising candidate for therapeutic applications.

10 Membrane-lytic Activities and Drug Resistance Mitigation

[00135] The predominant mechanism of action for AMPs lies in the rapid perturbation and destruction of microbial membranes, leading to the leakage of cytoplasmic contents and eventual cell death. As it is very challenging for microorganisms to repair extensive cell membrane damage, this bactericidal mode of action has been proposed to offer a highly attractive means to prevent and overcome drug resistance mechanisms. The present study first established that similar to its L-enantiomer, IK8-all D mediated a bactericidal mechanism of action, giving rise to more than three log reductions in viable colony counts (> 99.9% killing efficiencies) at the respective MIC values for each of the five microorganisms tested in this study (Figure 14). Under FE-SEM, significant membrane damage and corrugation were observed on the surface of Gram-positive *S. aureus* and Gram-negative *P. aeruginosa* after a short 2 h treatment with 125 mg L⁻¹ of IK8-all D as compared to the smooth surfaces of the respective controls treated with broth containing 10% (by volume) of water (Figure 15).

[00136] Prolonged repeated exposures of microorganisms to non-lethal doses of antibiotics are known to promote acquisition of drug resistance. In order to investigate if the membrane disruption mediated by IK8-all D can adequately prevent drug resistance development, *E. coli* and *S. aureus* treated with sub-MIC amounts of AMP were passaged daily and used for determination of MIC up to 20 passages. Various classes of clinically used antibiotics including the cell wall synthesis inhibitor penicillin G, bacterial topoisomerase inhibitor ciprofloxacin and protein synthesis inhibitor gentamicin sulfate were included as controls in this study. As shown in Figure 16, the MIC values of IK8-all D against *E. coli* and *S. aureus* remained unchanged at 3.9 mg L⁻¹ (MIC_n/MIC₀ = 1) despite repeated treatment with low doses of the designed AMP. Treatment of *E. coli* with the gentamicin sulfate

induced drug resistance as early as passage 2, as seen from the doubling of MIC value ($MIC_n/MIC_0 = 2$), which subsequently increases to 4-fold the original MIC value by passage 6 ($MIC_n/MIC_0 = 4$) (Figure 16a). Ciprofloxacin mediated a slightly delayed onset of drug resistance, with doubling of MIC occurring later at passage 4. This was followed by a quadrupling of MIC value by passage 7, which was followed by a drastic increase in MIC value by 32-fold at the end of the experiment. A similar trend was observed for *S. aureus*, with an early acquisition of drug resistance and an increase in MICs by 32- and 4-fold for penicillin G and ciprofloxacin, respectively by passage 20 (Figure 16b). These results, taken together, strongly suggest that the diverse antimicrobial mechanisms of action mediated by various antibiotics influenced their drug resistance development profiles to different extents. Notably, repeated treatment of *E. coli* and *S. aureus* with the synthetic β -sheet forming peptide did not induce any drug resistance development within the course of the experiment.

[00137] Besides being able to prevent drug resistance development, treatment of the gentamicin- and ciprofloxacin-resistant *E. coli* derived from this experiment with IK8-all D also revealed that the synthetic peptide effectively inhibited bacterial growth at the same concentration (i.e. 3.9 mg L^{-1}) as that in the wild type (non-drug resistant) bacteria (Figure 20). This result provides strong evidence that the membrane disrupting abilities of the β -sheet forming peptide could effectively overcome conventional mechanisms of antibiotics resistance to inhibit bacterial growth. The activities of the designed β -sheet forming peptides were further investigated against clinical isolates of drug resistant microorganisms. As seen in Table 5, the designed AMPs demonstrated broad-spectrum antimicrobial activities against MRSA, VRE, multidrug resistant *A. baumannii*, *P. aeruginosa* and yeast *C. neoformans*. Consistent with the results observed earlier, IK8-all D induced stronger antibacterial activities, with 4-8 folds reduction in MBCs as compared to its L-enantiomer. Additionally, IK8-all D, IK8-2D and IK12-all L also demonstrated excellent activities against clinically isolated *M. tuberculosis*, with MICs ranging from 15.6 to 125 mg L^{-1} (Table 6).

Table 5. Minimum microbicidal concentrations (MBCs) of synthetic antimicrobial peptides against clinically isolated drug resistant microorganisms.

Antimicrobial peptide	MBC (mg L^{-1})				
	Methicillin-resistant	<i>A. baumannii</i>	Vancomycin-resistant	<i>P. aeruginosa</i>	<i>C. neoformans</i>

	<i>S. aureus</i>		<i>Enterococci</i>		
IK8-all L	31.3	125	15.6	7.8	7.8
IK8-all D	3.9	31.3	3.9	15.6	7.8
IK12-all L	15.6	15.6	7.8	15.6	15.6

Table 6. Minimum inhibitory concentrations (MICs) of synthetic antimicrobial peptides against clinically isolated *Mycobacterium tuberculosis*.

Antimicrobial peptide	MIC (mg L ⁻¹)					
	Strain number					
	1140	1146	1173	1177	1178	1179
IK8-all D	15.6	15.6	—	—	—	—
IK8-2D	—	—	62.5	62.5	62.5	125
IK12-all L	15.6	> 31.3	—	—	—	—

5 Intracellular Killing of *S. aureus* in Infected Mouse Macrophages

[00138] The phagocytosis of microorganisms by alveolar macrophages could potentially create a source of persistent infections due to the survival and protection of bacteria from many extracellular antibiotics that penetrate poorly into cells. *S. aureus* is a clinically relevant example of an opportunistic pathogen that can survive within alveolar macrophages and is frequently implicated in community and nosocomial acquired cases of lung infections. As such, the ability of the synthetic AMP to eradicate intracellular *S. aureus* was investigated next. Infected RAW264.7 mouse alveolar macrophages were treated with various doses of IK8-all D for 4 and 8 h. As seen from Figure 17, there was a significant decrease in intracellular *S. aureus* colony counts after treatment with IK8-all D for 4 and 8 h when compared with that obtained for the control treated with media containing 10% (by volume) water ($P < 0.01$). For instance, the designed peptide effectively mediated 1.2-1.5 log reductions in colony counts at 8 h when compared to the control (Figure 17). Importantly, the intracellular killing of *S. aureus* mediated by IK8-all D was found to be independent of the cytotoxicity of the peptide as more than 80% cell viability was observed at bactericidal doses (Figure 21). The cytotoxicity of IK8-all D was also investigated in the human foetal lung fibroblast WI-38 cell line and it was demonstrated more than 77% cell viability up to 62.5 mg L⁻¹, which is well above its antimicrobial concentrations. Taken together, these results

demonstrated that besides its potent activities in extracellular bacterial killing, the designed β -sheet forming peptide could also effectively enter infected cells to reduce intracellular bacteria loads with minimal cytotoxicities.

5 [00139] Example 2 investigated the importance of secondary structure formation and effects of stereochemistry on antimicrobial activities and selectivities. It was demonstrated that the self-assembly of the synthetic peptide into β -sheets under microbial membrane-mimicking conditions was essential for its strong antimicrobial activities. Among the various peptides, IK8-all D exhibited the most potent antimicrobial activities, with a very high
10 selectivity index of 407.0. The D-enantiomer also displayed enhanced stability in the presence of broad spectrum proteases, trypsin and proteinase K. Additionally, membrane-lytic activities of IK8-all D provided an effective means to prevent drug resistance development and effectively mediated killing of various clinically isolated multidrug-resistant microorganisms. Besides its potency against extracellular microorganisms, the synthetic β -
15 sheet forming peptide IK8-all D also demonstrated efficient killing of intracellular *S. aureus*. Taken together, these results show that the D-amino acids substituted β -sheet forming peptide IK8-all D with its enhanced antimicrobial activities and improved protease stability, may be used to combat antibiotics resistance microorganisms in various clinical applications.

20 EXAMPLE 3 INVESTIGATION INTO THE ANTIPROLIFERATIVE PROPERTIES OF PEPTIDES OF THE PRESENT DISCLOSURE

Cell Culture

[00140] The human cervical cancer HeLa cell line, human dermal fibroblast HDF cell
25 line and rat macrophage NR8383 cell line were respectively maintained in RPMI, DMEM and FK15 growth media supplemented with 10% FBS, 100 U mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin and cultured at 37 °C under an atmosphere of 5% CO₂ and 95% humidified air.

30 Cell viability testing

[00141] HeLa, HDF and NR8383 cells were respectively seeded at a densities of 1×10^4 , 1×10^4 and 4×10^4 cells per well of a 96-well plate. Following overnight incubation, the

cells were treated with 3.9 to 500 mg L⁻¹ of (IRIK)₃-NH₂ (SEQ ID NO: 21) and (VRVK)₃-NH₂ (SEQ ID NO: 20) for 24 h. For the adherent HeLa and HDF cell lines, the incubation media in each well were replaced with 100 µL of growth media and 10 µL of MTT solution (5 mg·ml⁻¹ in PBS). The cells were further incubated for 4 h at 37°C according to the manufacturer's directions. Resultant formazan crystals formed in each well were solubilized using 150 µL of DMSO upon removal of growth media. A 100 µL aliquot from each well was then transferred to a new 96-well plate for determination of absorbance using a microplate spectrophotometer at wavelengths of 550 nm and 690 nm. Relative cell viability was expressed as $[(A_{550}-A_{690})_{\text{sample}}/(A_{550}-A_{690})_{\text{control}}] \times 100\%$. Data are expressed as mean $\pm$ standard deviations from two independent experiments performed in four replicates per concentration.

[00142] For the semi-adherent NR8383 cells, 20 µL of CellTitre-Blue[®] reagent was added into each well after treatment and the plate was incubated for a further 4 h. The fluorescence intensity readings of the wells were determined at excitation wavelength of 560 nm and emission wavelength of 590 nm using a microplate reader. Control wells containing peptide solutions in the absence of cells were included to determine background fluorescence.
$$= [(F_{\text{treated sample}} - F_{\text{corresponding background}})/(F_{10\% \text{ water control}} - F_{10\% \text{ water control background}})] \times 100.$$
 Data are expressed as mean $\pm$ standard deviations of 4 replicates.

Results

[00143] As seen from Figure 22, treatment of HeLa cervical cancer cell line with (IRIK)₃-NH₂ (SEQ ID NO: 21) resulted in a concentration-dependent decrease in cell viability to less than 70% from 125 mg/L onwards. On the other hand, more than 80% of the non-cancerous HDF and NR8383 cells were viable at 125 mg/L. Similarly, treatment of HeLa cells with (VRVK)₃-NH₂ (SEQ ID NO: 20) resulted in only 30% cell viability at 250 mg/L while HDF and NR8383 cells had more than 53% cell viabilities. These results thus demonstrate that the β -sheet forming peptides have greater selectivities for the more anionic cancer cell membranes over the normal cell membranes.

Claims

1. An amphiphilic peptide, wherein the peptide comprises: $(X_1Y_1X_2Y_2)_n$ (Formula I),
wherein
the C-terminal end of the peptide is amidated;
5 X_1 and X_2 is independently of each other a hydrophobic amino acid
 Y_1 and Y_2 is independently of each other a cationic amino acid; and
 n is at least 1.5,
wherein the peptide is capable of self-assembly into β -sheet structure.
- 10 2. The peptide of claim 1, wherein n is 2 or 2.5 or 3 or 3.5 or 4 or 4.5 or 5.
3. The peptide of any one of claims 1 to 2, wherein $(X_1Y_1X_2Y_2)_n$ is $(X_1Y_1X_2Y_2)_n\text{-NH}_2$.
4. The peptide of any one of claims 1 to 3, wherein the hydrophobic amino acid is
15 selected from the group consisting of alanine (A), valine (V), isoleucine (I), leucine (L),
methionine (M), phenylalanine (F), tryptophan (W) and cysteine (C).
5. The peptide of claim 4, wherein the hydrophobic amino acid is isoleucine (I), or
valine (V).
- 20 6. The peptide of any one of claims 1 to 5, wherein the cationic amino acid is selected
from the group consisting of lysine (K), arginine (R) and histidine (H).
7. The peptide of any one of claims 1 to 6, wherein each repeating unit n of Formula I
25 comprises independently of each other 1 or 2 or 3 or 4 or 6 or 8 D-amino acids with the
remaining amino acids being L-amino acids.
8. The peptide of any one of claims 1 to 6, wherein each repeating unit n of Formula I
comprises independently of each other 2 or 4 or 6 or 8 D-amino acids with the remaining
30 amino acids being L-amino acids.

