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(54) Title: MODIFIED ANTIMICROBIAL PEPTIDE DERIVED FROM AN ARGinine-RICH DOMAIN

FIG. 1

(57) Abstract: An antimicrobial peptide, the peptide comprising 2 to 20 variable domains, each variable domain is a sequence of 2 to 20 consecutive basic amino acids, wherein (a) the variable domains are separated from each other by a variable linker, (b) the variable linker can have 1 to 20 any amino acids other than two or more consecutive basic amino acids, and (c) the peptide has no more than 100 amino acids.



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Modified Antimicrobial Peptide Derived From An Arginine-Rich Domain

BACKGROUND

Antibiotics have been used for the treatment of bacterial infection for more than 60 years. Recently, the increasing number of antibiotics-resistant bacteria has become a major threat to public health. The development of new antibiotics for clinical treatment is an urgent need. Antimicrobial peptides (AMPs) from various species can serve as a defense weapon of the host against pathogenic microbes. Because they can kill bacteria and fungi via different mode of actions, they have been considered as potential candidates to overcome the problem of antibiotic-resistance.

SUMMARY

In one aspect, described herein is an antimicrobial peptide. The peptide contains 2 to 20 variable domains, each variable domain is a sequence of 2 to 20 consecutive basic amino acids, wherein (a) the variable domains are separated from each other by a variable linker, (b) the variable linker can have 1 to 20 any amino acids other than two or more consecutive basic amino acids, and (c) the peptide has no more than 100 amino acids. In one embodiment, peptide has at least 3 or 4 variable domains. The peptide can have a C-terminal cysteine. In some embodiments, at least one of the basic amino acids in the variable domains is an arginine. For example, all of the basic amino acids in each variable domain in the peptide can be arginine residues. Alternatively or in addition, at least one variable domain in the antimicrobial peptide has a lysine. In one embodiment, at least one variable domain has a histidine. The peptide can have a cyclic structure.

At least one of the basic amino acids in the peptide can be a chemically-modified amino acid. In one embodiment, the chemically-modified amino acid is a D-amino acid, e.g., D-arginine. The variable domains and the variable linkers can be derived from the arginine-rich domain of a hepadnavirus core protein (HBcARD). In one embodiment, the HBcARD contains a sequence from residue 147 to the C-terminal residue of a hepadnavirus core protein. Each variable domain in the peptide can have three or four arginine residues and each variable linker in the peptide can have 2 to 4 amino acids. The peptide can exhibit a broad spectrum antimicrobial activity against a gram-positive bacterium, gram-negative bacterium, fungus, parasite, or virus.

In one embodiment, the antimicrobial peptide contains a consensus sequence selected from the group consisting of:

(i) (X₁)GRX₂P(X₃)X₄PX₅P(X₆)X₇QX₈P(X₉) (SEQ ID NO: 1), wherein each of X₁, X₃, X₆, and X₉, individually, is a variable domain, and each of X₂, X₄, X₅, X₇, and X₈, individually, is any amino acid or absent,

5 (ii) (X₁)GRX₂P(X₃)X₄PX₅P(X₆) (SEQ ID NO: 2), wherein each of X₁, X₃, and X₆, individually, is a variable domain, and each of X₂, X₄, and X₅, individually, is any amino acid or absent, and

10 (iii) (X₁)X₂PX₃P(X₄)X₅QX₆P(X₇) (SEQ ID NO: 3), wherein each of X₁, X₄, and X₇, individually, is a variable domain, and each of X₂, X₃, X₅, and X₆, individually, is any amino acid or absent. Each variable domain is a sequence of 2 to 20 consecutive basic amino acids.

The peptide can have a consensus sequence selected from the group consisting of:

(i) (X₁)GRX₂P(X₃)X₄PX₅P(X₆)X₇QX₈P(X₉)X₁₀C (SEQ ID NO: 4), and

(ii) (X₁)GRX₂P(X₃)X₄PX₅P(X₆)X₇QX₈P(X₉)X₁₀Q (SEQ ID NO: 5),

15 wherein each of X₁, X₃, X₆, and X₉, individually, is a variable domain, and each of X₂, X₄, X₅, X₇, X₈, and X₁₀, individually, is any amino acid or absent.

In one embodiment, the antimicrobial peptide contains a sequence selected from the group consisting of:

(i) TVVRRRGRSPRRRTPSPRRRSQSPRRRSQSRESQC (SEQ ID NO: 6), in which at least one of the arginine residues is D-arginine,

20 (ii) RRRGRSPRRRTPSPRRRSQSPRRRS (SEQ ID NO: 7),

(iii) RRRGRSPRRRTPSPRRRSQSPRRRSQ (SEQ ID NO: 8),

(iv) RRRGRPRRRPPRRRQPRRRRC (SEQ ID NO: 9),

(v) RRRGRSPRRRTPSPRRRC (SEQ ID NO: 10),

25 (vi) RRRGRPRRRPPRRRC (SEQ ID NO: 11),

(vii) RRRTPSPRRRSQSPRRRC (SEQ ID NO: 12), and

(viii) RRRPPRRRQPRRRRC (SEQ ID NO: 13).

For example, the antimicrobial peptide can contain the sequence of RRRGRSPRRRTPSPRRRSQSPRRRS (SEQ ID NO: 7), in which each of the arginine residues in the sequence is L-arginine. Alternatively, the peptide can have the sequence of RRRGRSPRRRTPSPRRRSQSPRRRS (SEQ ID NO: 7), in which at least one of the arginine residues in the sequence is D-arginine.

In one embodiment, the antimicrobial peptide contains the sequence of rRrGRSPrRrTPSPPrRrRSQSPrRrRSC (SEQ ID NO: 7), in which R is L-arginine and r is D-arginine. Alternatively, the peptide can contain the sequence of RrRGRSPRrRTPSPPrRrSQSPRrRrSC (SEQ ID NO: 7), in which R is L-arginine and r is D-arginine.

5 The antimicrobial peptide can include the sequence of RRRGRPRRRPPRRRQPRRRRC (SEQ ID NO: 9), in which at least one of the arginine residues (e.g., 20%, 30%, 40%, or 50% of the arginine residues) in the sequence is D-arginine. For example, the sequence can be rRrGRPrRrPPrRrRQPrRrRC (SEQ ID NO: 9), in 10 which R is L-arginine and r is D-arginine.

In one embodiment, the antimicrobial peptide further contains a non-HBcARD peptide (e.g., an affinity tag, a signal sequence, a ligand, or another antimicrobial peptide or fragment thereof). The non-HBcARD peptide can be a poly-histidine or an analog thereof.

In one embodiment, the peptide has a sequence selected from the group consisting of:

15 (i) RRRGRSPRRRTPSPRRRSQSPRRRSHHHHHH (SEQ ID NO: 14),
(ii) HHHHHHRRGRSPRRRTPSPRRRSQSPRRRS (SEQ ID NO: 15),
(iii) RRRGRPRRRPPRRRQPRRRHHHHHH (SEQ ID NO: 16), and
(iv) HHHHHHRRGRPRRRPPRRRQPRRR (SEQ ID NO: 17).

20 In another aspect, described herein is an antimicrobial peptide conjugate, which contains the antimicrobial peptide disclosed herein and a non-peptide moiety.

In yet another aspect, a pharmaceutical composition is described herein. The composition includes the antimicrobial peptide or the antimicrobial conjugate, and a pharmaceutically acceptable carrier.

25 Also contemplated herein is a method of treating an infection in a subject in need thereof. The method includes administering to the subject an antimicrobial peptide, an antimicrobial peptide conjugate, or a pharmaceutical composition described herein.

The details of one or more embodiments are set forth in the accompanying drawing and the description below. Other features, objects, and advantages of the embodiments will be apparent from the description and drawing, and from the claims.

HBcARD sequences are highly conserved among human (SEQ ID NO: 6), wooly monkey (SEQ ID NO: 18), ground squirrel (SEQ ID NO: 19), woodchuck (SEQ ID NO: 20), and bat (SEQ ID NO: 21). (B) There are also four positive charge clusters separated in the HBc C-terminus of duck (SEQ ID NO: 22), heron (SEQ ID NO: 23), parrot (SEQ ID NO: 24), Ross's goose (SEQ ID NO: 25) and snow goose hepatitis B virus (SEQ ID NO: 26).

FIG. 2 is a set of graphs showing comparison of serum resistance between L- and D-HBcARD peptides. Peptides (L-HBcARD and D-HBcARD) were incubated with MBC buffer (10 mM sodium phosphate and 50 mM sodium chloride, pH 7.2) containing 5% fetal bovine serum (A), 5% mouse serum (B) or human serum (male and female) (C) at 37°C for 3 hours. The amounts of peptides were determined using SDS PAGE electrophoresis and green angel staining. (D) Peptide D-HBcARD exhibited 10,000-fold higher potency than that of L-HBcARD in MBC assay. Peptides (L-HBcARD and D-HBcARD) were incubated with *S. aureus* ATCC19636 with or without 5% mouse serum at 37°C for 3 hours. The antimicrobial activity was determined by colony formation assay. ***P<0.0001.

FIG. 3 is a graph showing comparison of hemolytic effect between L- and D-HBcARD peptides. Human red blood cells were incubated with different concentrations of the peptides. Hemolysis is presented as the percentage of Triton X-100-induced hemolysis. ***P<0.0001.

FIG. 4 is a set of graphs showing comparison of *in vivo* protection activities between L- and D-form HBcARD peptides in a mouse sepsis model infected with *S. aureus*. (A) Three week-old ICR mice were inoculated with *S. aureus* (4×10^6 CFU/mouse) and i.p. injected with PBS, L-HBcARD, or D-HBcARD at 2 hours post-inoculation. Each group contained ten mice. (B) and (C) Three week-old ICR mice were immunized with L- and D-HBcARD peptides (5 mg/kg), respectively, at day 0, 3 and 6. On day 14, the mice were inoculated with *S. aureus* (4×10^6 CFU/mouse) and i.p. injected with PBS, L-HBcARD or D-HBcARD (10 mg/kg) at 1 hour post-inoculation, respectively. Another group of mice were treated with PBS in parallel as a control. Each group of animals contained five mice. *P<0.05; ***P<0.0001; ns, no significance.

FIG. 5 is a graph showing *in vivo* protection efficacies of various modified HBcARD peptides at different doses in an ICR mouse sepsis model.

FIG. 6 is a graph showing *in vivo* protection efficacies of 150-177C and 150-177Q peptides in a BALB/c mouse lung infection model. Colistin-resistant *A. baumannii* was

inoculated via intra-tracheal route.

