The Synergistic Antimicrobial Effects of Novel Bombinin and Bombinin H Peptides from the Skin Secretion of Bombina orientalis

Xiang, J., Zhou, M., Wu, Y., Chen, T., Shaw, C., & Wang, L. (2017). The Synergistic Antimicrobial Effects of Novel Bombinin and Bombinin H Peptides from the Skin Secretion of Bombina orientalis. Bioscience Reports. https://doi.org/10.1042/BSR20170967

Published in:
Bioscience Reports

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Publisher rights
Copyright 2017 the authors.
This is an open access article published under a Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution and reproduction in any medium, provided the author and source are cited.

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person’s rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Open Access
This research has been made openly available by Queen's academics and its Open Research team. We would love to hear how access to this research benefits you. – Share your feedback with us: http://go.qub.ac.uk/oa-feedback

Download date: 26. Oct. 2023
The Synergistic Antimicrobial Effects of Novel Bombinin and Bombinin H Peptides
from the Skin Secretion of Bombina orientalis

Jie Xiang, Mei Zhou, Yuxin Wu*, Tianbao Chen, Chris Shaw, Lei Wang

97 Lisburn Road, Natural Drug Discovery Group, School of Pharmacy, Queen's University
Belfast, Belfast BT9 7BL, Northern Ireland, UK.

The nucleotide sequence of the cDNA encoding BHL-bombinin and bombinin HL precursor
from the skin secretion of Bombina orientalis, has been deposited in the EMBL Nucleotide
Sequence Database under the accession code: LT615078

Corresponding: yuxin.wu@qub.ac.uk
Abstract

Bombinin and bombinin H are two antimicrobial peptide (AMP) families initially discovered from the skin secretion of Bombina that share the same biosynthetic precursor-encoding cDNAs, but have different structures and physicochemical properties. Insight into their possible existing relationship lead us to perform the combination investigations into their anti-infectious activities. In this work, we report the molecular cloning and functional characterisation of two novel AMPs belonging to bombinin and bombinin H families from secretions of Bombina orientalis. Their mature peptides (BHL-bombinin and bombinin HL), coded by single open-reading frame (ORF), were chemically synthesised along with an analogue peptide that replaced L-leucine with D-leucine from the 2nd position of the N-terminus (bombinin HD). Circular dichroism analysis revealed that all of them displayed well-defined α-helical structures in membrane mimicking environments. Furthermore, BHL-bombinin displayed broad-spectrum bactericidal activities on a wide range of micro-organisms, while bombinin H only exhibited a mildly bacteriostatic effect on the Gram-positive bacteria Staphylococcus aureus. The combination potency of BHL-bombinin with either bombinin HL or bombinin HD showed the synergistic inhibition activities against S. aureus (FICI 0.375). A synergistic effect has also been observed between bombinin H and ampicillin, which was further systematically evaluated and confirmed by in vitro time-killing investigations. Haemolytic and cytotoxic examinations exhibited a highly synergistic selectivity and low cytotoxicity on mammalian cells of these three peptides. Taken together, the discovery of the potent synergistic effect of AMPs in a single biosynthetic precursor with superior functional selectivity provides a promising strategy to combat multidrug-resistant pathogens in clinical therapy.

Keyword: peptide; skin secretion; antimicrobial; synergism; selectivity

1. Introduction

Bombinin, one of the typical cationic AMPs, was firstly isolated from the skin secretion of the yellow-bellied toad B. variegata [1]. The nucleotide sequence analysis of bombinin-related peptides
prompted the existence of a class of structurally differentiated peptides, which were named as bombinin H [2,3]. Importantly, the presence of a subtle and inconspicuous single D-amino acid (D-alloisoleucine or D-leucine) at the second position from N-terminus of bombinin H, as a consequence of post-translational modification, was observed. This type of modification may contribute to the versatile antimicrobial mechanisms of frog skin peptides, and may be beneficial to the prevention of bacterial resistance [4-6]. However, since the initial discovery of bombinin, bombinin H and D-isoform bombinin H, research have been focused on the study of the individual peptide’s antimicrobial property, instead of their synergistic potencies. Combined effects of bombinin peptides with conventional antibiotics, and their antimicrobial selectivity towards pathogens have very rarely been reported [7].

Here, we report the structural and functional characterisation of two novel, linear cationic α-helical AMPs, initially identified in a single ORF from the skin secretion of Bombina orientalis. These peptides belong to the bombinin and bombinin H families. The potent synergistic relationship of the novel bombinin and bombinin H peptides highlights the significance of combinational utility of AMPs in the treatment of infections caused by drug-resistant bacteria, this continues to provide researchers with novel approaches for prospective innovation in clinical studies.