9. The peptide of claim 7 or 8, wherein the distribution of D-amino acids in each repeating unit n of Formula I is identical or different from each other.

10. The peptide of any one of claims 7 to 9, wherein n is 2 and amino acids in position 4 and 6 are D-amino acids while the remaining amino acids are L-amino acids.

11. The peptide of any one of claims 1 to 10 comprising the sequence $(IY_1IY_2)_n-NH_2$.

12. The peptide of any one of claims 1 to 10 comprising the sequence $(IRX_2K)_n-NH_2$.

13. The peptide of any one of claims 1 to 12, wherein the peptide is selected from the group consisting of VRVKVRVK-NH₂ (SEQ ID NO: 11), VRVKVRVKVRVK-NH₂ (SEQ ID NO: 20), IRIRIRIR-NH₂ (SEQ ID NO: 12), IKIKIKIK-NH₂ (SEQ ID NO: 13), IRVKIRVK-NH₂ (SEQ ID NO: 14), FRFKFRFK-NH₂ (SEQ ID NO: 15), WRWKWRWK-NH₂ (SEQ ID NO: 16), IRIKIRIK-NH₂ (SEQ ID NO: 17), IRIKIRIKIRIK-NH₂ (SEQ ID NO: 21), irikir-NH₂ (SEQ ID NO: 10), irikirik-NH₂ (SEQ ID NO: 18), IRIkIRIK-NH₂ (SEQ ID NO: 19), IRVKIRVKIRVK-NH₂ (SEQ ID NO: 22) and irvkirvkirvk-NH₂ (SEQ ID NO: 23), wherein small underlined residues represent D-amino acids while capital non-underlined represent L-amino acid.

14. A peptide of any one of claims 1 to 13 for use as a medicament.

15. A method of treating a bacterial infection or removing bacteria comprising administration of a pharmaceutically effective amount of a peptide according to any one of claims 1 to 13.

16. The method of claim 15, wherein the bacteria are Gram-positive or Gram-negative bacteria.

17. The method of claim 15 or 16, wherein the bacteria are selected from the genus selected from the group of *Acetobacter*, *Acinetobacter*, *Actinomyces*, *Agrobacterium* spp., *Azorhizobium*, *Azotobacter*, *Anaplasma* spp., *Bacillus* spp., *Bacteroides* spp., *Bartonella* spp.,

Bordetella spp., *Borrelia*, *Brucella* spp., *Burkholderia* spp., *Calymmatobacterium*,
Campylobacter, *Chlamydia* spp., *Chlamydophila* spp., *Clostridium* spp., *Corynebacterium*
spp., *Coxiella*, *Ehrlichia*, *Enterobacter*, *Enterococcus* spp., *Escherichia*, *Francisella*,
Fusobacterium, *Gardnerella*, *Haemophilus* spp., *Helicobacter*, *Klebsiella*, *Lactobacillus* spp.,
5 *Lactococcus*, *Legionella*, *Listeria*, *Methanobacterium* extroquens, *Microbacterium*
multiforme, *Micrococcus luteus*, *Moraxella catarrhalis*, *Mycobacterium* spp., *Mycoplasma*
spp., *Neisseria* spp., *Pasteurella* spp., *Peptostreptococcus*, *Porphyromonas*, *Pseudomonas*,
Rhizobium, *Rickettsia* spp., *Rochalimaea* spp., *Rothia*, *Salmonella* spp., *Serratia*, *Shigella*,
Staphylococcus spp., *Stenotrophomonas*, *Streptococcus* spp., *Treponema* spp., *Vibrio* spp.,
10 *Wolbachia*, and *Yersinia* spp.

18. The method of claim 17, wherein the bacteria are selected from the group consisting
of *Acetobacter aurantius*, *Acinetobacter baumannii*, *Actinomyces Israelii*, *Agrobacterium*
radiobacter, *Agrobacterium tumefaciens*, *Azorhizobium caulinodans*, *Azotobacter vinelandii*,
15 *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Bacillus anthracis*, *Bacillus brevis*,
Bacillus cereus, *Bacillus fusiformis*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus*
mycoides, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacteroides fragilis*, *Bacteroides*
gingivalis, *Bacteroides melaminogenicus* (*Prevotella melaminogenica*), *Bartonella henselae*,
Bartonella quintana, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Borrelia burgdorferi*,
20 *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Burkholderia mallei*, *Burkholderia*
pseudomallei, *Burkholderia cepacia* complex, *Burkholderia cenocepacia*,
Calymmatobacterium granulomatis, *Campylobacter coli*, *Campylobacter fetus*,
Campylobacter jejuni, *Campylobacter pylori*, *Chlamydia trachomatis*, *Chlamydophila*. (such
as *C. pneumoniae*, *Chlamydophila psittaci*, *Clostridium botulinum*, *Clostridium difficile*,
25 *Clostridium perfringens*, *Clostridium tetani*), *Corynebacterium diphtheriae*,
Corynebacterium fusiforme, *Coxiella burnetii*, *Ehrlichia chaffeensis*, *Enterobacter cloacae*,
Enterococcus avium, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*,
Enterococcus gallinarum, *Enterococcus maloratus*, *Escherichia coli*, *Francisella tularensis*,
Fusobacterium nucleatum, *Gardnerella vaginalis*, *Haemophilus ducreyi*, *Haemophilus*
30 *influenzae*, *Haemophilus parainfluenzae*, *Haemophilus pertussis*, *Haemophilus vaginalis*,
Helicobacter pylori, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Lactobacillus casei*,
Lactococcus lactis, *Legionella pneumophila*, *Listeria monocytogenes*, *Methanobacterium*

extroquens, *Microbacterium multiforme*, *Micrococcus luteus*, *Moraxella catarrhalis*,
Mycobacterium avium, *Mycobacterium bovis*, *Mycobacterium diphtheriae*, *Mycobacterium*
intracellulare, *Mycobacterium leprae*, *Mycobacterium lepraemurium*, *Mycobacterium phlei*,
Mycobacterium smegmatis, *Mycobacterium tuberculosis*, *Mycoplasma fermentans*,
5 *Mycoplasma genitalium*, *Mycoplasma hominis*, *Mycoplasma penetrans*, *Mycoplasma*
pneumoniae, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pasteurella multocida*,
Pasteurella tularensis, *Peptostreptococcus*, *Porphyromonas gingivalis*, *Pseudomonas*
aeruginosa, *Rhizobium*, *Radiobacter*, *Rickettsia prowazekii*, *Rickettsia psittaci*, *Rickettsia*
quintana, *Rickettsia rickettsii*, *Rickettsia trachomae*, *Rochalimaea henselae*, *Rochalimaea*
10 *quintana*, *Rothia dentocariosa*, *Salmonella enteritidis*, *Salmonella typhi*, *Salmonella*
typhimurium, *Serratia marcescens*, *Shigella dysenteriae*, *Staphylococcus aureus*,
Staphylococcus epidermidis, *Stenotrophomonas maltophilia*, *Streptococcus agalactiae*,
Streptococcus avium, *Streptococcus bovis*, *Streptococcus cricetus*, *Streptococcus faecium*,
Streptococcus faecalis, *Streptococcus ferus*, *Streptococcus gallinarum*, *Streptococcus lactis*,
15 *Streptococcus mitior*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*,
Streptococcus pneumoniae, *Streptococcus pyogenes*, *Streptococcus rattus*, *Streptococcus*
salivarius, *Streptococcus sanguis*, *Streptococcus sobrinus*, *Treponema pallidum*, *Treponema*
denticola, *Vibrio cholerae*, *Vibrio comma*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*,
Wolbachia, *Yersinia enterocolitica*, *Yersinia pestis* and *Yersinia pseudotuberculosis*.

20
 19. The method of any one of claims 16 to 18, wherein the bacterial infection is selected
 from the group consisting of pneumonia, tuberculosis, meningitis, diarrhoeal diseases,
 formation of biofilm, sepsis, listeriosis, gastroenteritis, toxic shock syndrome, hemorrhagic
 colitis; hemolytic uremic syndrome, Lyme Disease, gastric and duodenal ulcers, human
 25 ehrlichiosis, pseudomembranous colitis, cholera, salmonellosis, cat scratch fever, necrotizing
 fasciitis (GAS), streptococcal toxic shock syndrome, nosocomial and community associated
 infections, atherosclerosis, sudden infant death syndrome (SIDS), ear infections, respiratory
 tract infections, urinary tract infections, skin and soft tissue infections, nail bed infections,
 wound infection, septicemia, gastrointestinal disease, hospital-acquired endocarditis and
 30 blood stream infections.

20. A method of neutralizing endotoxins comprising administration of a pharmaceutically effective amount of a peptide according to any one of claims 1 to 13.

21. The method of claim 20, wherein the endotoxins are bacterial endotoxins or fungal endotoxins.

22. A method of treating viral based infectious diseases comprising administration of a pharmaceutically effective amount of a peptide according to any one of claims 1 to 13.

23. The method of claim 22, wherein the viral infectious disease is selected from the common cold, influenza, chickenpox, cold sores, Ebola, AIDS, avian influenza, SARS, dengue, herpes, shingles, measles, mumps, rubella, rabies, human papillomavirus, sexually transmitted diseases, viral meningitis, viral hepatitis, coxsackie virus and Epstein Barr virus.

24. A method of treating a fungal infection or infestation, or removing fungus comprising administration of a pharmaceutically effective amount of a peptide according to any one of claims 1 to 13.

25. The method of claim 24, wherein the fungus is selected from the group consisting of the genus of *Absidia*, *Ajellomyces*, *Arthroderma*, *Aspergillus*, *Blastomyces*, *Candida*, *Cladophialophora*, *Coccidioides*, *Cryptococcus*, *Cunninghamella*, *Epidermophyton*, *Exophiala*, *Filobasidiella*, *Fonsecaea*, *Fusarium*, *Geotrichum*, *Histoplasma*, *Hortaea*, *Issatschenkia*, *Madurella*, *Malassezia*, *Microsporum*, *Microsporidia*, *Mucor*, *Nectria*, *Paecilomyces*, *Paracoccidioides*, *Penicillium*, *Pichia*, *Pneumocystis*, *Pseudallescheria*, *Rhizopus*, *Rhodotorula*, *Scedosporium*, *Schizophyllum*, *Sporothrix*, *Trichophyton*, and *Trichosporon*.

26. The method of claim 25, wherein the fungus is selected from the group consisting of *Absidia corymbifera*, *Ajellomyces capsulatus*, *Ajellomyces dermatitidis*, *Arthroderma benhamiae*, *Arthroderma fulvum*, *Arthroderma gypseum*, *Arthroderma incurvatum*, *Arthroderma otae* and *Arthroderma vanbreuseghemii*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus niger*, *Blastomyces dermatitidis*, *Candida albicans*, *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*

and *Candida pelliculosa*, *Cladophialophora carrionii*, *Coccidioides immitis* and *Coccidioides posadasii*, *Cryptococcus neoformans*, *Cunninghamella* Sp, *Epidermophyton floccosum*, *Exophiala dermatitidis*, *Filobasidiella neoformans*, *Fonsecaea pedrosoi*, *Fusarium solani*, *Geotrichum candidum*, *Histoplasma capsulatum*, *Hortaea werneckii*,
 5 *Issatschenkia orientalis*, *Madurella grisae*, *Malassezia furfur*, *Malassezia globosa*, *Malassezia obtusa*, *Malassezia pachydermatis*, *Malassezia restricta*, *Malassezia slooffiae*, *Malassezia sympodialis*, *Microsporum canis*, *Microsporum fulvum*, *Microsporum gypseum*, *Microsporidia*, *Mucor circinelloides*, *Nectria haematococca*, *Paecilomyces variotii*, *Paracoccidioides brasiliensis*, *Penicillium marneffei*, ~~*Pichia anomala*~~, *Pichia guilliermondii*,
 10 *Pneumocystis jiroveci*, *Pneumocystis carinii*, *Pseudallescheria boydii*, *Rhizopus oryzae*, *Rhodotorula rubra*, *Scedosporium apiospermum*, *Schizophyllum commune*, *Sporothrix schenckii*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton verrucosum* and *Trichophyton violaceum*, and *Trichosporon asahii*, *Trichosporon cutaneum*, *Trichosporon inkin* and *Trichosporon mucoides*.

15

27. A method of removing a biofilm comprising administration of an effective amount of a peptide according to any one of claims 1 to 13.