FIG. 7 is a set of graphs showing lower *in vivo* toxicity of HBcARD peptide D-150-177C in comparison with polymyxin B. At day 0, male ICR mice (5 mice/group) were ip. injected with different doses of D-150-177C peptide (20-80 mg/kg) and polymyxin B (50 mg/kg), respectively. (A) The survival rates of all groups were monitored for 7 days. (B) Serum samples collected from mice treated with D-150-177C and polymyxin B were determined for alanine aminotransferase activity (ALT) at day 1. The dash line represents the mean of ALT value (45 U/L) of ICR mice (Charles River Laboratories).

FIG. 8 is a graph showing *in vivo* protection efficacies of all-D-arginine and partial-D-arginine substituted 150-177C peptides in a mouse sepsis model. Two hours after the inoculation, the mice (n=10/group) were treated with 5 mg/kg of various peptides D-, DL-, LD-150-177C, and PBS, respectively. Peptides D- and DL-150-177C protected all *S. aureus*-infected mice from death, while peptide LD-150-177C protected only 80% of mice.

FIG. 9 is a graph showing that deletion of serine and threonine residues from peptide DL-150-177C improved the *in vivo* protection efficacies at different doses in a mouse sepsis model.

DETAILED DESCRIPTION

It was unexpectedly discovered that certain modified peptides derived from an arginine-rich domain of HBV core protein exhibited broad spectrum antimicrobial activities.

Described herein is an antimicrobial peptide. It contains at least two (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) variable domains. The variable domains are in tandem and each separated from another by a variable linker. The antimicrobial peptide can have a maximum length of 100 amino acids (e.g., less than 10, 10, 14, 15, 20, 21, 22, 25, 28, 30, 35, 37, 40, 45, 47, 50, 55, 57, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acids). In one embodiment, the peptide has a C-terminal cysteine.

Each of the variable domains, individually, is a sequence of at least two (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) consecutive basic amino acids, e.g., arginine, histidine, and lysine. Each variable domain can contain a sequence of identical basic amino acids or different basic amino acids. For example, the antimicrobial peptides can contain 2 to 20 identical or different variable domains, each being X₁X₂X₃X₄X₅X₆X₇X₈X₉X₁₀X₁₁X₁₂X₁₃X₁₄X₁₅X₁₆X₁₇X₁₈X₁₉X₂₀ (SEQ ID NO: 27), wherein

each of X_1 - X_{20} , individually, is an arginine, histidine, or lysine (natural or chemically modified) and any of X_3 - X_{20} can be present or absent. For example, each variable domain can have 2 to 10 basic amino acids.

In one embodiment, at least one variable domain in the peptide consists solely of 5 arginine residues. In another embodiment, all of the variable domains in the peptide contain only arginine residues. Alternatively, the peptide can contain at least one variable domain that has one or more histidine or lysine residues.

Each variable linker has at least one amino acid (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 10 12, 13, 14, 15, 16, 17, 18, 19, or 20) and can contain any amino acid. It cannot have two or more consecutive basic amino acids.

The term "amino acid" refers to any of the 20 standard amino acids (i.e., alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine). The term can also refer to a non-standard, non-proteinogenic, or 15 chemically-modified amino acid, or an amino acid analog. An amino acid can be in the L- or D-form stereoisomer.

The term "basic amino acid" refers to arginine, lysine, or histidine, the L- or D-form thereof, or an analog thereof.

Non-standard amino acids include selenocysteine, pyrrolysine, N-formylmethionine, 20 non-proteinogenic amino acids, amino acid analogs, and chemically-modified amino acids. A chemically-modified amino acid or amino acid analog typically has a different side chain from its naturally-occurring counterpart. Amino acid analogs and methods of incorporating them into a polypeptide are known in the art. See, e.g., Nguyen et al., *Biochimica et Biophysica Acta* 1808 (2011), 2297-2303; Knappe, *Antimicrobial Agents and Chemotherapy* 54(9) 2010, 4003-4005; US7879979; US5972940; US8835162; and US20080199964.

Amino acid analogs are also commercially available. Amino acid analogs can be incorporated in the antimicrobial peptide to improve its stability, bioavailability, pharmacokinetics, tissue distribution, safety, tolerability, and/or efficacy.

Any of the antimicrobial peptides described herein can contain one or more residues 30 that are not one of the twenty standard amino acids. In particular, one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 2%, 3%, 5%, 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) of the

basic amino acids in the variable domains or in the entire antimicrobial peptide can be a D-form of natural arginine, lysine, or histidine, or an analog of natural arginine, lysine, or histidine.

The antimicrobial peptide can be derived from the arginine-rich domain of a hepadnavirus core protein (HBcARD). HBcARD refers to a highly conserved arginine-rich C-terminal region of a core protein (HBc). See, Fig. 1. The HBc can be a mammalian HBc or an avian HBc. A mammalian HBc can be human HBc, woolly monkey HBc, ground squirrel HBc, woodchuck HBc, and bat HBc. An avian HBc can be a duck, heron, parrot, Ross's goose, or snow goose HBc. The HBc can be from a hepadnavirus of any genotype.

For example, the antimicrobial peptide can include a fragment of the HBcARD or a variant thereof (e.g., containing one or more amino acid substitutions, deletions, or insertions). The variable domains and the linkers can be derived from an HBcARD. For example, the sequence between any two arginine repeats in an HBcARD or a variant thereof can be used as a linker.

In one embodiment, the antimicrobial peptide contains a consensus sequence selected from the group consisting of:

(i) (X₁)GRX₂P(X₃)X₄PX₅P(X₆)X₇QX₈P(X₉) (SEQ ID NO: 1), wherein each of X₁, X₃, X₆, and X₉, individually, is a variable domain, and each of X₂, X₄, X₅, X₇, and X₈, individually, is any amino acid or absent,

(ii) (X₁)GRX₂P(X₃)X₄PX₅P(X₆) (SEQ ID NO:2), wherein each of X₁, X₃, and X₆, individually, is a variable domain, and each of X₂, X₄, and X₅, individually, is any amino acid or absent, and

(iii) (X₁)X₂PX₃P(X₄)X₅QX₆P(X₇) (SEQ ID NO: 3), wherein each of X₁, X₄, and X₇, individually, is a variable domain, and each of X₂, X₃, X₅, and X₆, individually, is any amino acid or absent. Each variable domain is a sequence of 2 to 20 consecutive basic amino acids.

The peptide can have a consensus sequence selected from the group consisting of:

(i) (X₁)GRX₂P(X₃)X₄PX₅P(X₆)X₇QX₈P(X₉)X₁₀C (SEQ ID NO: 4), and

(ii) (X₁)GRX₂P(X₃)X₄PX₅P(X₆)X₇QX₈P(X₉)X₁₀Q (SEQ ID NO: 5),

wherein each of X₁, X₃, X₆, and X₉, individually, is a variable domain, and each of X₂, X₄, X₅, X₇, X₈, and X₁₀, individually, is any amino acid or absent.

The antimicrobial peptide can be a fusion or chimeric peptide that further contains a non-HBcARD peptide. A non-HBcARD peptide is not derived from any HBcARD and does

not contain at least two variable domains connected by a linker as described above. A non-HBcARD peptide can be a peptide derived from another source (e.g., from a protein other than an HBcARD), an engineered peptide (e.g., another antimicrobial peptide), an affinity tag (e.g., a FLAG, poly-His, Myc, HA, CBP, HBH, or V5 tag), a signal sequence (e.g., a leader sequence or a localization signal), or a ligand (e.g., a receptor ligand).
5

For example, the antimicrobial peptide can have up to 100 amino acids and contain a sequence selected from the group consisting of:

(i) TVVRRRGRSPRRRTPSPRRRRSQSPRRRSQSRESQC (SEQ ID NO: 6), in which at least one of the arginine residues is D-arginine,

10 (ii) RRRGRSPRRRTPSPRRRRSQSPRRRS (SEQ ID NO: 7),

(iii) RRRGRSPRRRTPSPRRRRSQSPRRRSQ (SEQ ID NO: 8),

(iv) RRRGRPRRRPPRRRQPRRRRC (SEQ ID NO: 9),

(v) RRRGRSPRRRTPSPRRRRC (SEQ ID NO: 10),

(vi) RRRGRPRRRPPRRRC (SEQ ID NO: 11),

15 (vii) RRRTSPRRRSQSPRRRC (SEQ ID NO: 12),

(viii) RRRPPRRRQPRRRRC (SEQ ID NO: 13).

(ix) RRRGRSPRRRTPSPRRRRSQSPRRRSHHHHH (SEQ ID NO: 14),

(x) HHHHHHRRGRSPRRRTPSPRRRRSQSPRRRS (SEQ ID NO: 15),

(xi) RRRGRPRRRPPRRRQPRRRRHHHHH (SEQ ID NO: 16), and

20 (xii) HHHHHHRRGRPRRRPPRRRQPRRRR (SEQ ID NO: 17).

As described herein, the antimicrobial peptide can have one or more modified amino acids. For example, in any of the above-described sequences or consensus sequences, one or more of the basic amino acids in the variable domains or in the entire antimicrobial peptide can be a D-form of natural arginine, lysine, or histidine, or an analog of natural arginine, lysine, or histidine.
25

Any of the above-described sequences or consensus sequences can be at the N- or C-terminus of the antimicrobial peptide.

The antimicrobial peptide described herein can also be conjugated to a non-peptide moiety at the N- or C-terminus to form a peptide conjugate. A non-peptide moiety can be a polymer (e.g., a polyethylene glycol polymer), oligosaccharide, lipid, glycolipid, solid support (e.g., a bead or nanoparticle), small molecule drug, biotin, nucleic acid molecule, antibody, vitamin, carrier protein (e.g., KLH, BSA, or OVA), or detectable label (e.g.,
30

fluorescent, radioactive, or enzymatic label). The peptide conjugate also exhibits an antimicrobial activity. Methods of generating peptide conjugates are known in the art.

The antimicrobial peptide or peptide conjugate described herein can be mixed with a pharmaceutically acceptable carrier to form a pharmaceutical composition.

5 The composition can be formulated with a pharmaceutically acceptable carrier such as a phosphate buffered saline, a bicarbonate solution, and/or an adjuvant. Suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use, are known in the art. This composition may be prepared as an injectable, liquid solution, emulsion, or another suitable formulation.

10 An effective amount of the composition described above may be administered by intranasal inhalation, topical application, or parenteral routes, e.g., intravenous injection, subcutaneous injection or intramuscular injection. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or 15 triglycerides. Oral formulations may include normally employed incipients such as pharmaceutical grades of saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders.

15 The above-described composition can be administered to a subject (e.g., a human, another mammal, or a laboratory animal) to treat a microbial infection or to inhibit growth of a microbe in the subject. The composition can be used to treat an infection caused by a Gram-positive or Gram-negative bacteria, fungus, parasite, or virus, e.g., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Clostridium difficile*, *Candida*, *Aspergillus*, *Blastomyces*, 20 *Cryptococcus neoformans*, *Cryptococcus gattii*, *Coccidioides*, *Histoplasma*, *Pneumocystis jirovecii*, *ringworm*, *Sporothrix*, *Exserohilum*, or *Cladosporium*.