28. The method of claim 27, wherein the biofilm occur on surfaces.

20

29. The method of claim 28, wherein the surfaces is selected from the group consisting of a ship, dockyard, food processors, mixers, machines, containers, water tanks, water filtrations, purification systems, preservatives in food industries, personal care products, scalpels, needles, scissors and other devices used in invasive surgical, therapeutic or
 25 diagnostic procedures; implantable medical devices, including artificial blood vessels, catheters and other devices for the removal or delivery of fluids to patients, artificial hearts, artificial kidneys, orthopaedic pins, plates and implants, catheters, urological and biliary tubes, endotracheal tubes, peripherally insertable central venous catheters, dialysis catheters, long term tunnelled central venous catheters, peripheral venous catheters, short term central
 30 venous catheters, arterial catheters, pulmonary catheters, Swan-Ganz catheters, urinary catheters, peritoneal catheters, long term urinary devices, tissue bonding urinary devices, artificial urinary sphincters, urinary dilators, ventricular or arterio-venous shunts; breast

implants, penile prostheses, vascular grafting prostheses, heart valves, artificial joints, artificial larynxes, otological implants, vascular catheter ports, wound drain tubes, hydrocephalus shunts, pacemakers and implantable defibrillators, dental implants, fillings, dentures, medical equipment, medical gear worn or carried by personnel in the health care setting, counter tops and fixtures in areas used for medical procedures or for preparing medical apparatus, tubes and canisters used in respiratory treatments, nebulisers, aesthetic agents, gloves, aprons and face-shields.

30. A method of treating proliferative diseases comprising administration of a pharmaceutically effective amount of a peptide according to any one of claims 1 to 13.

31. The method of claim 30, wherein the proliferative disease is selected from the group consisting of tumour, cancer, malignancy or combinations thereof.

32. The method of claim 31, wherein the proliferative disease is selected from the group consisting of colorectal cancer, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangio-endotheliosarcoma, synovioma, mesothelioma, Ewing's tumour, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, gastric cancer, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma of the head and neck, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, liver metastases, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, thyroid carcinoma such as anaplastic thyroid cancer, Wilms' tumour, cervical cancer, testicular tumour, lung cancer, small cell cancer of the lung, non-small cell cancer of the lung, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

33. Use of a peptide of any one of claims 1 to 13 in the manufacture of a medicament for treating cancer, a bacterial infection, or removing bacteria, or neutralizing endotoxins, or

treating viral based infectious diseases, or treating a fungal infection or infestation, or removing fungus, or treating proliferative diseases.

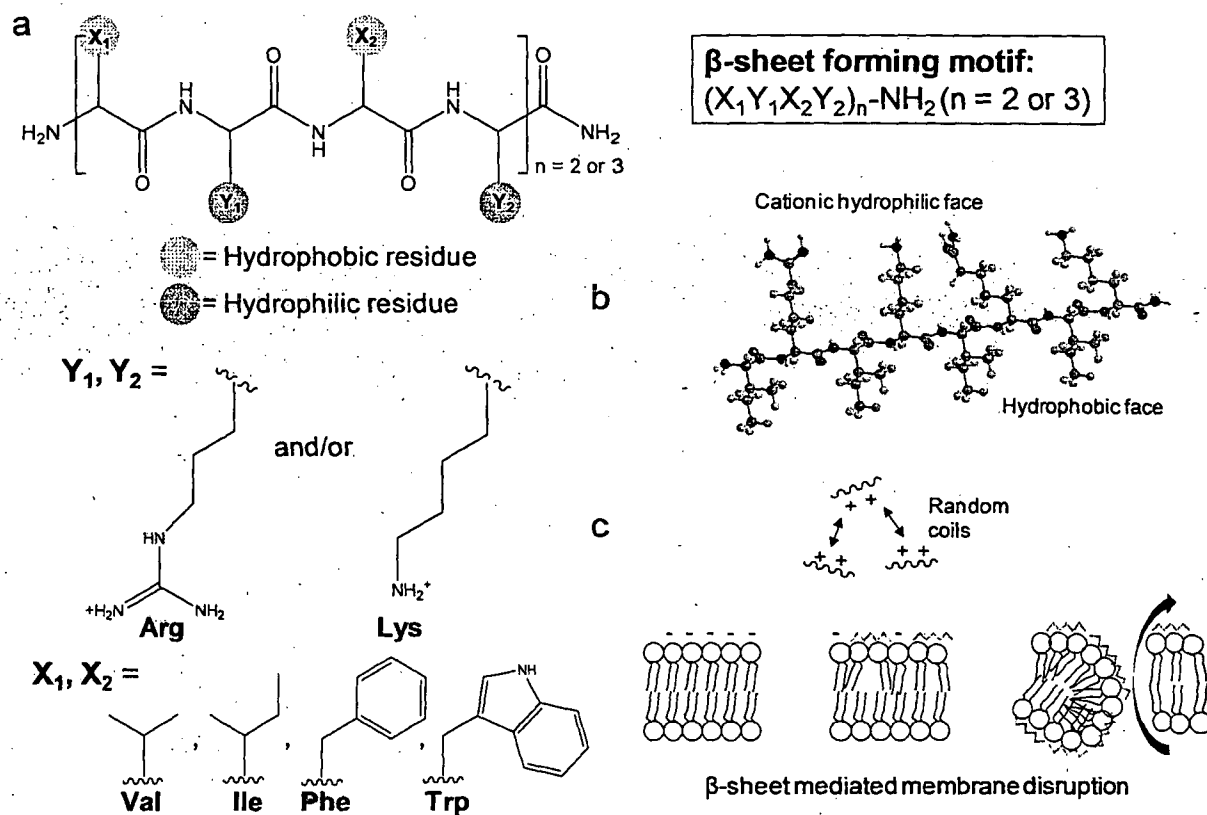


Figure 1

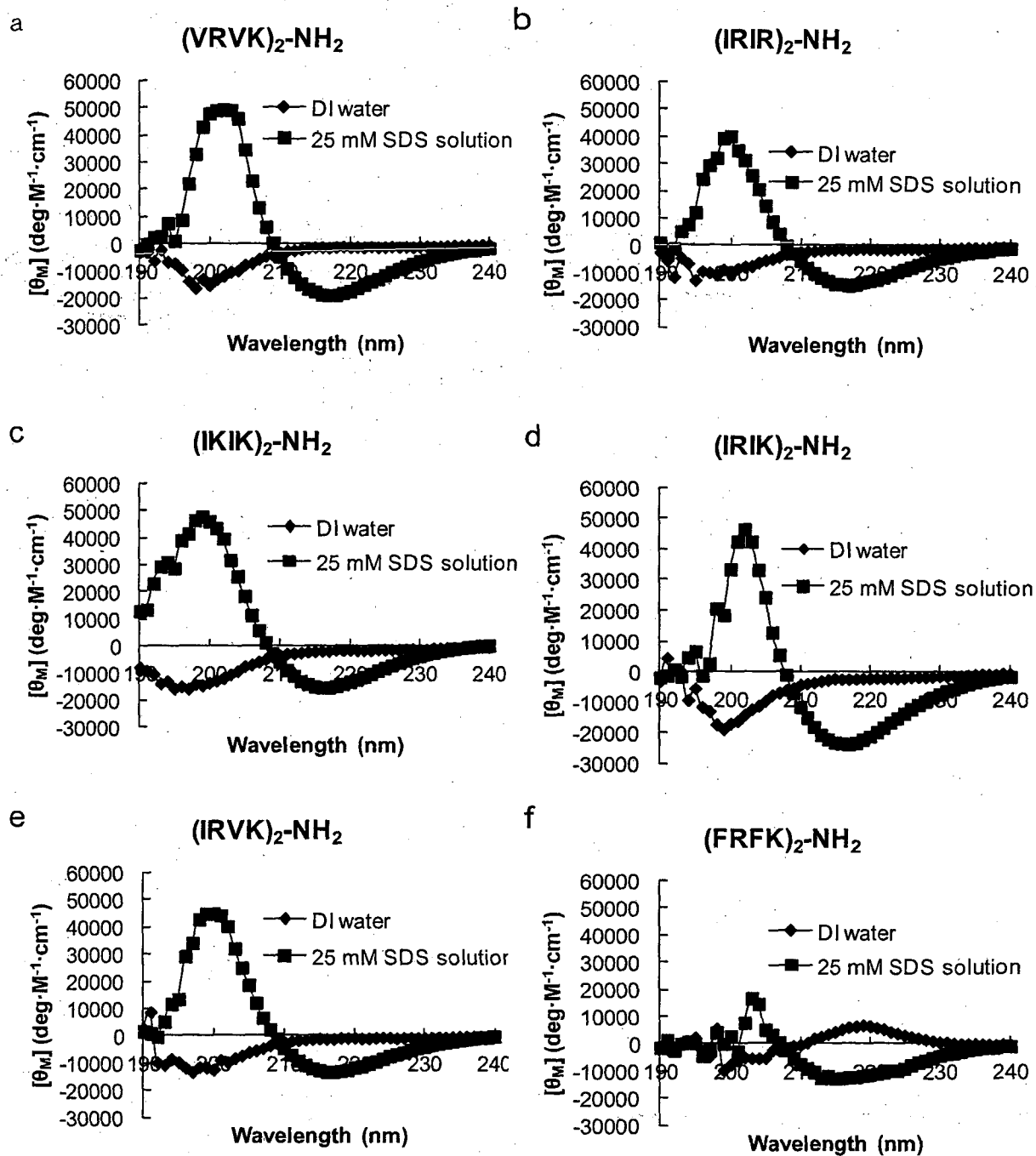


Figure 2

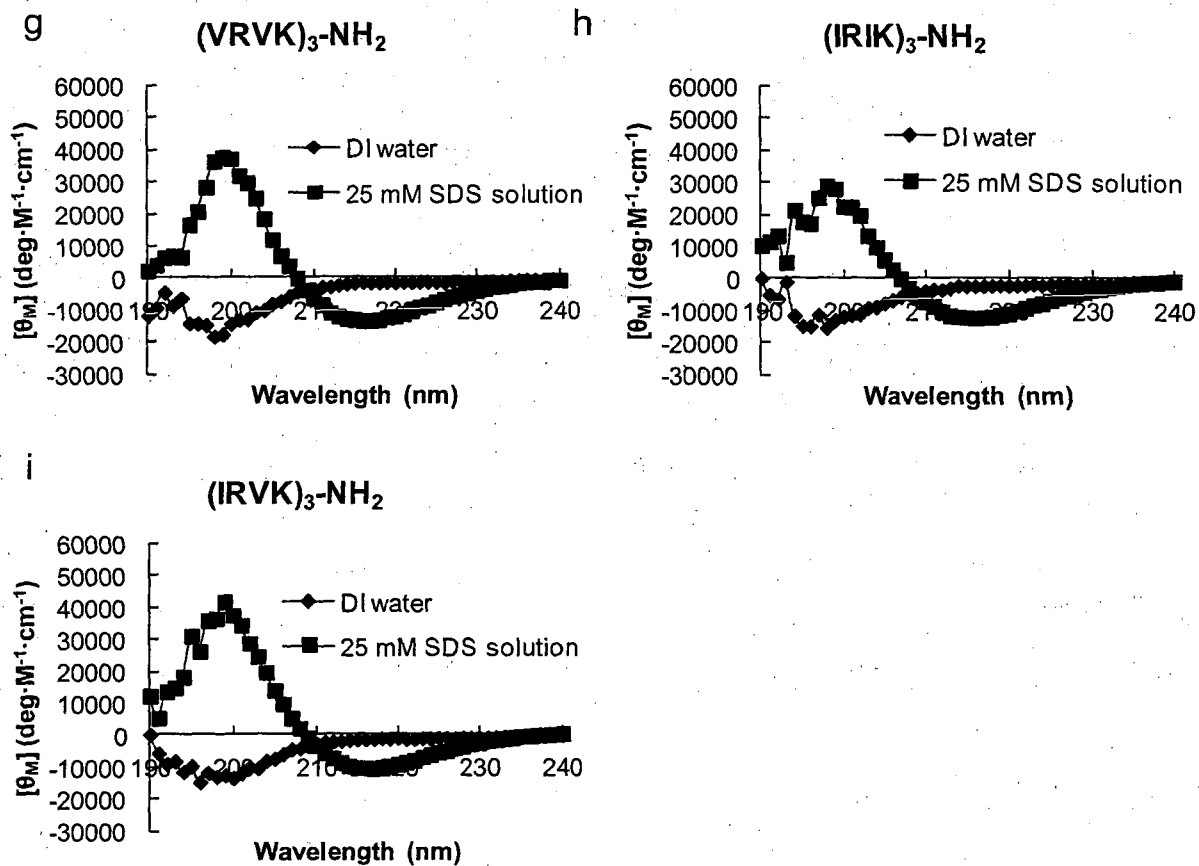


Figure 2 (continued)

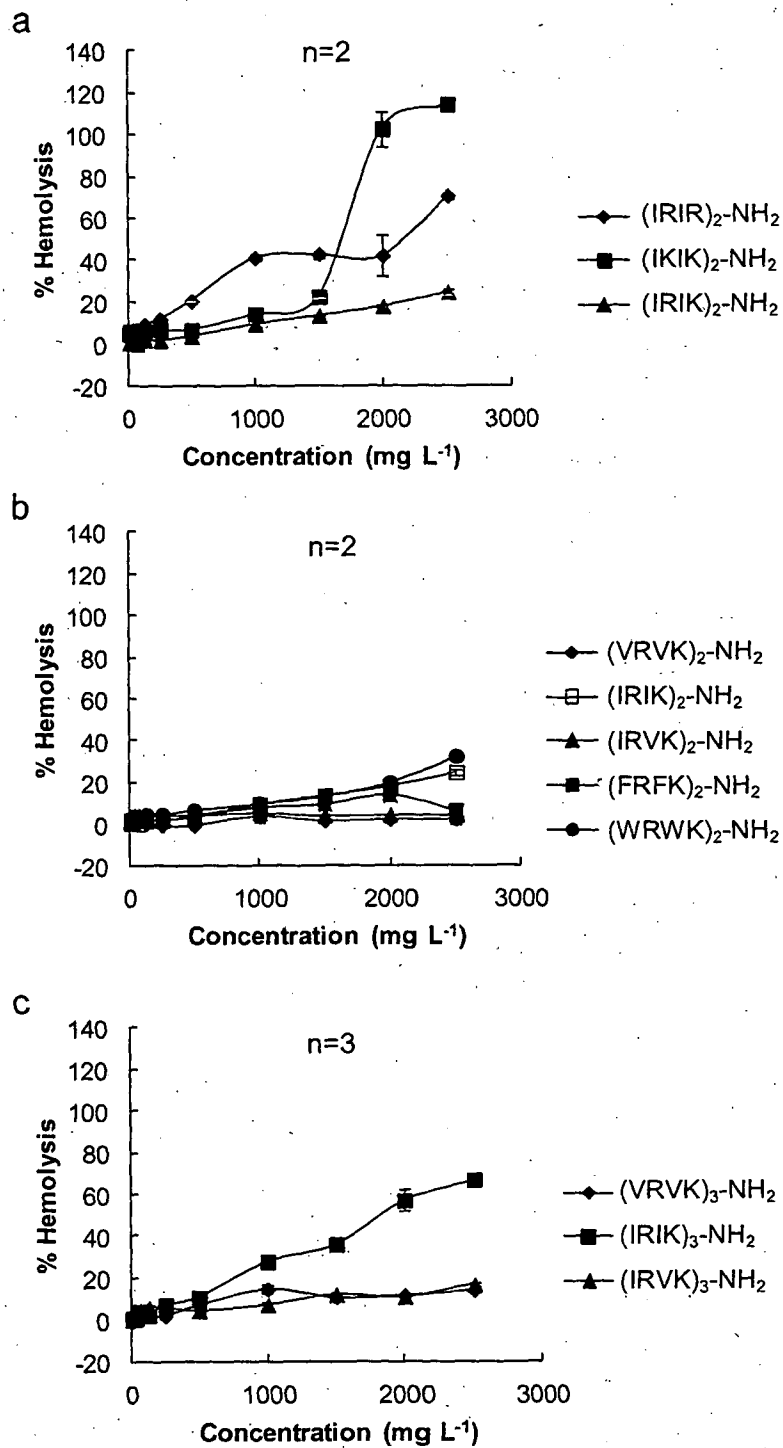


Figure 3

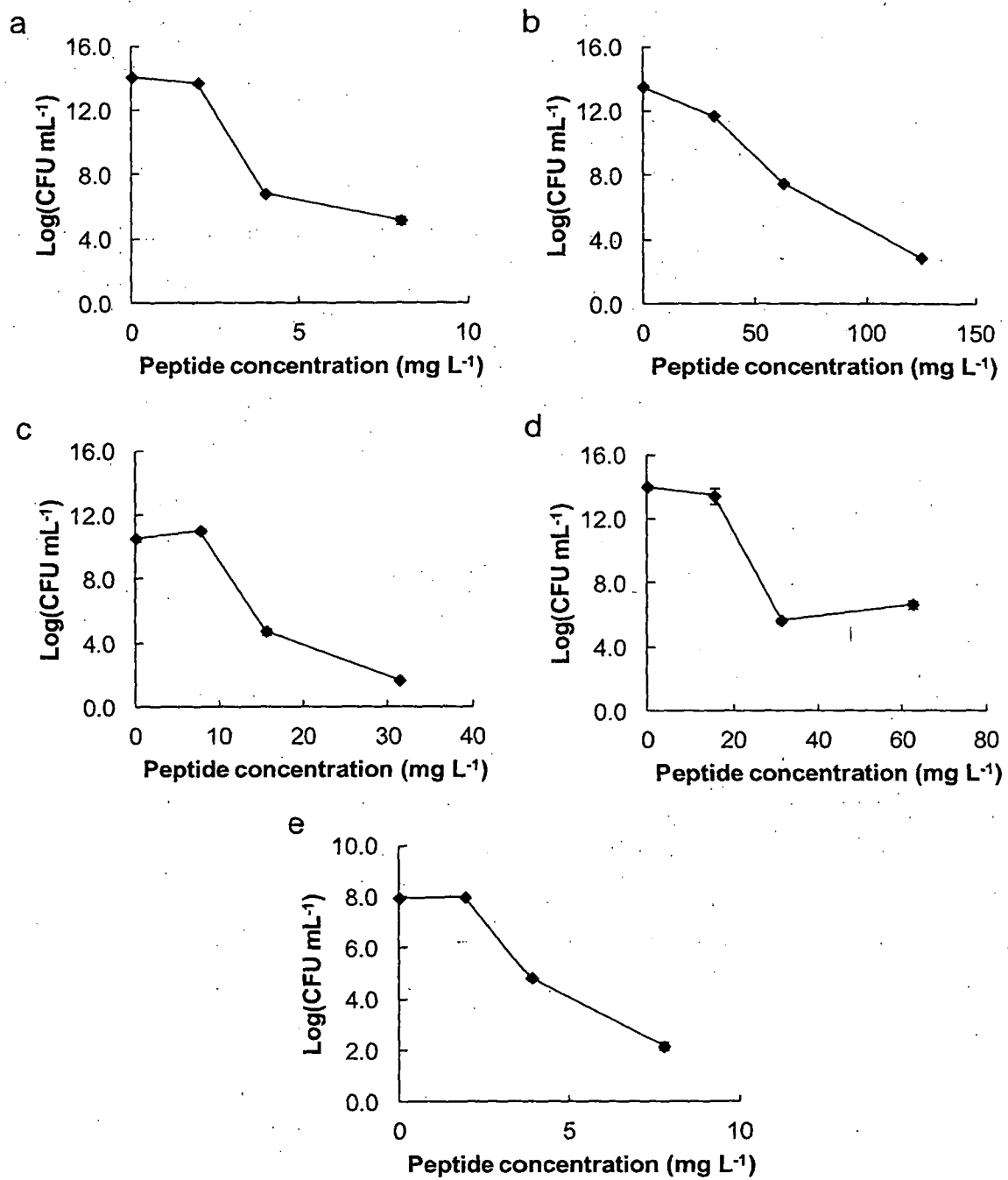


Figure 4

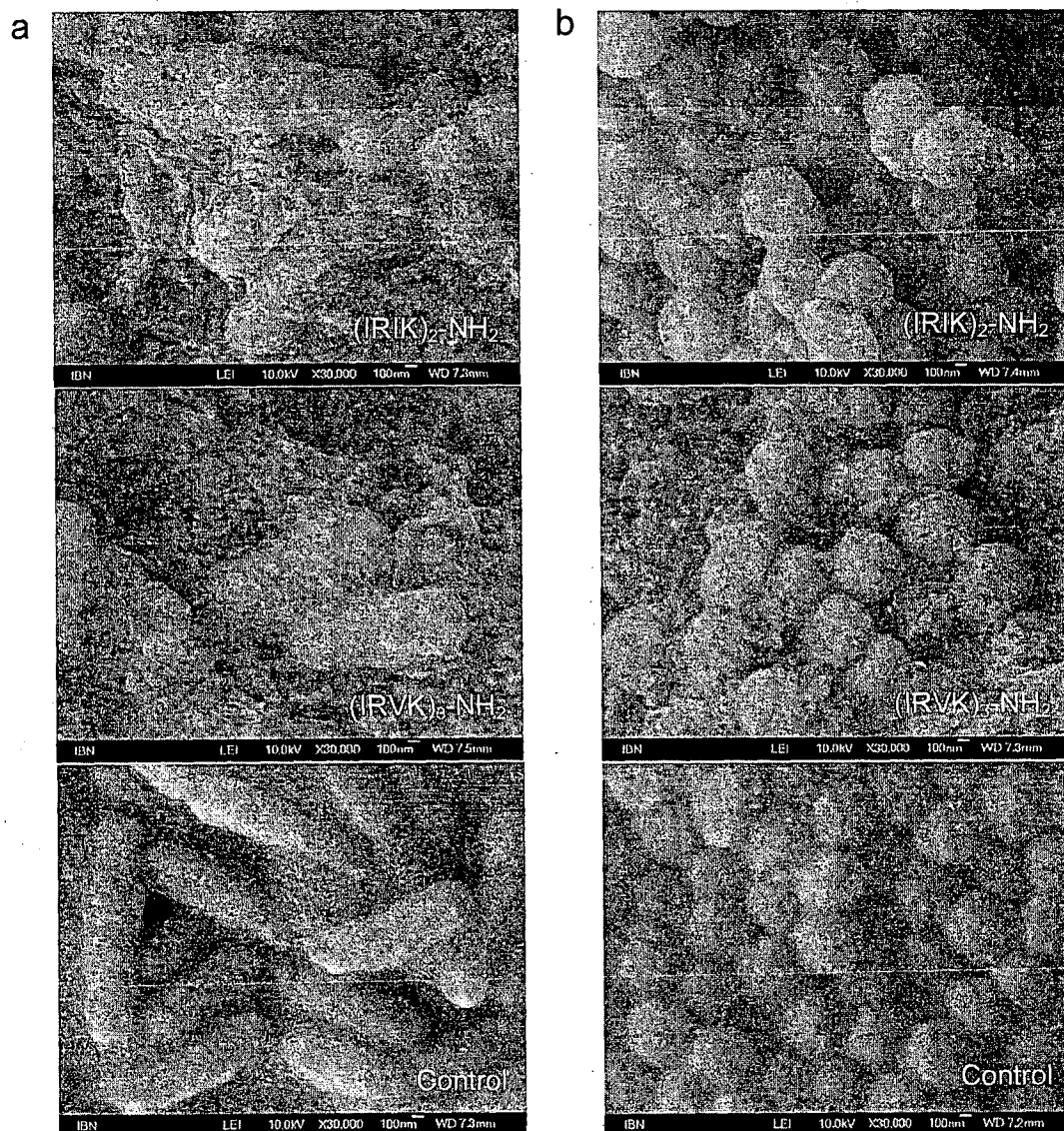
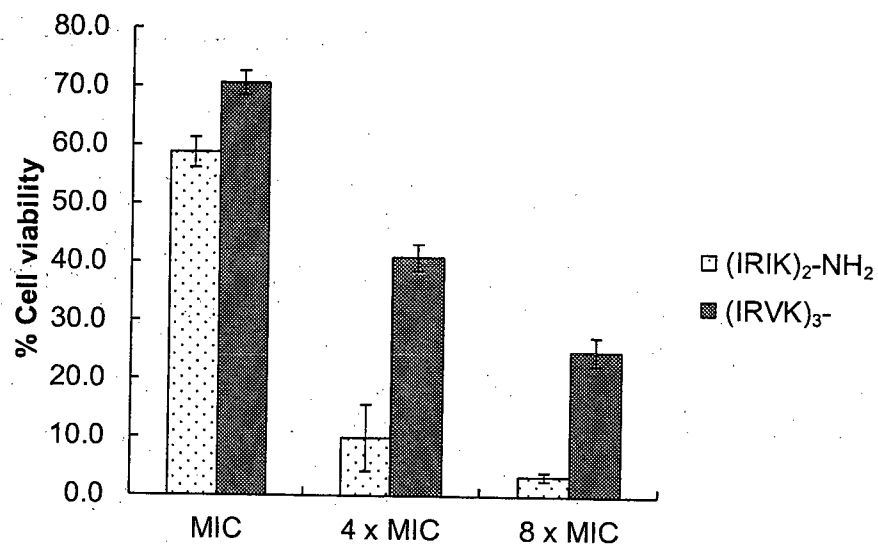
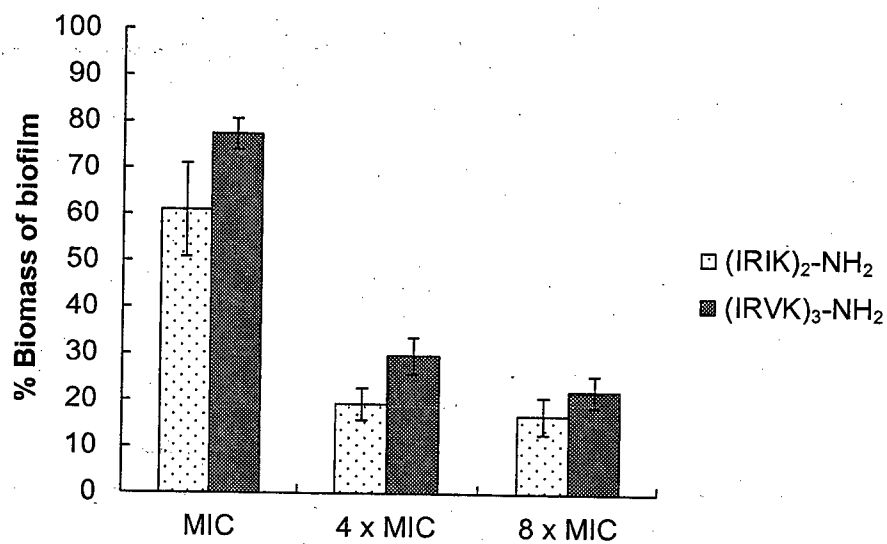