25 The specific disclosure below is to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present disclosure to its fullest extent. All publications cited herein are hereby 30 incorporated by reference in their entirety.

Modified antimicrobial peptides derived from human HBcARD

A novel antimicrobial peptide from human hepatitis B virus (HBV) core protein (HBc) arginine-rich domain (ARD) was previously identified. See Chen et al. (2013), PLoS Pathog 9:e1003425 doi:10.1371/journal.ppat.1003425, and Chen et al., (2016), Appl Microbiol Biotechnol. 100(21): 9125-9132. This HBcARD peptide showed a broad spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria. In our mouse sepsis model, injection of HBcARD peptide at 2 hour postinoculation can protect 40% of *S. aureus*-infected mice from death. See Chen et al. (2013).

The HBcARD peptides are highly conserved in the hepadnaviruses of different species. To improve the antimicrobial efficacy of HBcARD, we compared the sequences and antimicrobial activities of various HBcARD peptides of mammalian, rodent, and avian hepadnaviruses. HBcARD peptide of human hepadnaviruses displayed the strongest antimicrobial activity.

To further improve the potency of our lead compound HBcARD, we tested the antimicrobial activity of each of several HBcARD derivatives, including peptides modified by truncation and D-arginine substitution for L-arginine. See Table 1.

Table 1. Sequences of various HBcARD peptides tested for their bactericidal activity

HBcARD peptide	amino acid sequence
L-147-183	TVVRRGRSPRRRTPSPRRRSQSPRRRSQSRESQC (SEQ ID NO: 6)
D-147-183	TVVrrrGrSPrrrTPSPrrrrSQSPrrrrSQSrESQC (SEQ ID NO: 6)
150-177C	RRRGRSPRRRTPSPRRRSQSPRRRSQC (SEQ ID NO: 7)
150-177Q	RRRGRSPRRRTPSPRRRSQSPRRRSQ (SEQ ID NO: 8)
150-171C	RRRGRSPRRRTPSPRRRSQC (SEQ ID NO: 10)
157-177C	RRRTPSPRRRSQSPRRRSQC (SEQ ID NO: 12)
164-177C	RRRRSQSPRRRSQC (SEQ ID NO: 28)
D-150-177C	rrrGrSPrrrTPSPrrrrSQSPrrrrSC (SEQ ID NO: 7)
DL-150-177C	rRrGRSPPrRrTPSPPrRrRSQSPPrRrRSC (SEQ ID NO: 7)
LD-150-177C	RrRGRSPPrRTPSPPrRrRSQSPPrRrRSC (SEQ ID NO: 7)
DL-dST-150-177C	rRrGRPrRrPPrRrRQPrRrRC (SEQ ID NO: 9)

R: L-arginine; r: D-arginine

Comparison of sequences and antimicrobial activities of human and non-human HBcARD peptides

By multiple sequence alignment, we compared the sequences of HBcARD peptides of various hepadnaviruses, including human (AAP31571.1), wooly monkey (AAO74859.1),

ground squirrel (AAB08031.1), woodchuck (AAA46761.1), bat (AGT17576.1), duck (AAO49490.1), heron (AAA45737.1), parrot (AFY97786.1), Ross's goose (AAR89928.1) and snow goose (AAD22001.1). See Fig. 1. Similar to the HBcARD peptide of human hepatitis B virus, HBcARD peptides of other mammalian hepadnaviruses (woolly monkey, 5 ground squirrel, woodchuck and bat) contained four clustering arginine-rich domains. While the sequence homology of HBcARD peptides between avian and mammalian hepadnaviruses was low, they both contained four highly positive-charged domains. To compare their respective antimicrobial activities, we determined the minimal bactericidal concentrations of four HBcARD peptides derived from bat, woodchuck, duck and heron hepadnaviruses. The 10 results indicated that peptides derived from bat and woodchuck hepadnaviruses exhibited potent antimicrobial activity comparable to that from human hepatitis B virus. See Table 2. In contrast, peptides derived from avian hepadnaviruses displayed lower antimicrobial activity. Because HBcARD peptides from human HBV is shorter in length (147-183; 37 amino acids), we focused on this peptide in our subsequent modification and optimization 15 experiments.

Table 2. Antimicrobial activity of human, bat, woodchuck, duck and heron HBcARD peptides against Gram-positive and Gram-negative bacteria

Bacterial strain ^a	Minimal bactericidal concentration (mg/L)				
	Human HBcARD	Bat HBcARD	Woodchuck HBcARD	Duck HBcARD	Heron HBcARD
<i>P. aeruginosa</i> ATCC 9027	9.2	10	9.8	>70.6	>69.3
<i>K. pneumoniae</i> ATCC 13884	9.2	5	9.8	>70.6	>69.3
<i>E. coli</i> ATCC 25922	18.4	20	19.6	ND	ND
<i>A. baumannii</i> ATCC 17978	2.3	2.5	4.9	70.6	69.3
<i>S. aureus</i> ATCC 19636	18.4	20	19.6	ND	ND

^a ATCC: American Type Culture Collection; ND: not detectable

20 Comparison of antimicrobial activity of L- and D-HBcARD peptides

We used PeptideCutter to predict the potential protease cleavage sites on the HBcARD 147-183 peptide (37-mer). The results showed that more than 70% of the protease cleavage sites were mapped to the arginine residues of HBcARD peptide (data not shown).

To improve the peptide stability in the serum, we synthesized a modified peptide, D-HBcARD 147-183 peptide, by D-amino acid replacement, in which all arginine residues were replaced with its D-isomer.

To investigate whether D-arginine substitution will improve the antimicrobial activity, 5 L- and D-HBcARD peptides (37-mer) were tested side-by-side against a wide variety of bacteria, including *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, *E. coli*, *S. dysenteriae* and *S. aureus*. Relative to the L-HBcARD, the D-HBcARD peptide displayed similar antimicrobial activity against *A. baumannii* (MBC=2.3-4.6 mg/L), and better antimicrobial activity against *P. aeruginosa* (MBC=4.6 mg/L), *K. pneumoniae* (MBC=4.6 mg/L), and *S. aureus* 10 (MBC=4.6-9.2 mg/L). However, for reasons that remain unclear, the antimicrobial activity of D-HBcARD peptide against *E. coli* and *S. dysenteriae* was decreased from 18.4 to 73.6 mg/L. See Table 3.

Table 3. Comparison of minimal bactericidal concentration (MBC) between wild type HBcARD 147-183 peptide L-HBcARD and D-HBcARD

15

Bacterial strain ^a	Minimal bactericidal concentration (mg/L)	
	L-HBcARD (37-mer)	D-HBcARD (37-mer)
Gram-negative		
<i>P. aeruginosa</i> ATCC 9027	9.2	4.6
<i>P. aeruginosa</i> ATCC 27853	9.2-18.4	4.6
<i>K. pneumoniae</i> ATCC 13884	9.2	4.6
<i>E. coli</i> ATCC 25922	18.4	73.6
<i>A. baumannii</i> ATCC 17978	2.3	2.3
<i>A. baumannii</i> ATCC 17978CR ^b	2.3	2.3
<i>A. baumannii</i> ATCC 19606	2.3	2.3
<i>A. baumannii</i> ATCC 19606CR ^b	2.3	2.3
<i>A. baumannii</i> TCGH 45530 ^b	2.3	2.3
<i>A. baumannii</i> TCGH 46709 ^b	4.6	4.6
<i>S. dysenteriae</i> Xen27 ^c	18.4	73.6
Gram-positive		
<i>S. aureus</i> ATCC 19636	18.4	9.2
<i>S. aureus</i> ATCC 25923	18.4	4.6
<i>S. aureus</i> ATCC 29213	18.4	~4.6

^a ATCC: American Type Culture Collection

^b Colistin-resistant *A. baumannii* (Laboratory induced and Clinical isolates from Tzu Chi General Hospital (Chen et al. PLoS Pathog. 2013;9(6):e1003425.)

^c Caliper Life Sciences, Inc.

Comparison of serum resistance between L-HBcARD and D-HBcARD

To compare the stability of the 37-mer L- and D-HBcARD peptides against serum protease degradation, we performed a protease resistance assay. After incubation with 5% bovine serum at 37°C for 3 hours, more than 80% of L-HBcARD peptide was no longer detectable by staining with Green Angel on SDS-PAGE. In contrast, the signal intensity of the D-HBcARD peptide was not reduced under the same condition. See Fig. 2, A. Similar results were obtained, when L-HBcARD was incubated with 5% mouse or human (male and female) sera. See Fig. 2, B and C. These results suggested that D-HBcARD was more resistant than L-HBcARD to protease digestion of fetal bovine, mouse and human serums.

Furthermore, we determined the *in vitro* antimicrobial activity of L- and D-HBcARD peptides in the presence of serum. The results showed that both L-HBcARD and D-HBcARD peptides at 18.4 mg/L concentration were able to kill *S. aureus* in the MBC assay. See Fig. 2, D. Upon the addition of 5% mouse serum, there was a significant difference in the antimicrobial activities between L- and D-HBcARD peptides (P<0.0001). While the treatment with L-HBcARD peptide in the presence of 5% mouse serum produced 10⁷ bacterial colonies, treatment with D-HBcARD peptide produced around 10³ colonies. Therefore, our D-HBcARD peptide exhibited higher antimicrobial activity than that of the L-HBcARD peptide in the presence of 5% mouse serum. This observation suggested that D-HBcARD was more stable and resistant to proteolytic degradation.

Hemolytic activity of D-HBcARD peptide

In the hemolysis assay, human RBCs were incubated with serially-diluted doses (0 to 460 mg/L) of D-HBcARD, L-HBcARD, or melittin, at 37°C for one hour. As shown in Fig. 3, melittin caused 100% hemolysis at the concentration of 8.9 mg/L. In contrast, no hemolytic activity of both D- and L-HBcARD peptides was detected up to 460 mg/L (P<0.0001).

Comparison of *in vivo* protection efficacies between L- and D-form HBcARD peptides in a mouse sepsis model infected with *S. aureus*

To investigate the *in vivo* protection efficacy of the 37-mer L- and D-HBcARD peptides, we performed a mouse sepsis model infected with *S. aureus*. ICR mice were first i.p. inoculated with *S. aureus* (4x10⁶ CFU/mouse), followed by one single i.p. injection of L-HBcARD, D-HBcARD and PBS at two hours post-inoculation, respectively. All mice (n=10)

administered with PBS died at day 1. See Fig. 4, A. At 7 days post-inoculation, 40% of mice treated with L-HBcARD (10 mg/kg) survived. See Fig. 4, A. In contrast, D-HBcARD peptide was able to achieve a much higher survival rate (60% for 5 mg/kg dose, and 100% for 10 mg/kg dose). See Fig. 4, A. These results indicated that the *in vivo* efficacy of HBcARD 5 can be improved by the D-arginine replacement strategy (P<0.0001).