Figure 5

**Figure 6****Figure 7**

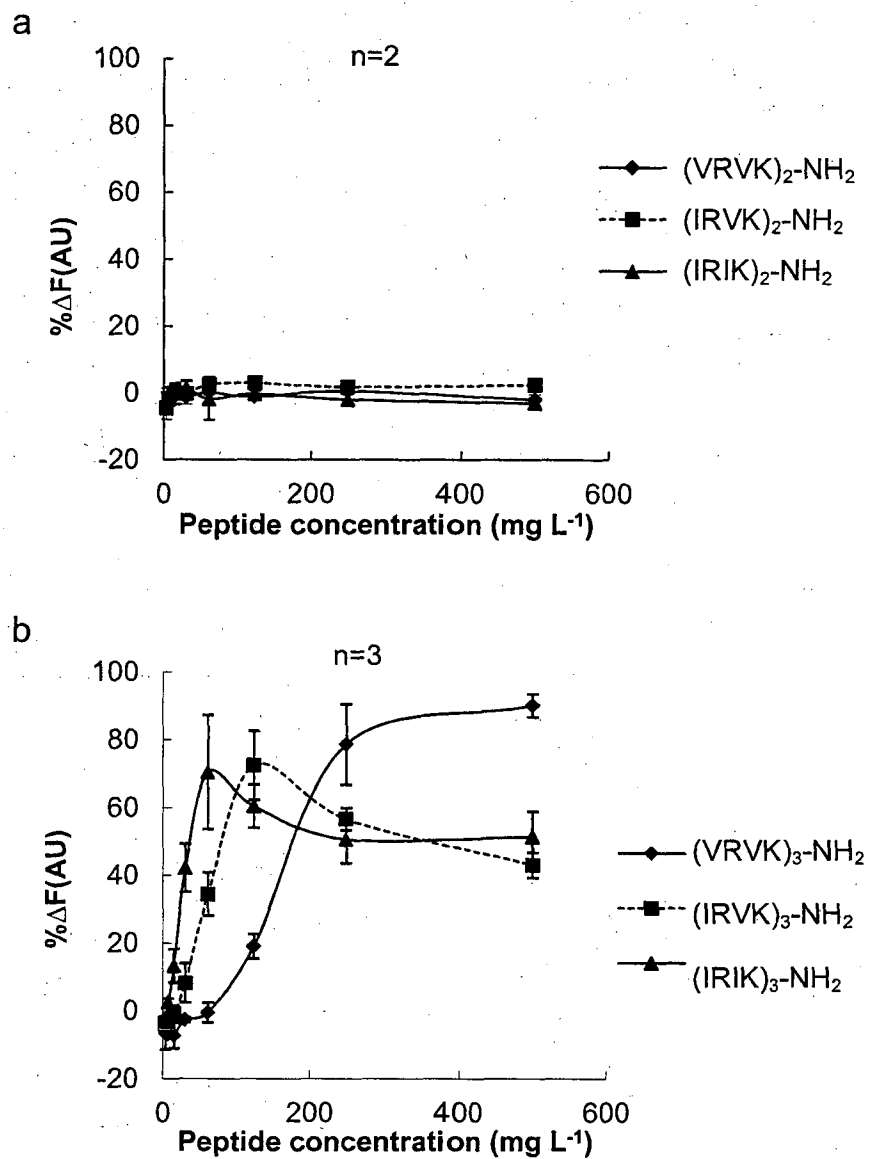


Figure 8

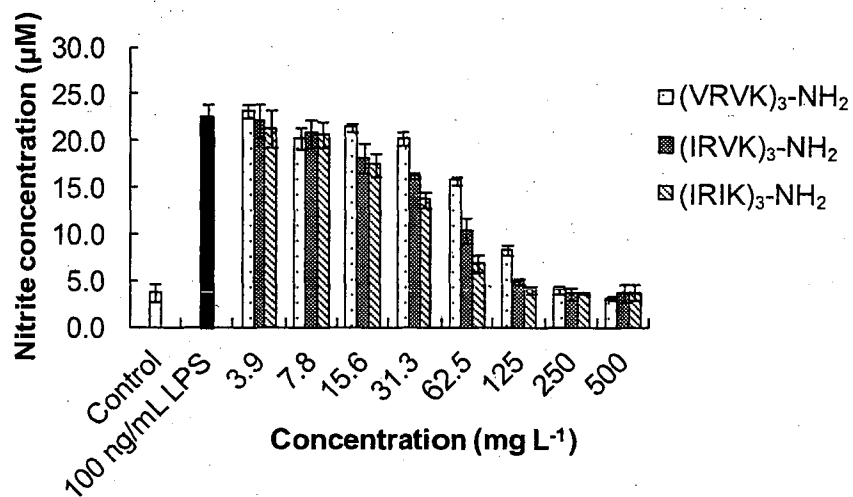


Figure 9

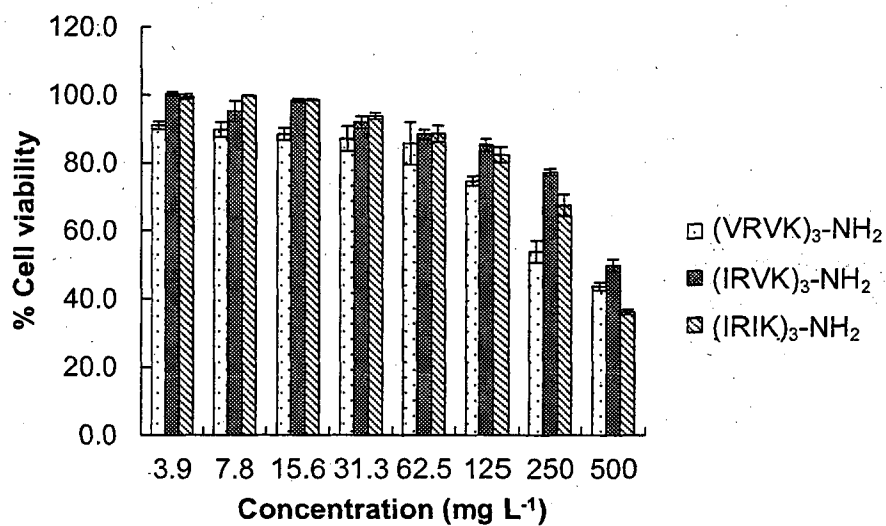


Figure 10

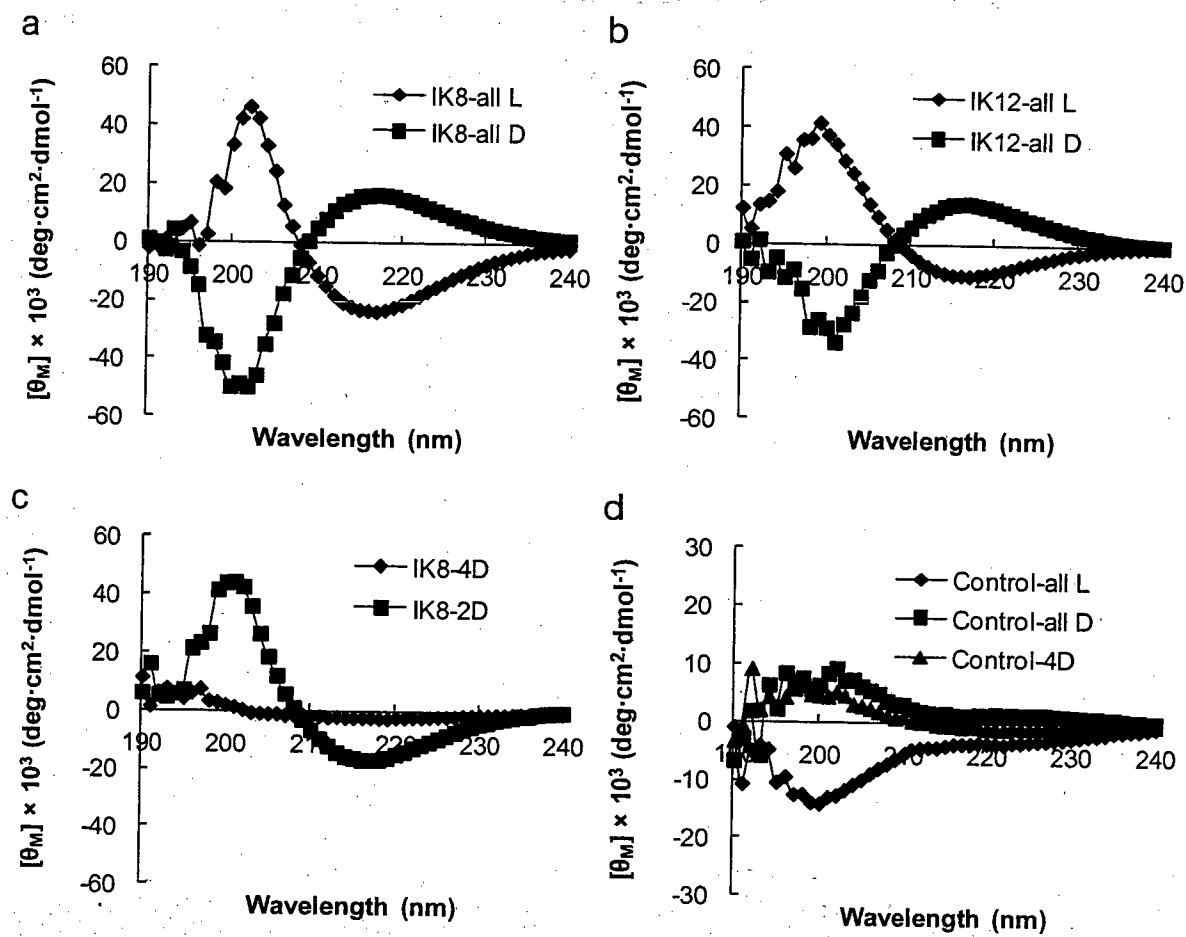


Figure 11

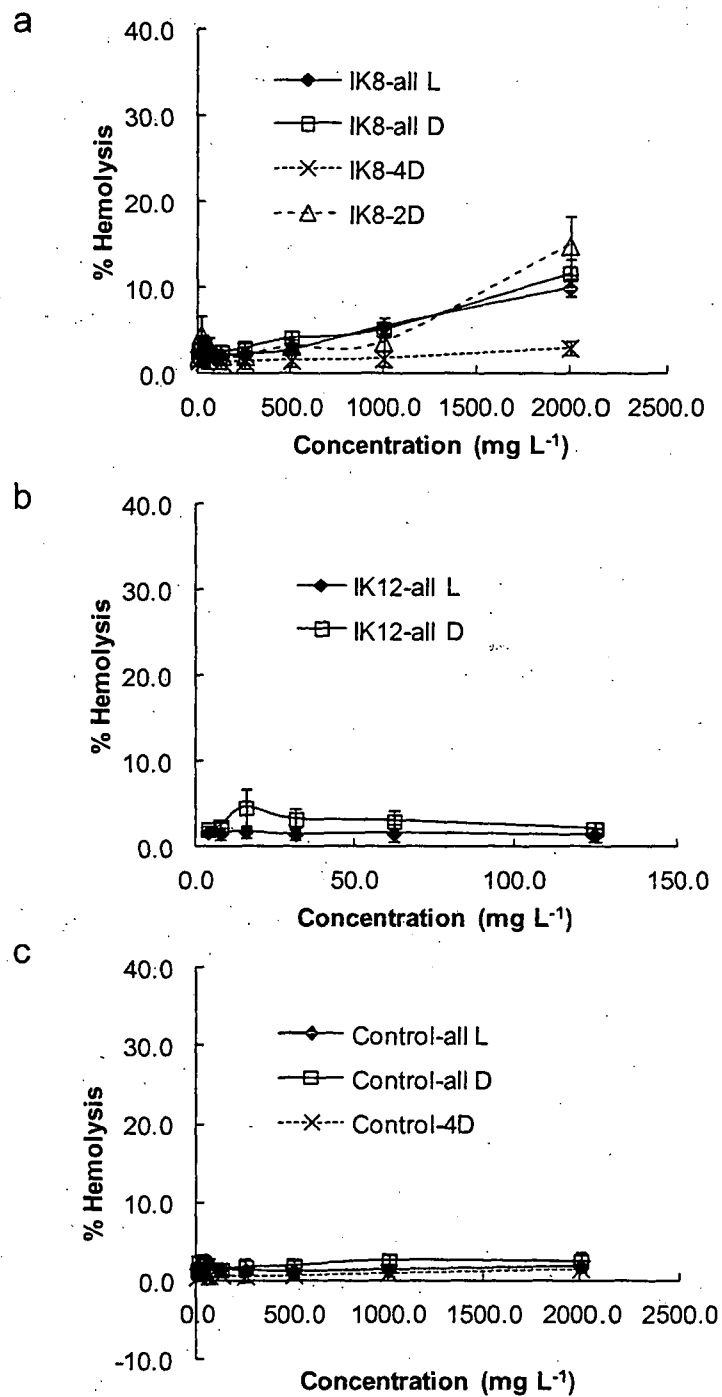


Figure 12

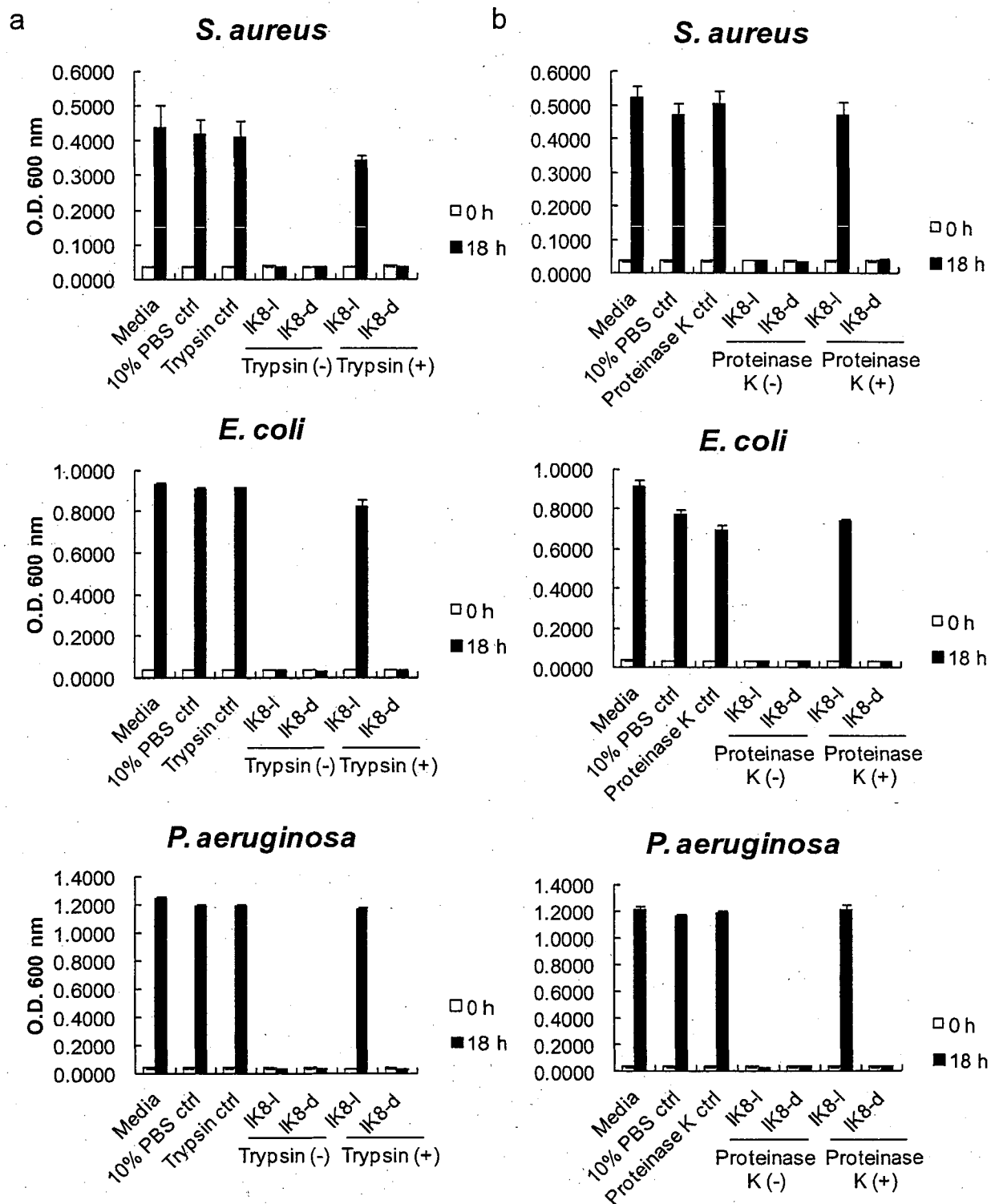


Figure 13

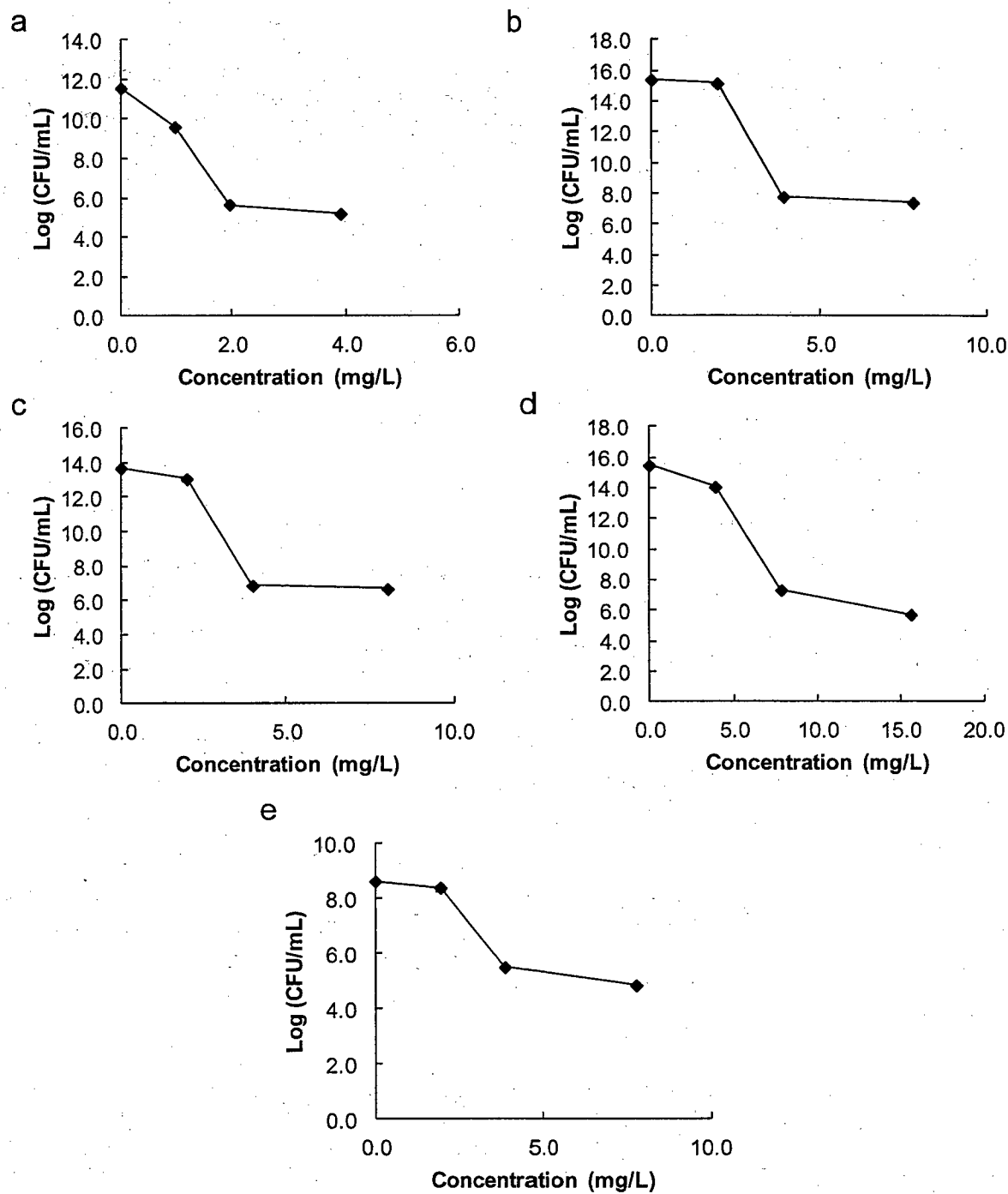


Figure 14

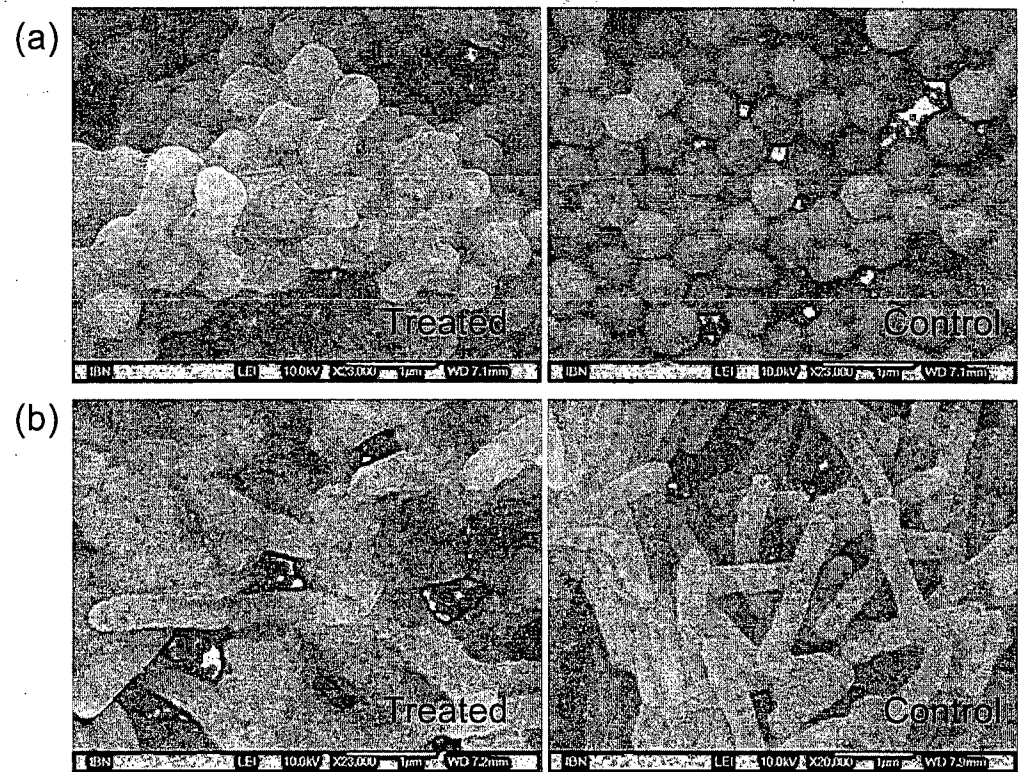


Figure 15