Comparison of the immunogenicity between L- and D-form HBcARD peptides in a mouse sepsis model infected with *S. aureus*

To investigate whether repeated treatments with the 37-mer HBcARD peptides may compromise the efficacy by inducing neutralization antibody *in vivo*, we immunized 3-week 10 old mice three times with L- and D-HBcARD peptides, respectively, prior to bacterial infection. Two weeks after the first immunization, immunized mice were then inoculated with *S. aureus*, followed by i.p. injection with either L- or D-HBcARD peptides 1 hour post-inoculation. Consistent with our previous report (Chen et al., 2013), L-HBcARD still 15 protected all mice from death, irrespective of the prior immunizations with or without L-HBcARD peptide. See Fig. 4, B. Control mice immunized with PBS and treated with PBS showed a mortality near 80% within 24 hours after bacterial challenge (P<0.05). Seven days post-inoculation, all mice treated with L- and D-HBcARD (10 mg/kg) survived bacterial 20 challenge, irrespective of the prior immunization with or without D-HBcARD. See Fig. 4, C. Therefore, prior immunizations with either D-form or L-form peptides induced no neutralization activity against the *in vivo* antimicrobial activity of subsequent treatments with HBcARD peptides.

Significance of terminal cysteine

We tested the antimicrobial activity of additional HBcARD derivatives. See Table 1. The antimicrobial activities of these peptides were determined by minimal bactericidal 25 concentration (MBC). Unlike the parental peptide HBcARD 147-183 (37-mer) (see Chen et al., 2013), the derivative peptide HBcARD 150-177Q (28-mer) showed no detectable bactericidal activity against *S. aureus*. See Table 4. To mimic the parental peptide HBcARD 147-183 with a cysteine at the carboxyl terminus, we designed another 28-mer derivative HBcARD 150-177C by replacing the terminal Q (glutamine) residue of HBcARD 150-177Q 30 with a C (cysteine) residue. See Table 1. Interestingly, this Q-to-C substitution effectively rescued the bactericidal activity against *S. aureus*. See Table 4.

Table 4. Minimal bactericidal concentrations (MBC) of modified HBcARD peptides against various Gram-negative and Gram-positive bacteria

Bacteria strains	MBC (uM)				
	150-177C	150-177Q	150-171C	157-177C	164-177C
Gram-negative					
<i>P. aeruginosa</i> ATCC9027	2	—	—	—	—
<i>P. aeruginosa</i> ATCC27853	2	—	—	—	—
<i>E. coli</i> ATCC25922	2	—	—	—	—
<i>A. baumannii</i> ATCC17978	0.5	—	—	—	—
<i>A. baumannii</i> ATCC19606	0.25	—	—	—	—
<i>A. baumannii</i> ATCC45530	0.5	—	—	—	—
<i>A. baumannii</i> ATCC46709	1	—	—	—	—
Gram-positive					
<i>S. aureus</i> ATCC19636	2	ND	ND	ND	ND

—, not determined; ND, no detectable antimicrobial activity

5 Significance of length or arginine content

Given the fact that the terminal cysteine appears to be important for the bactericidal activity, we asked if the total length of the HBcARD 150-177C peptide (28-mer) can be further reduced. We compared the potencies against *S. aureus* among peptides 150-171C (20-mer), 157-177C (21-mer), and 164-177C (14-mer). As shown in Table 4, none of these peptides showed detectable activity. The results here indicated that shortened peptides with a reduced number of arginines exhibited no antimicrobial activity against *S. aureus*. See Table 4. On the other hand, we have shown that shorter peptides containing 3 variable domains are sufficient to kill other bacteria (data not shown).

The 28-mer peptide D-150-177C improved protection efficacy in a sepsis mouse model

15 We compared the *in vivo* protection efficacy of HBcARD 150-177C peptides (28-mer) containing either D-form or L-form arginines. See Table 1 and Fig. 5. ICR mice (n=10/group) were ip. inoculated with *S. aureus* ATCC19636 (4x10⁶/mouse), followed by treatment with modified peptides at two hours post-inoculation. All PBS control mice died

on day 1, and only 20% of the mice treated with control peptide (150-177Q) survived. Administration of L-150-177C peptide at the dose of 5 and 10 mg/kg protected 30% and 70% mice from death, respectively. See Fig. 5. When treated with 5 mg/kg of D-150-177C, all mice (100%) survived.

5 **The 28-mer peptide D-150-177C protected mice from lung infection with colistin-resistant *A. baumannii***

BALB/c mice (n=8/group) were intra-tracheally inoculated with colistin -resistant *A. baumannii* TCGH 46709 (3.4×10^8 cfu/mouse). These lung-infected mice were ip. treated with colistin (5 mg/kg/day) or D-150-177C (5 and 10 mg/kg/day), respectively. All mice treated with colistin died at 60 hours post-inoculation with drug-resistant *A. baumannii*. See Fig. 6. In contrast, there was a dose-dependent protection effect from D-150-177C. A significant difference was observed when the mice were treated with 10 mg/kg/day of D-150-177C peptide ($p < 0.05$). See Fig. 6.

15 **Comparison of *in vivo* toxicity between peptide D-150-177C and polymyxin B**

Using the sepsis mouse model, we examined the survival rates of ICR mice intra-peritoneally (i.p.) injected with the 28-mer D-150-177C peptide (20 – 80 mg/kg body weight) and polymyxin B (a colistin-related compound) (50 mg/kg body weight). See Fig. 7, A. All mice treated with polymyxin B (50 mg/kg) died at day one, as expected from the notorious nephrotoxicity of colistin and polymyxin B to kidney. See Fig. 7, A. In contrast, no acute toxicity was observed in the mice treated with 20 and 40 mg/kg D-150-177C. See Fig. 7, A. When the doses were increased to 60 and 80 mg/kg, survival rates of mice were decreased to 80% and 40%, respectively. See, Fig. 7, A. Liver injury can be detected by the serum ALT level. Mice treated with D-150-177C in the dose range of 20 to 40 mg/kg showed a higher ALT level than polymyxin B, but the p value is insignificant. See Fig. 7, B. The ALT levels of mice treated with 60 mg/kg were significantly higher than those treated at 20 mg/kg polymyxin B ($p < 0.01$). See Fig. 7, B.

20 **Comparison of *in vivo* protection efficacies among various modified 150-177C peptides**

30 The 28-mer peptide D-150-177C contains a total of 14 L-arginines substituted with 14 D-arginines. See Table 1 and Fig. 6. D-arginine is far more expensive than L-arginine. To reduce the cost of peptide synthesis, we compared the *in vivo* protection efficacies between peptides containing complete or partial D-arginine substitution. See Table 1 and Fig. 8.

Peptide DL-150-177C is only partially D-arginine substituted, and exhibited very similar protection efficacy to all-D-arginine substituted peptide D-150-177C (100% substitution). See Fig. 8. In contrast, the protection efficacy of peptide LD-150-177C (also partially substituted) appeared to be less than DL-150-177C. See Fig. 8.

5 Serine and threonine are dispensable

We engineered a new 22-mer peptide, DL-dST-150-177C (see Table 1), with two major modifications: (1) partial-D-arginine substitution by using the backbone of peptide DL-150-177C; and (2) deletion of 5 serine and one threonine residues from the parental 28-mer peptide DL-150-177C. This 22-mer peptide DL-dST-150-177C showed remarkably improved protection efficacy compared to the 28-mers DL-150-177C and L-150-177C. See Fig. 9.

Bacterial isolates

The antimicrobial activities of HBcARD peptides were tested using a number of bacterial strains, including *Pseudomonas aeruginosa* Migula strains (ATCC 27853, ampicillin-resistant and ATCC 9027, ampicillin-resistant), *Klebsiella pneumoniae* strain (ATCC 13884), *Shigella dysenteriae* Xen27 (Caliper Co.), *Escherichia coli* strain (ATCC 25922), *Staphylococcus aureus* subsp. strains (ATCC 25923, methicillin-resistant ATCC 29213, methicillin-resistant and ATCC 19636, methicillin-resistant) and *Acinetobacter baumannii* strains (ATCC 17978, ATCC 17978 CR, ATCC 19606, ATCC 19606 CR, TCGH 45530 and TCGH 46709). Clinical isolates TCGH 45530 and TCGH 46709 were obtained from Tzu-Chi Buddhist General Hospital (TCGH) in Taiwan, and were identified using the Vitek system (Biomerieux Vitek, Inc., Hazelwood, MO, USA). See Chang et al. (2012), J Microbiol Immunol Infect 45:37-42 doi:10.1016/j.jmii.2011.09.019.

Antimicrobial assay

25 L- and D-HBcARD peptides were purchased from Yao-Hong Biotechnology Inc. (Taipei, Taiwan). Antimicrobial activity was determined as described. See, Chen et al., 2013. Briefly, bacteria were grown in MH broth (Difco) to mid-logarithmic phase at 37°C, and were diluted to 10⁶ CFU (colony formation unit)/ml in phosphate buffer (10 mM sodium phosphate and 50 mM sodium chloride, pH 7.2). Peptides were serially diluted in the same buffer. Fifty microliters (μl) of bacteria were mixed with fifty μl of peptides at varying

concentrations, followed by incubation at 37°C for 3 hours without shaking. At the end of incubation, bacteria were placed on Mueller-Hinton broth agar plates, and allowed to grow at 37°C overnight for measurement of minimal bactericidal concentration (MBC). The lowest peptide concentration that displayed no bacterial growth (zero colony) was defined as MBC.

5 All peptides were tested in triplicate.

Stability to proteases

L- and D-HBcARD peptides (0.5 nmol) were mixed with MBC buffer in the presence or absence of 5% serum collected from bovine, mouse and human origins. After incubation at 37°C for 3 hours, the amounts of peptides surviving the protease digestion were

10 determined by SDS-PAGE electrophoresis and staining with Green Angel. The images were quantified using image J software and the intensities were normalized with the no serum control. To investigate the effect of serum on the antimicrobial activity of HBcARD peptides, L- and D-HBcARD peptides at their respective MBC concentrations were incubated with *S. aureus* ATCC19636 strain (10⁶ CFU/ml) at 37°C for three hours. Bacteria were plated on

15 MH agar and the antimicrobial activity was determined by colony formation.

Hemolytic activity

The hemolytic activities of peptides were determined by hemolysis against human red blood cells (hRBCs). Human blood was obtained in EDTA-containing tube and was centrifuged at 450 g for 10 min. The pellet was washed three times with PBS buffer, and a solution of 10% hRBCs was prepared. hRBCs solution was mixed with serial dilutions of peptides in PBS buffer, and the reaction mixtures were incubated for 1 h at 37°C. After centrifugation at 450 g for 10 min, the percentage of hemolysis was determined by measuring the absorbance at the wavelength of 405 nm of the supernatant. Blank and 100% hemolysis were determined in PBS buffer and in the presence of 1% Triton X-100, respectively.