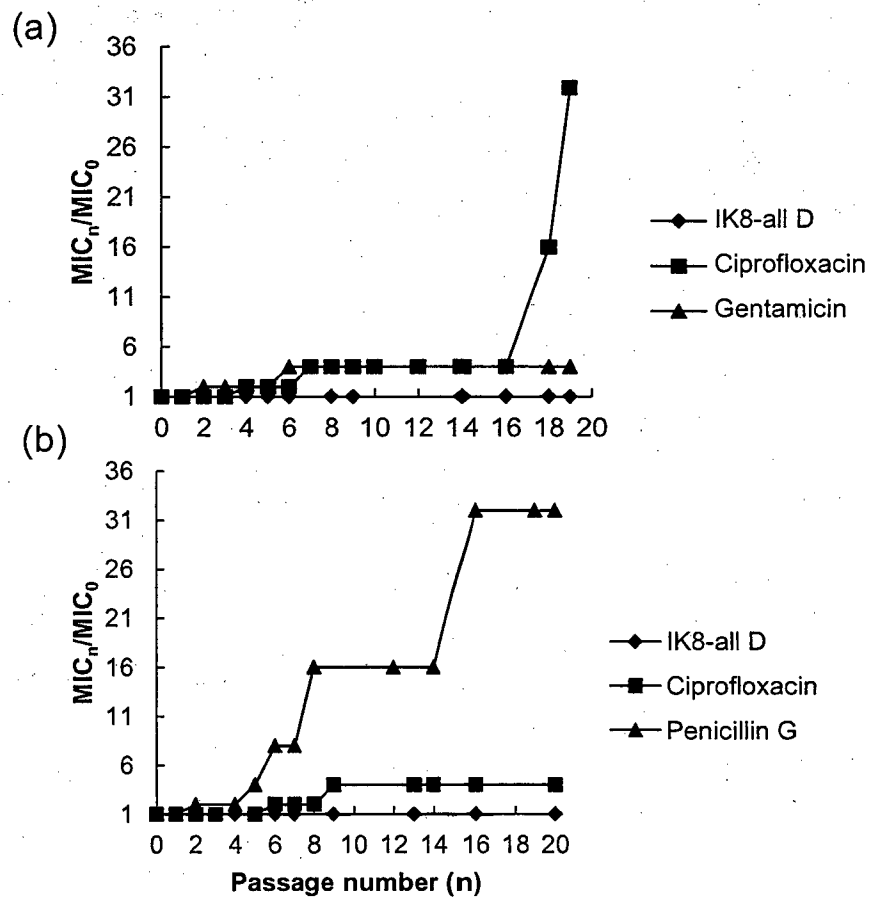


Figure 16

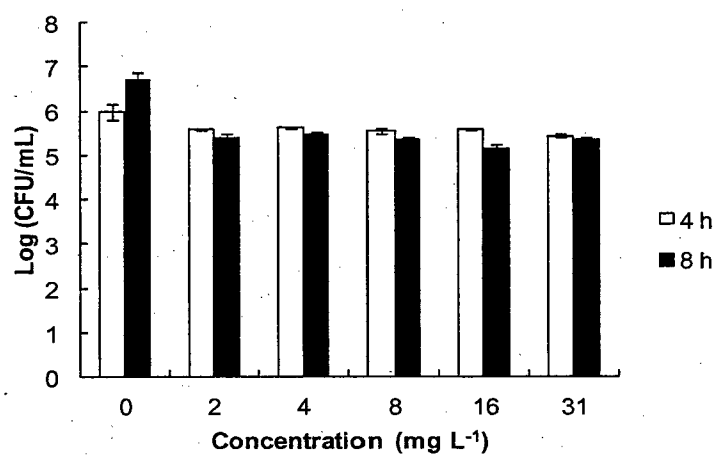


Figure 17

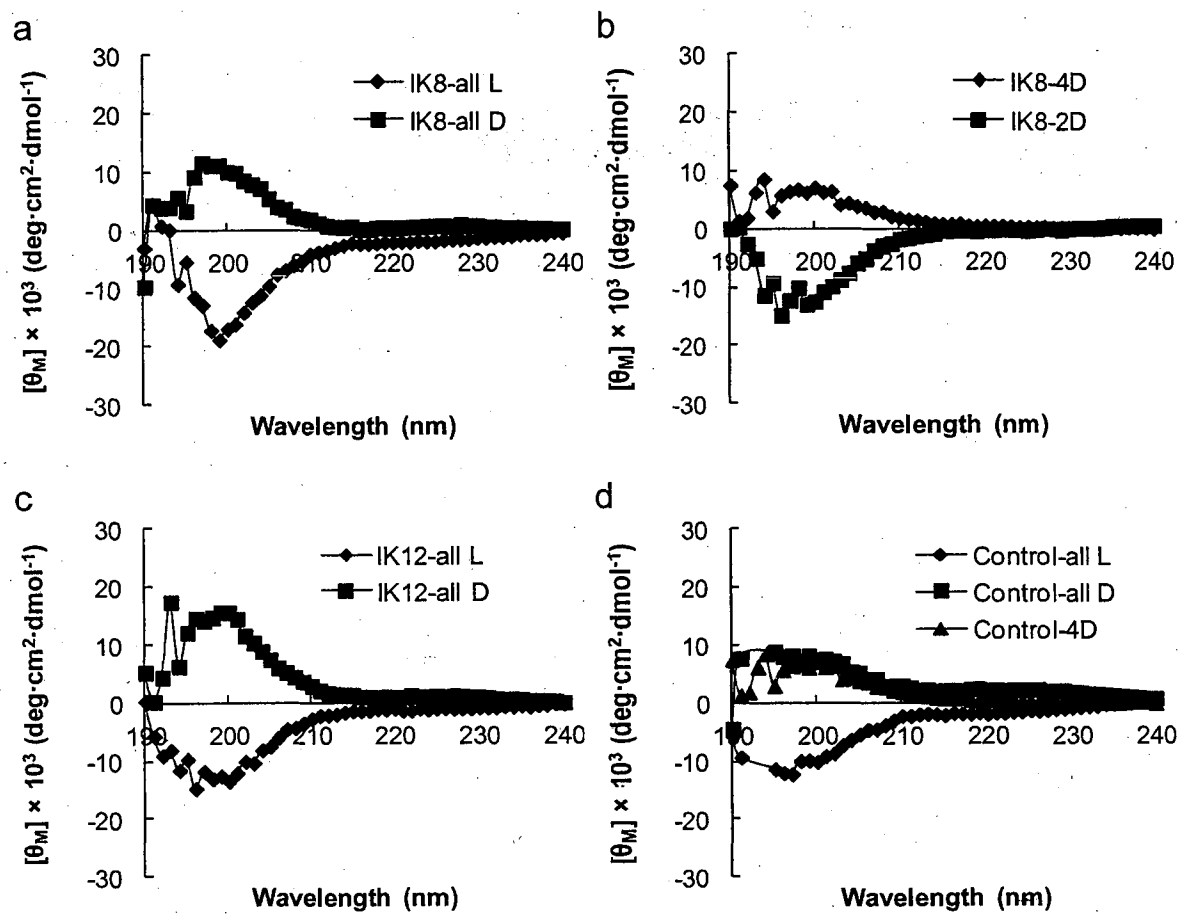


Figure 18

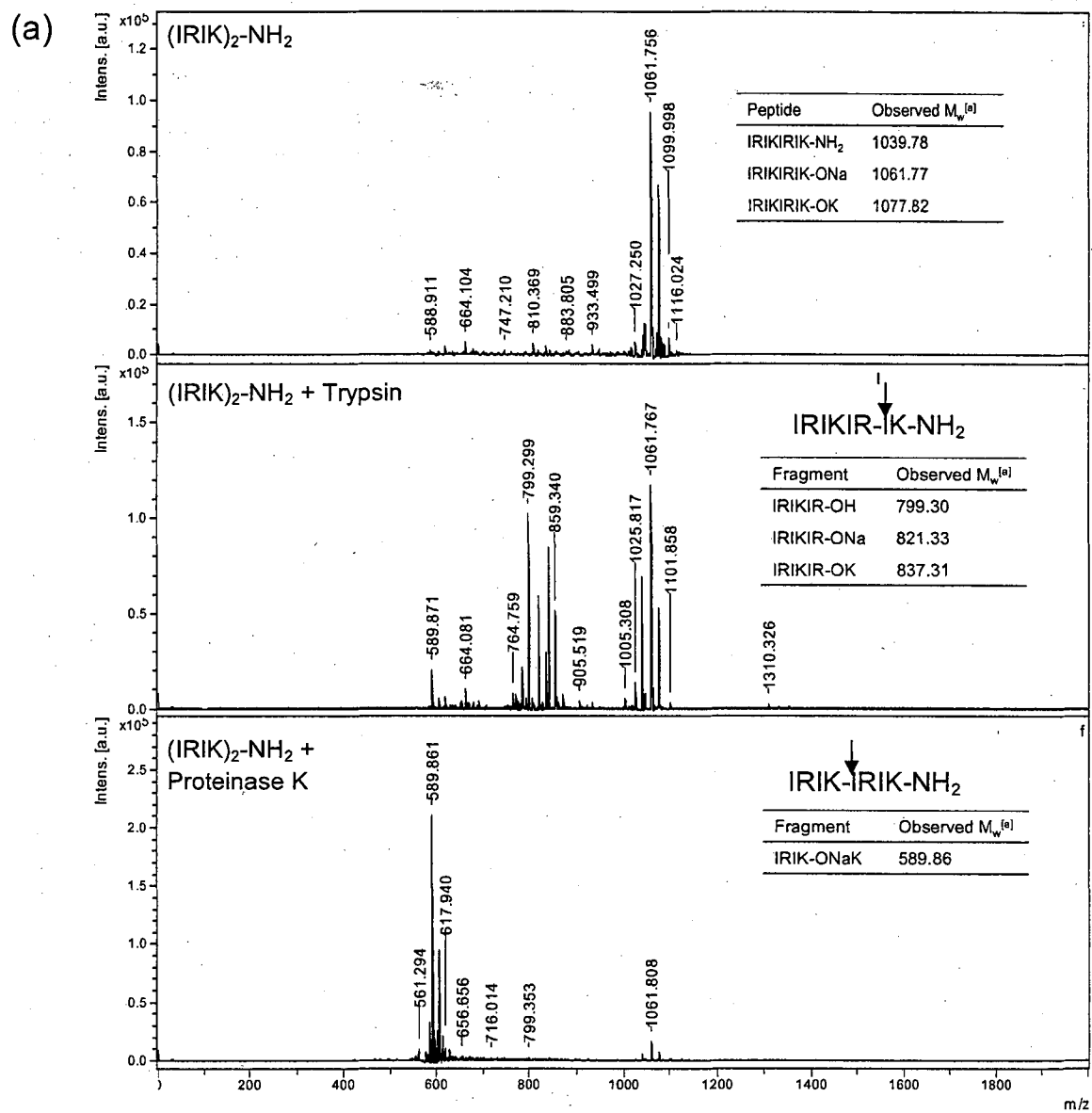


Figure 19 (continued)