25 ***In vivo* animal studies**

Three-week old male ICR mice (19 to 21 g) were purchased from BioLASCO (Taiwan). To test the *in vivo* protection efficacy of the HBcARD peptides, all mice were inoculated intraperitoneally with *S. aureus* ATCC 19636 (4 x 10⁶ CFU/mouse). HBc147-183 (L- or D-HBcARD) or the PBS control was administered intraperitoneally at 2 hours after bacterial inoculation, respectively. Each group contained 10 mice. Mortality was monitored

daily for 7 days following the bacterial inoculation.

To investigate the potential immunogenicity of HBcARD peptides, we determined the *in vivo* antimicrobial activity of HBcARD peptide in peptide-immunized mice. Briefly, three week-old male mice were immunized three times with 0.2 ml of L- and D-HBcARD peptides (5 mg/kg) at day 0, 3, 6, respectively. Immunized mice were inoculated with *S. aureus* ATCC 19636 (4 x 10⁶ CFU/mouse) at day 14, and were administered with 0.2 ml of PBS, or L- and D-HBcARD peptide (10 mg/kg) one-hour postinoculation. Another group of mice received the identical protocol with PBS as a control. Each group contained 5 mice. Mortality was monitored daily for 7 days following the bacterial inoculation.

10 **Statistical analysis**

Statistical analysis was performed using Graphpad software. The results were shown in mean ± SD, and the difference between individual groups was analyzed by student t test. Survival curves were plotted by Kaplan-Meier method and analyzed by log-rank test.

15

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless explicitly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

20

From the above description, one skilled in the art can easily ascertain the essential characteristics of the described embodiments, and without departing from the spirit and scope thereof, can make various changes, derivatives, and modifications of the embodiments to adapt it to various usages and conditions. Thus, other embodiments are also within the 25 claims.

WHAT IS CLAIMED IS:

1. An antimicrobial peptide, the peptide comprising 2 to 20 variable domains, each variable domain is a sequence of 2 to 20 consecutive basic amino acids, wherein (a) the 5 variable domains are separated from each other by a variable linker, (b) the variable linker can have 1 to 20 any amino acids other than two or more consecutive basic amino acids, and (c) the peptide has no more than 100 amino acids.

2. The antimicrobial peptide of claim 1, wherein the peptide has at least 3 10 variable domains.

3. The antimicrobial peptide of claim 1, wherein the peptide has a C-terminal cysteine.

4. The antimicrobial peptide of claim 1, wherein at least one of the basic amino acids in the variable domains is an arginine.

5. The antimicrobial peptide of claim 4, wherein all of the basic amino acids in each variable domain are arginine residues.

20 6. The antimicrobial peptide of claim 1, wherein at least one of the basic amino acids is a chemically-modified amino acid.

7. The antimicrobial peptide of claim 6, wherein the chemically-modified amino 25 acid is a D-amino acid.

8. The antimicrobial peptide of claim 7, wherein the D-amino acid is D-arginine.

9. The antimicrobial peptide of any of claims 1-8, wherein the variable domains 30 and the variable linkers are derived from the arginine-rich domain of a hepadnavirus core protein (HBcARD).

10. The antimicrobial peptide of claim 9, wherein the HBcARD contains a sequence from residue 147 to the C-terminal residue of a hepadnavirus core protein.

11. The antimicrobial peptide of claim 10, wherein each variable domain has three 5 or four arginine residues and each variable linker has 2 to 4 amino acids.

12. The peptide of claim 11, wherein the peptide contains a consensus sequence selected from the group consisting of:

(i) (X₁)GRX₂P(X₃)X₄PX₅P(X₆)X₇QX₈P(X₉), wherein each of X₁, X₃, X₆, and X₉,

10 individually, is a variable domain, and each of X₂, X₄, X₅, X₇, and X₈, individually, is any amino acid or absent,

(ii) (X₁)GRX₂P(X₃)X₄PX₅P(X₆), wherein each of X₁, X₃, and X₆, individually, is a variable domain, and each of X₂, X₄, and X₅, individually, is any amino acid or absent, and

(iii) (X₁)X₂PX₃P(X₄)X₅QX₆P(X₇), wherein each of X₁, X₄, and X₇, individually, is a variable domain, and each of X₂, X₃, X₅, and X₆, individually, is any amino acid or absent,

13. The antimicrobial peptide of claim 12, wherein the peptide has a C-terminal cysteine.

20 14. The antimicrobial peptide of claim 12, wherein the peptide has a consensus sequence selected from the group consisting of:

(i) (X₁)GRX₂P(X₃)X₄PX₅P(X₆)X₇QX₈P(X₉)X₁₀C, and

(ii) (X₁)GRX₂P(X₃)X₄PX₅P(X₆)X₇QX₈P(X₉)X₁₀Q,

wherein each of X₁, X₃, X₆, and X₉, individually, is a variable domain, and each of X₂, X₄,

25 X₅, X₇, X₈, and X₁₀, individually, is any amino acid or absent.

15. The antimicrobial peptide of claim 12, wherein the peptide contains a sequence selected from the group consisting of:

30 (i) TVVRRRGRSPRRRTPSPRRRSQSPRRRSQSRESQC, in which at least one of the arginine residues is D-arginine,

(ii) RRRGRSPRRRTPSPRRRSQSPRRRSQC,

(iii) RRRGRSPRRRTPSPRRRSQSPRRRSQ,

- (iv) RRRGRPRRRPPRRRQPRRRRC,
- (v) RRRGRSPRRRTPSPRRRRC,
- (vi) RRRGRPRRRPPRRRRC,
- (vii) RRRTPSPRRRSQSPRRRRC, or
- (viii) RRRPPRRRQPRRRRC.

5

16. The antimicrobial peptide of claim 15, wherein the peptide contains the sequence of RRRGRSPRRRTPSPRRRSQSPRRRSC, in which each of the arginine residues in the sequence is L-arginine.

10

17. The antimicrobial peptide of claim 15, wherein the peptide contains the sequence of RRRGRSPRRRTPSPRRRSQSPRRRSC, in which at least one of the arginine residues in the sequence is D-arginine.

15

18. The antimicrobial peptide of claim 17, wherein each of the arginine residues in the sequence is D-arginine.

19. The antimicrobial peptide of claim 17, wherein the sequence is rRrGRSPrRrTPSPrRrRSQSPrRrRSC, in which R is L-arginine and r is D-arginine.

20

20. The antimicrobial peptide of claim 17, wherein the sequence is RrRGRSPRrRTPSPRrRrSQSPRrRrSC, in which R is L-arginine and r is D-arginine.

25

21. The antimicrobial peptide of claim 15, wherein the peptide contains the sequence of RRRGRPRRRPPRRRQPRRRRC, in which at least one of the arginine residues in the sequence is D-arginine.

22. The antimicrobial peptide of claim 15, wherein the sequence is rRrGRPrRrPPrRrRQPrRrRC, in which R is L-arginine and r is D-arginine.

30

23. The antimicrobial peptide of any of claims 15-22, wherein the sequence is at the C-terminus of the peptide.

24. The antimicrobial peptide of any of claims 1-23, further comprising a non-HBcARD peptide.

25. The antimicrobial peptide of claim 24, wherein the non-HBcARD peptide is an affinity tag, a signal sequence, a ligand, or another antimicrobial peptide or fragment thereof.

26. The antimicrobial peptide of claim 25, wherein the non-HBcARD peptide is a poly-histidine or an analog thereof.

10

27. The antimicrobial peptide of claim 26, wherein the peptide has a sequence selected from the group consisting of:

- (i) RRRGRSPRRRTPSPRRRSQSPRRRSHHHHHH,
- (ii) HHHHHHRRGRSPRRRTPSPRRRSQSPRRRS,
- (iii) RRRGRPRRPPRRRQPRRRHHHHHH, and
- (iv) HHHHHHRRGRPRRPPRRRQPRRRR

28. The antimicrobial peptide of any of claims 1-4, wherein at least one variable domain has a lysine.

20

29. The antimicrobial peptide of any of claims 1-4, wherein at least one variable domain has a histidine.

30. The peptide of any of claims 1-29, wherein the peptide has a cyclic structure.

25

31. The antimicrobial peptide of any of claims 1-30, wherein the peptide exhibits a broad spectrum antimicrobial activity against a gram-positive bacteria, gram-negative bacteria, fungus, parasite, or virus.

30

32. An antimicrobial peptide conjugate, the conjugate comprising the peptide of any of claims 1-31 and a non-peptide moiety.

33. A pharmaceutical composition comprising the antimicrobial peptide of any of claims 1-31 and a pharmaceutically acceptable carrier.

34. A pharmaceutical composition comprising the antimicrobial peptide conjugate 5 of claim 32 and a pharmaceutically acceptable carrier.

35. A method of treating a microbial infection, the method comprising administering the composition of claim 33 or 34 to a subject in need thereof.

FIG. 1

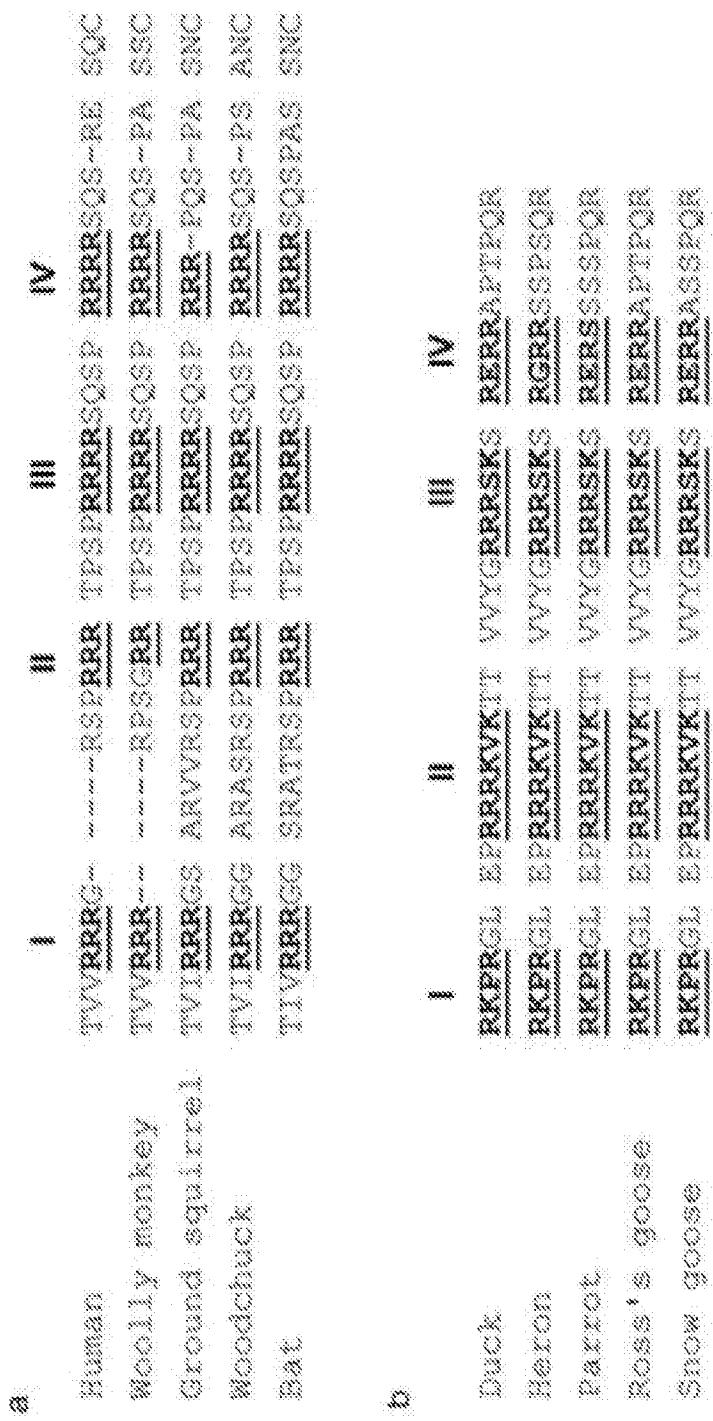
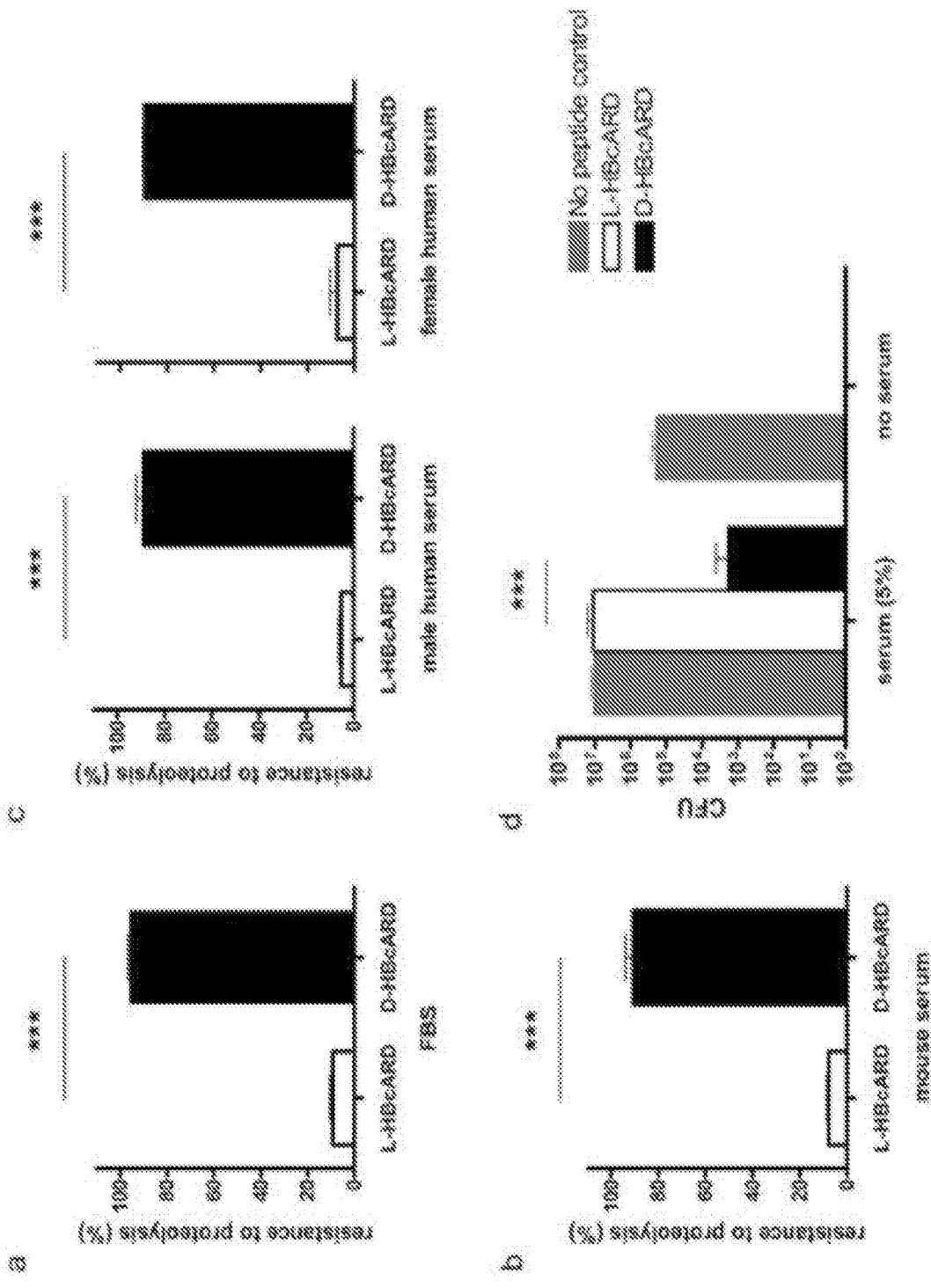