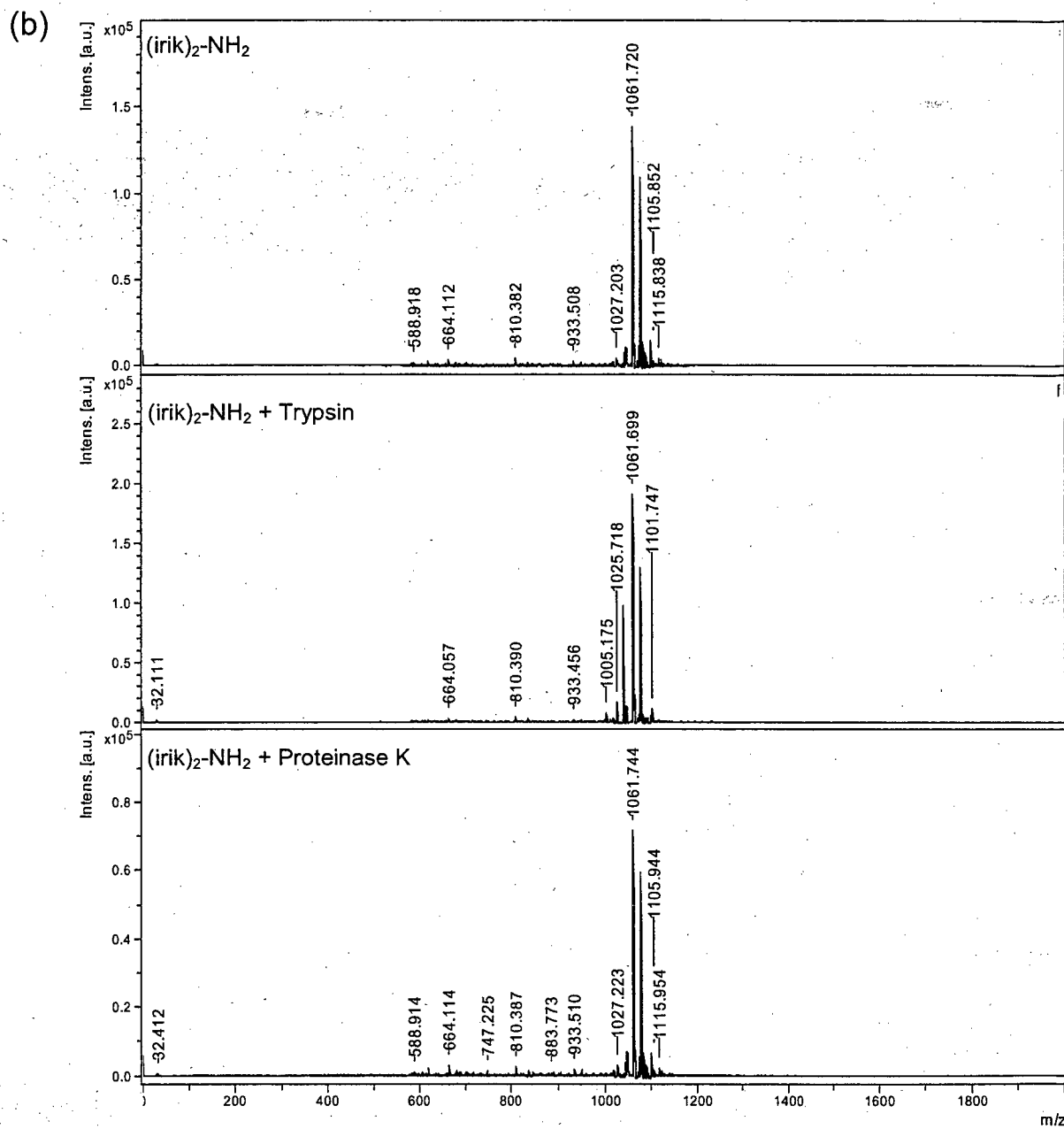
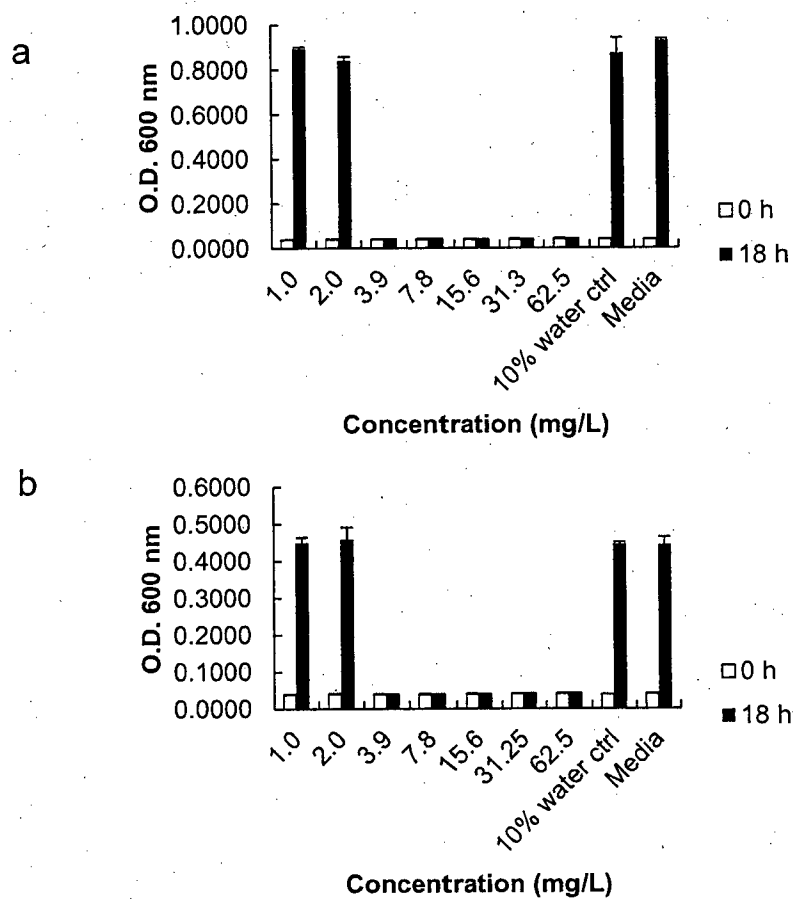
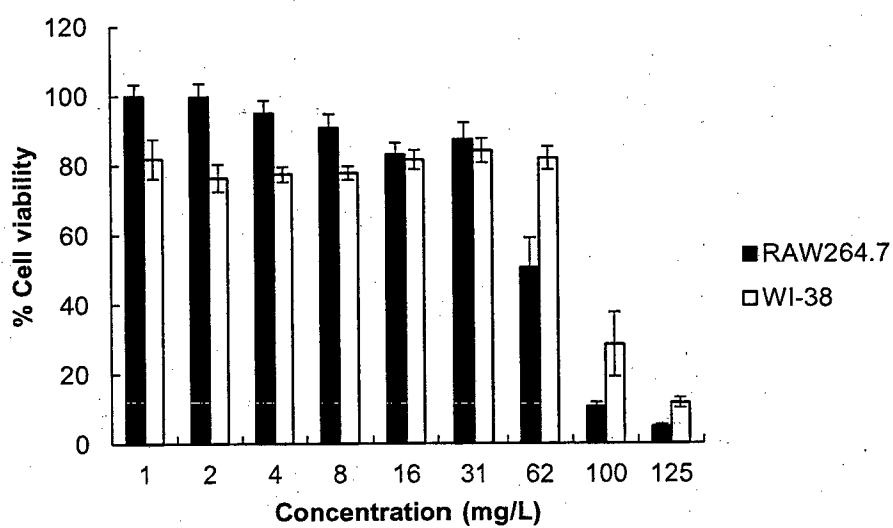


Figure 19

**Figure 20****Figure 21**

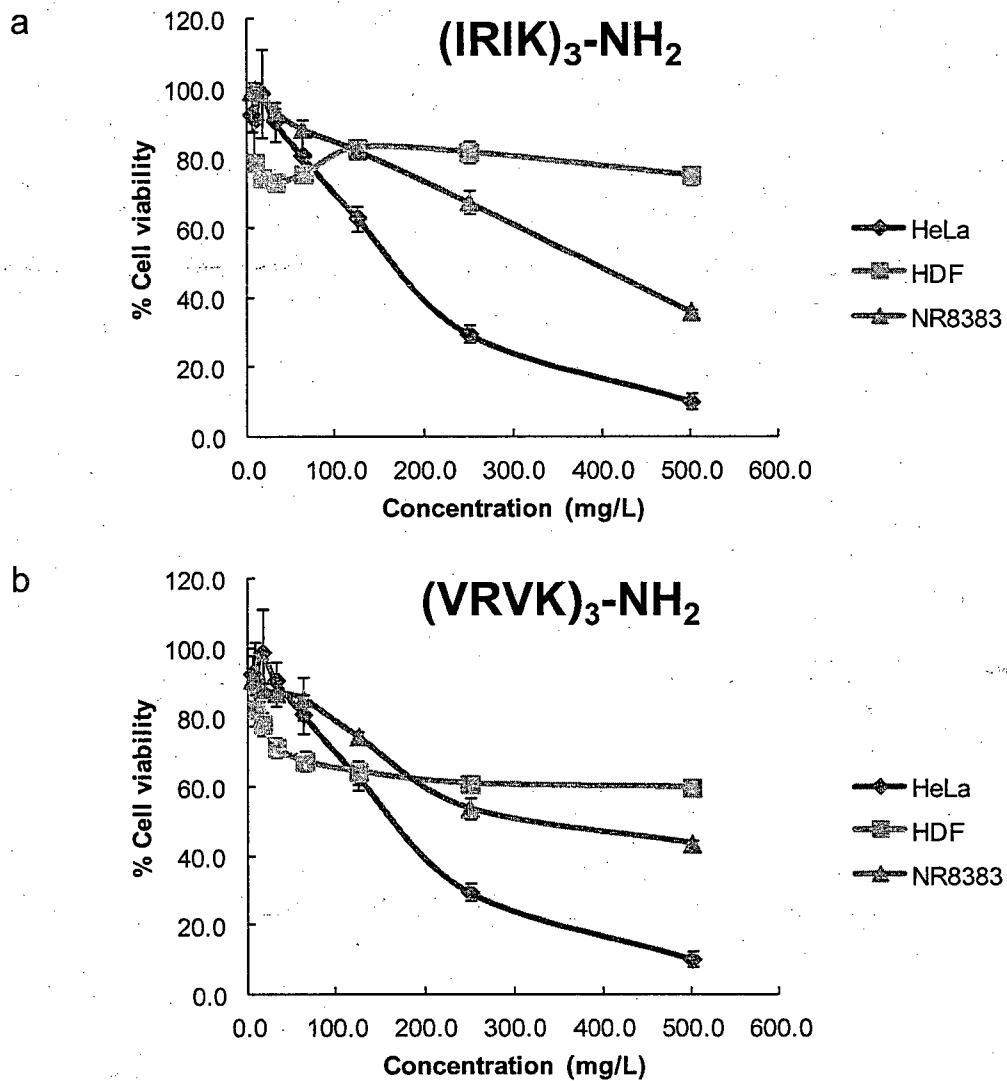


Figure 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2013/000378

A. CLASSIFICATION OF SUBJECT MATTER

**C07K 7/06 (2006.01) C07K 7/08 (2006.01) A61K 38/08 (2006.01) A61K 38/10 (2006.01) A61P 31/00 (2006.01)
A61P 35/00 (2006.01)**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Google: beta sheet AND antibacterial
REGISTRY (through STN): exact family protein searches of sequences listed in claim 13, then documents retrieved from CAPLus.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
27 November 2013

Date of mailing of the international search report
27 November 2013

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2013/000378
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ONG, Z. Y. ET AL. "Short Synthetic β -Sheet Forming Peptide Amphiphiles as Broad Spectrum Antimicrobials with Antibiofilm and Endotoxin Neutralizing Capabilities" Advanced Functional Materials (2013), 23: 3682-3692 see whole document	1-33
X Y	LEE, D. L. ET AL. "Effects of single D-amino acid substitution on disruption of β -sheet structure and hydrophobicity in cyclic 14-residue antimicrobial peptide analogs related to gramicidin S" Journal of Peptide Research (2004) 63: 69-84 see page 73 (figure 1), page 78 (figure 5) and page 73 (figure 2) see whole document	1-21, 24-29 22-23, 30-33
Y	WO 2003/092631 A2 (THE SCRIPPS RESEARCH INSTITUTE) 13 November 2003 see whole document	22-23,33
Y	WO 2003/092632 A2 (THE SCRIPPS RESEARCH INSTITUTE) 13 November 2003 see whole document	30-33

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/SG2013/000378	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2003/092631 A2	13 Nov 2003	WO 03092631 A2	13 Nov 2003
WO 2003/092632 A2	13 Nov 2003	WO 03092632 A2	13 Nov 2003
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			