FIG. 2



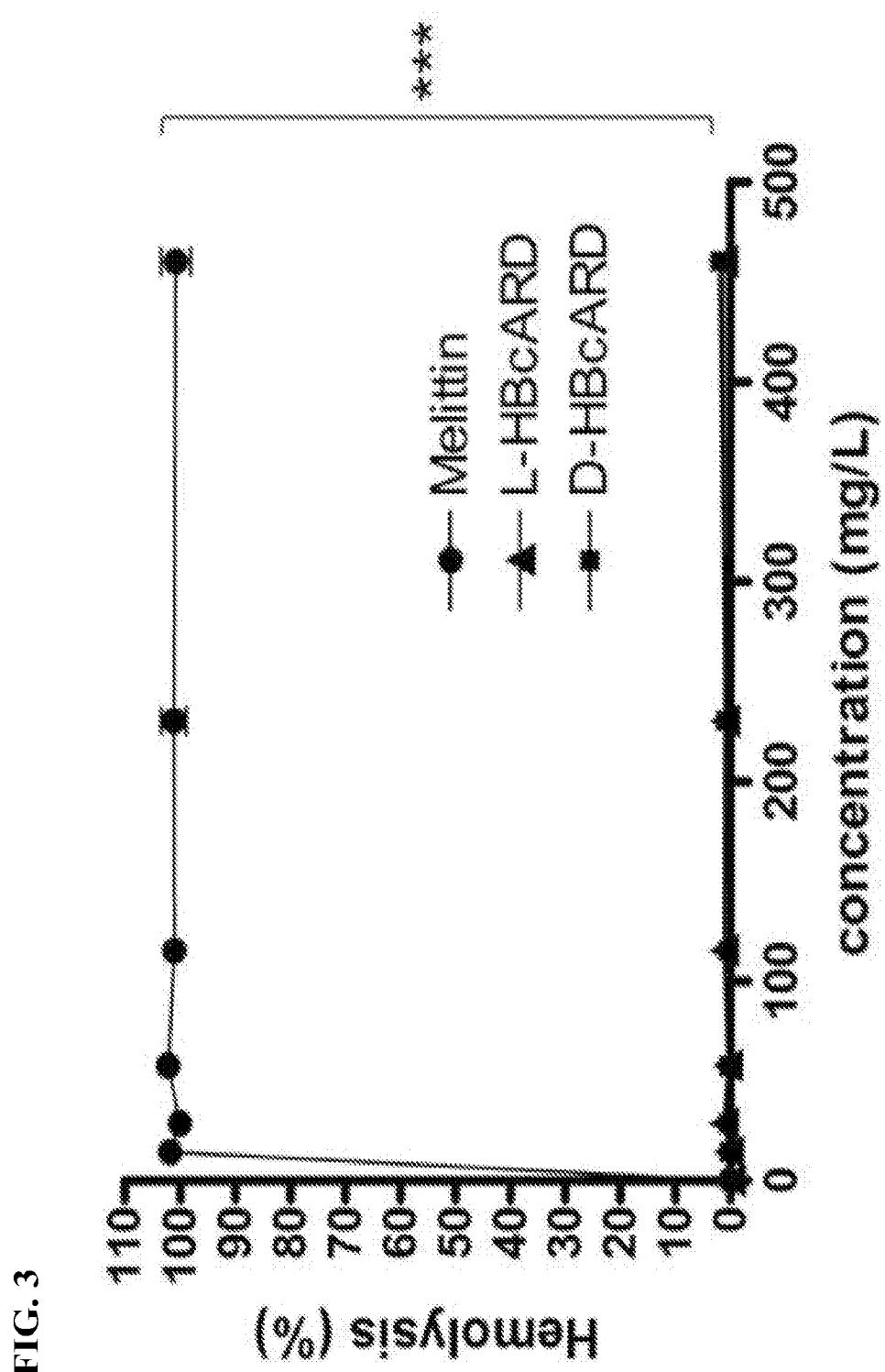
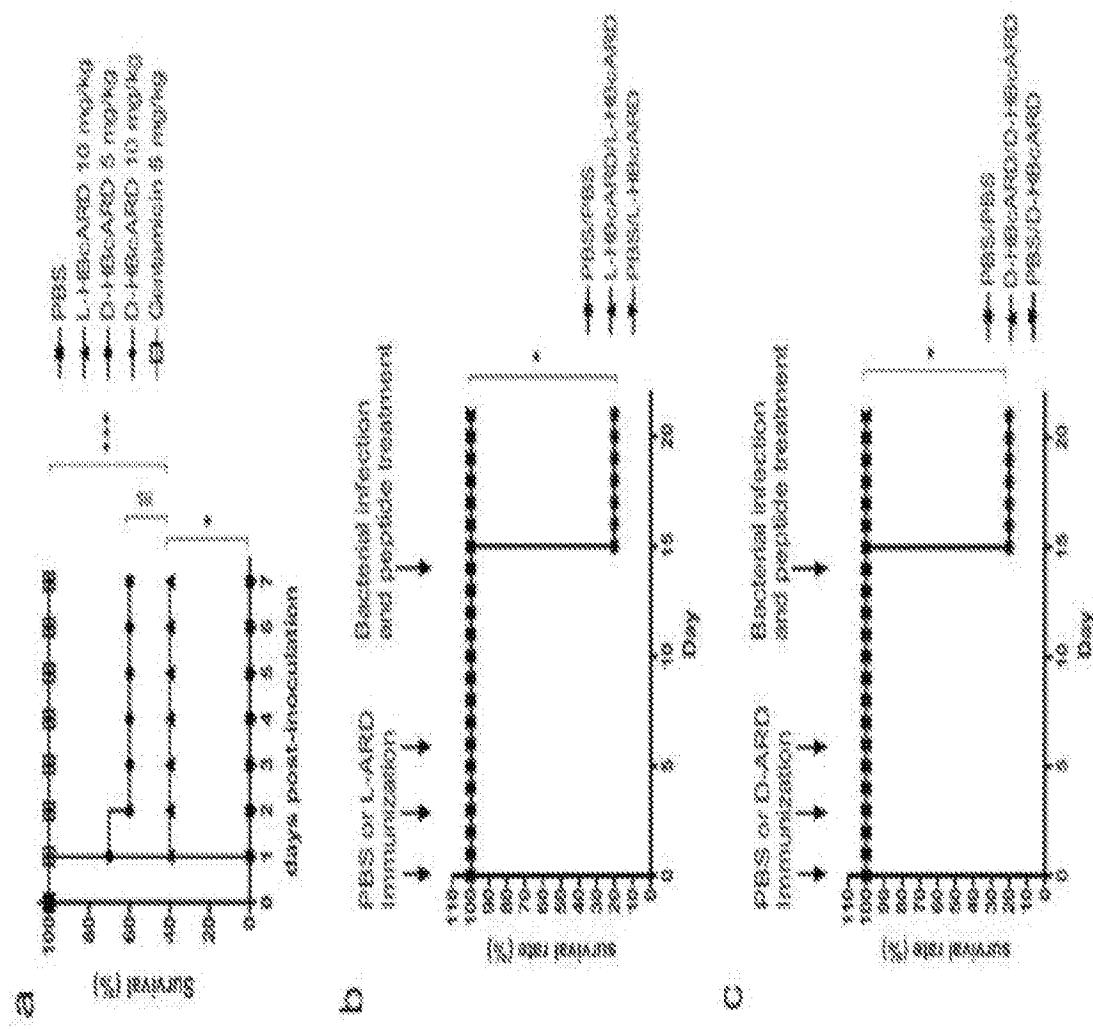
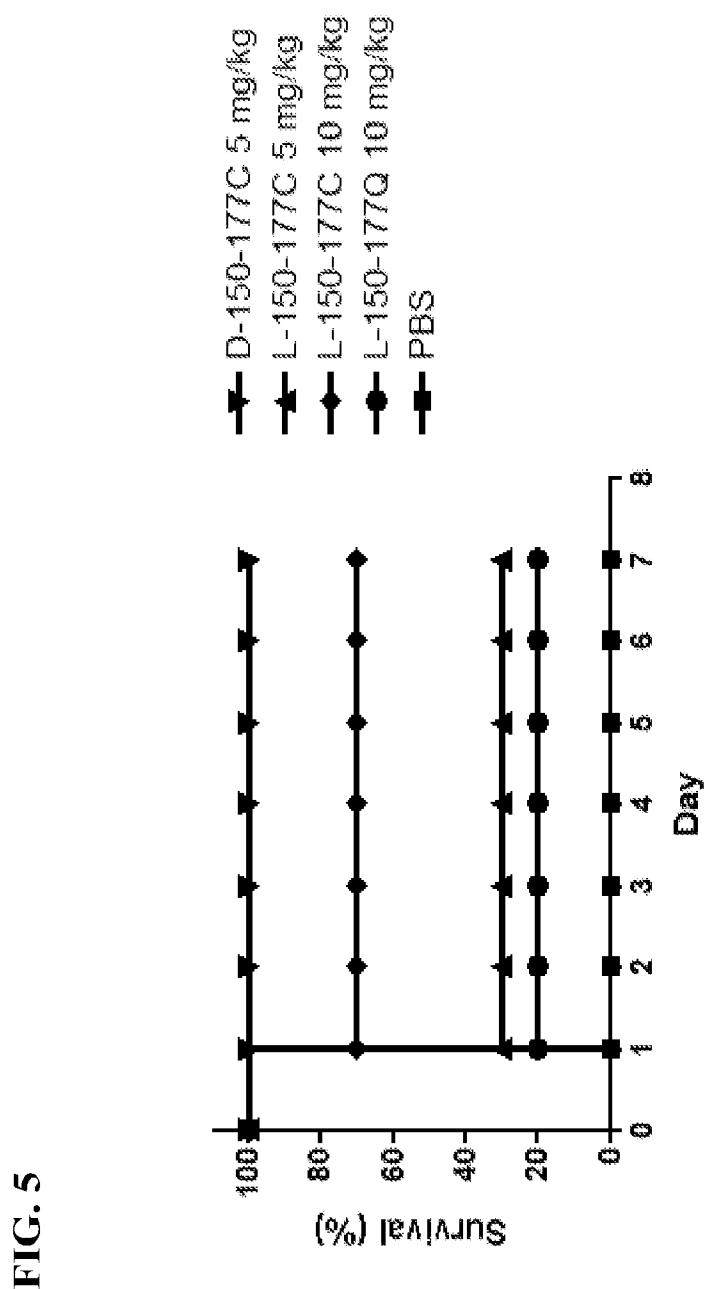


FIG. 3

FIG. 4





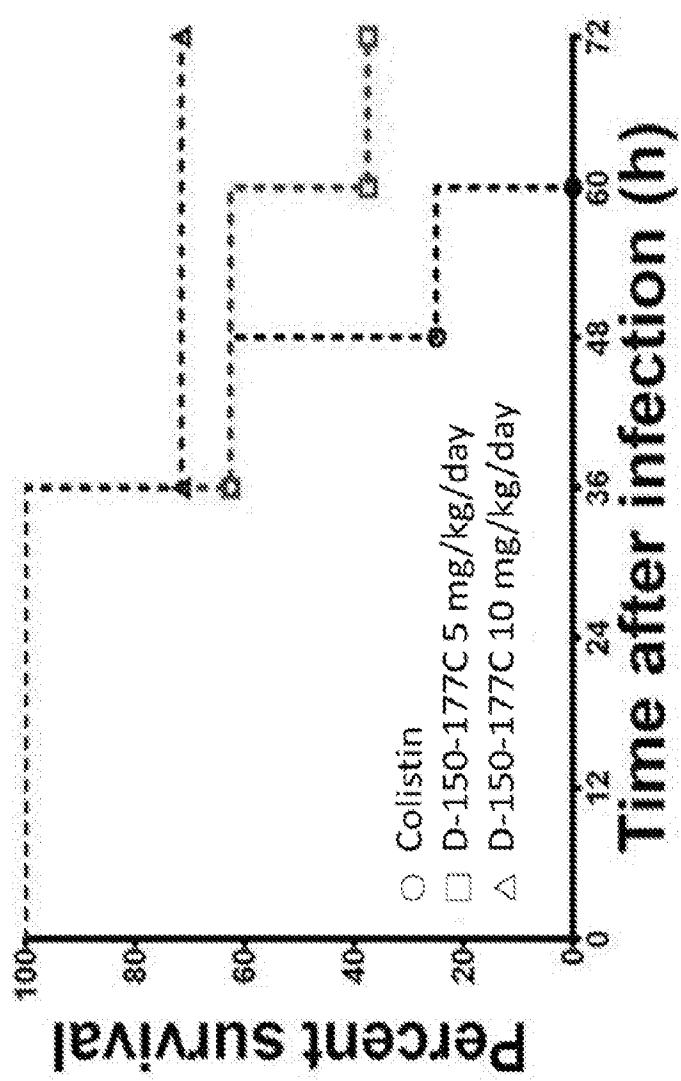
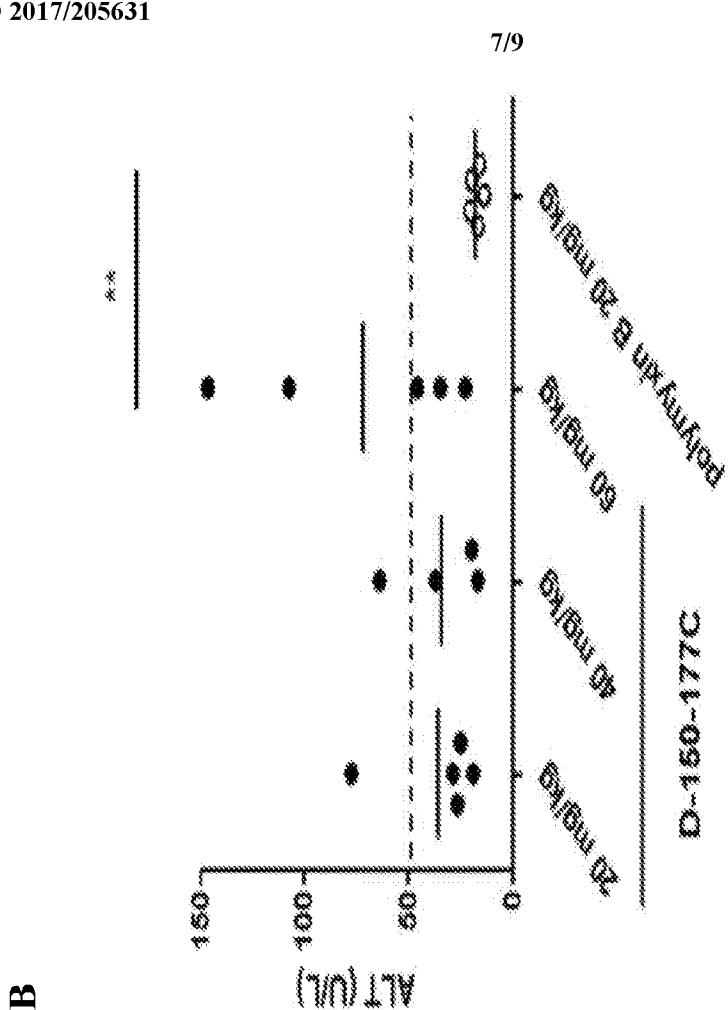
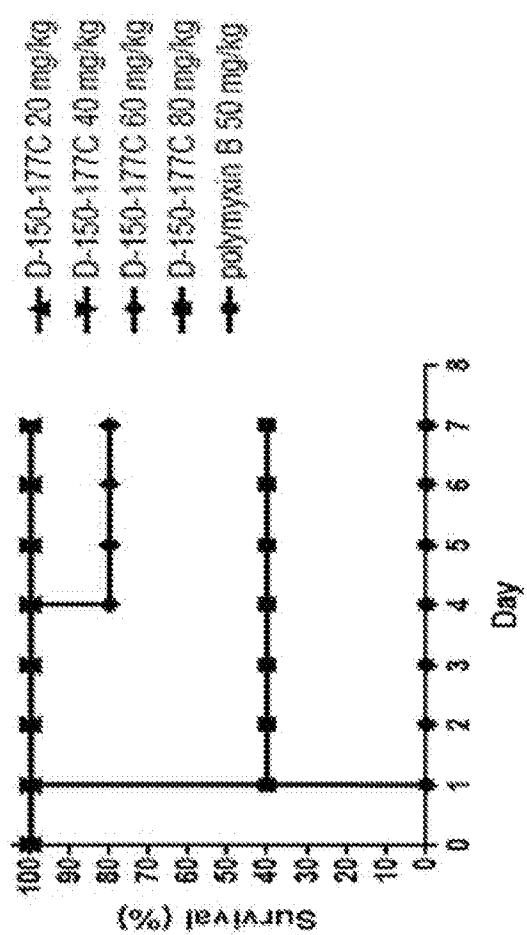
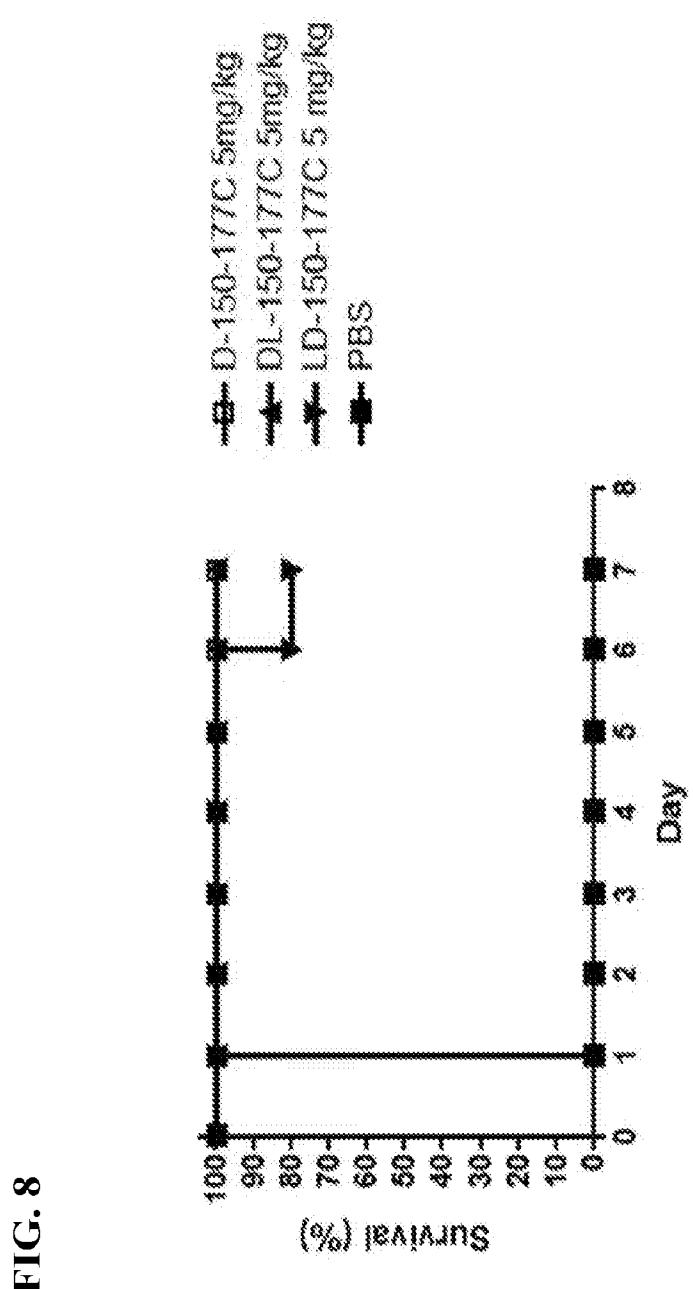


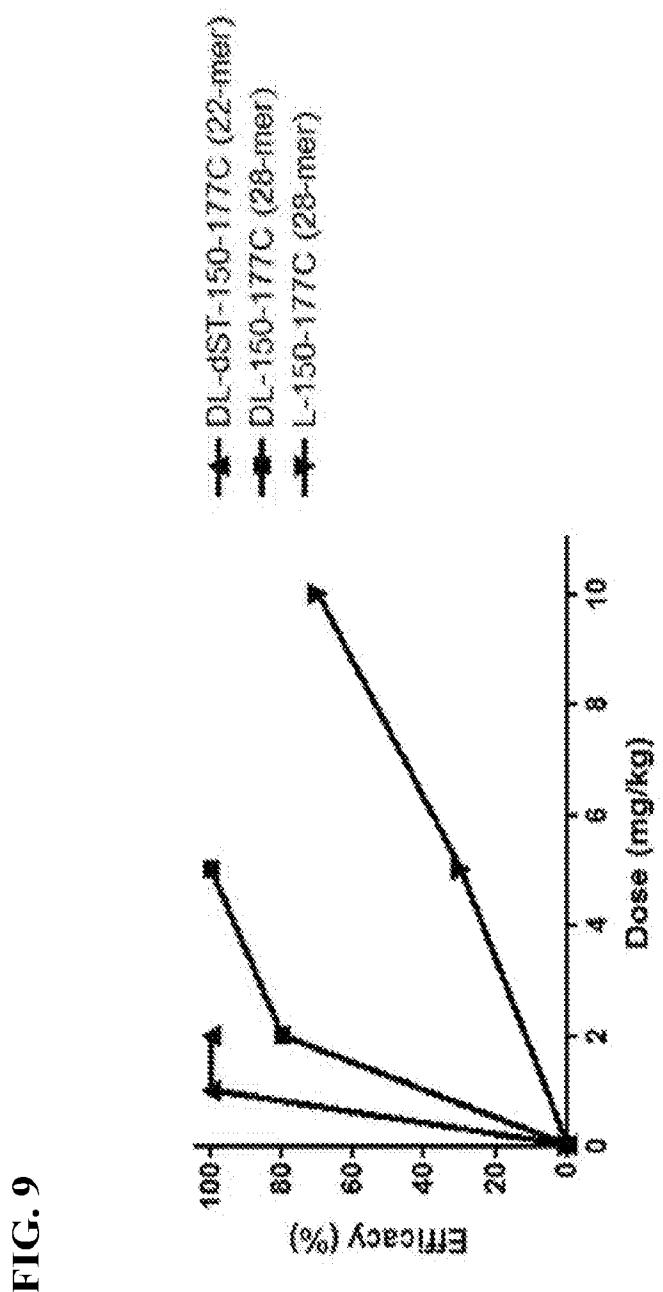
FIG. 6

FIG. 7

A







INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/034489

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/005(2006.01)i, C07K 7/08(2006.01)i, A01N 37/46(2006.01)i, A01N 63/00(2006.01)i

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)

C07K 14/005; C07K 7/08; C12N 7/00; A61K 38/16; A01N 37/46; A01N 63/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility modelsElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: antimicrobial peptide, hepatitis B virus core protein, arginine-rich domain, D-arginine replacement

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2016-0002300 A1 (LIANG et al.) 07 January 2016 See paragraphs [0038], [0115]; and claims 1, 8, 10.	1-6, 9-14, 28-29
Y		7-8, 15-22
Y	HMAMOTO et al., 'Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D□amino acid substitutions', Microbiology and Immunology, 2002, vol. 46, issue 11, pp. 741-749 See abstract; and pages 741-742.	7-8, 15-22
A	US 2003-0105006 A1 (MANN et al.) 05 June 2003 See abstract; claims 1, 3; and table 1.	1-22, 28-29
A	CHEN et al., 'Identification of a novel antimicrobial peptide from human hepatitis B virus core protein arginine-rich domain (ARD)', PLoS Pathogens, 13 June 2013, vol. 9, issue 6, article no. 1003425, internal pages 1-15 See abstract; pages 9-11; and figures 1, 3.	1-22, 28-29
A	ALBADA et al., 'Modulating the activity of short arginine-tryptophan containing antibacterial peptides with N-terminal metallocenoyl groups', Beilstein Journal of Organic Chemistry, 15 October 2012, vol. 8, pp. 1753-1764 See abstract; and table 1.	1-22, 28-29

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	
"A" 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 12 September 2017 (12.09.2017)	Date of mailing of the international search report 12 September 2017 (12.09.2017)
Name and mailing address of the ISA/KR International Application Division Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea Facsimile No. +82-42-481-8578	Authorized officer LEE GYONG CHEOL Telephone No. +82-42-481-8611

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/034489**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	CHEN et al., 'Improvement of in vivo antimicrobial activity of HBcARD peptides by D-arginine replacement', Applied Microbiology and Biotechnology, 30 May 2016 (online), vol. 100, no. 21, pp. 9125-9132 See the whole documents.	1-22, 28-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/034489

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 35
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 35 pertains to methods for treatment of the human body by therapy, and thus relates to a subject matter which this International Searching Authority is not required under PCT Article 17(2)(a)(i) and Rule 39.1(iv), to search.
2. Claims Nos.: 25-27,34
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 25-27 and 34 are not clear because they refer to multiple dependent claims, which do not comply with PCT Rule 6.4(a).
3. Claims Nos.: 23-24,30-33,35
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2017/034489

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2016-0002300 A1	07/01/2016	CN 105101796 A CN 105101796 B EP 2986115 A1 EP 2986115 A4 TW 201434855 A TW I495642 B WO 2014-124047 A1 WO 2014-124047 A4	25/11/2015 06/06/2017 24/02/2016 01/03/2017 16/09/2014 11/08/2015 14/08/2014 02/10/2014
US 2003-0105006 A1	05/06/2003	AT 373679 T AU 2822600 A AU 758150 B2 CA 2362153 A1 CA 2362153 C CN 1362969 A DE 60036456 T2 DK 1151009 T3 EP 1151009 A2 EP 1151009 B1 ES 2293892 T3 JP 2002-541066 A MX PA01007956 A US 6399570 B1 US 7244706 B2 WO 00-49040 A2 WO 00-49040 A3	15/10/2007 04/09/2000 13/03/2003 24/08/2000 24/11/2009 07/08/2002 19/06/2008 28/01/2008 07/11/2001 19/09/2007 01/04/2008 03/12/2002 19/08/2004 04/06/2002 17/07/2007 24/08/2000 14/12/2000