2. Materials and Methods

2.1 Specimen preparation and secretion harvesting

Specimens of the oriental fire-bellied toad B. orientalis were obtained from a commercial supplier and raised in a specially designed vivarium until mature, over a period of 4 months. The skin secretions were collected and lyophilised as previously described [8]. Sampling of skin secretion was performed by Mei Zhou under UK Animal (Scientific Procedures) Act 1986, project license PPL 2694, issued by the Department of Health, Social Services and Public Safety, Northern Ireland. Procedures had been vetted by the IACUC of Queen’s University, Belfast, and approved on 1 March 2011.
2.2 Molecular cloning of novel bombinin and bombinin H precursor-encoding cDNA from the skin secretion-derived cDNA library

A five-mg lyophilised secretion of *B. orientalis* was dissolved in 1 ml of mRNA protection buffer, the polyadenylated mRNA was obtained by using magnetic oligo-dT beads following the instructions of the manufacturer (Dynal Biotech, Wirral, UK), and subsequently reverse-transcribed. The cDNA was subjected to 3′-RACE PCR procedure to obtain the full-length prepro-bombinin and prepro-bombinin H nucleotide sequence using a SMART-RACE kit (Clontech, Oxford, UK) as described by the manufacturer. For 3′ RACE reaction, a nested universal primer (NUP) (supplied with the kit) and a degenerate sense primer were designed and performed as previously reported [9,10]. The 3′-RACE reactions were performed as previous description [11].

2.3 Identification and structural analysis of deduced mature peptides in the skin secretions

Another 5 mg of lyophilised secretion were dissolved in 1.0 ml of 0.05/99.95 (v/v) trifluoroacetic acid (TFA)/water and clarified by centrifugation. The rp-HPLC system was fitted with an analytical column (Phenomenex C-5, 0.46 × 25 cm and phenomenex C-18, 250×10mm), eluting with a linear gradient formed from trifluoroacetic acid (TFA) / dd water (0.05/99.95, v/v) to TFA / dd water / Acetonitrile) (0.05/19.95/80.0, v/v/v) in 240 min at 1 ml/min. The fractions were collected automatically at minute intervals and effluent absorbance was continuously monitored at λ 214 nm and λ 280 nm. Each reverse phase HPLC fraction was analysed with matrix-assisted, laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using α-cyano-4-hydroxycinnamic acid as the matrix. Fractions containing peptides with molecular masses coincident with predicted mature peptides from ‘shotgun’ cloning were infused into the LCQ Fleet™ ion-trap electrospray mass spectrometer for analysis (Thermo Quest, San Jose, CA, USA).

2.4 Peptides synthesis and purification

The two-novel identified bombinin peptides and one single residue D-isomer analogue were synthesised by Tribute® Peptide Synthesizer (Protein Technologies, Inc., Tucson, USA) with solid-
phase Fmoc chemistry methodology and amide resin. Their molecular masses were analysed and confirmed by MALDI-TOF. Then, synthetic replicates were purified with rp-HPLC to obtain high-purity of synthetic peptides.

2.5 Circular dichroism (CD) spectroscopy

CD spectra between 190 nm and 250 nm were performed on a Jasco J-815 CD spectrometer (Jasco, Essex, UK). The machine units of millidegrees ellipticity were converted to mean residue molar ellipticity using the following equation (n: the number of peptide bonds; Ellipticity is the raw data from the instrument):

$$\theta \text{ (deg.cm}^2\text{.dmol}^{-1}) = \text{Ellipticity (mdeg)}\times 10^6 / \text{Pathlength (mm)} \times [\text{Peptide}] \text{ (µM)} \times n$$

The spectra were recorded at 100 nm/min in ammonium acetate (10 mM) buffer or TFE (50%) solution. CD measurements were performed at 20 °C with 1 mm path length cuvette. An average of three scans were collected and automated analysed for each peptide. The final predicted percentage of secondary structure was calculated using the K2D3 CD spectra web server [12].

2.6 Antimicrobial activity and minimal biofilm eradication concentration (MBEC) assays

The minimal inhibitory concentrations (MICs) of the synthetic replicates of the AMPs were determined using quality control strains, the Gram-positive bacterium, *Staphylococcus aureus* (NCTC 10788), the Gram-negative bacteria *Escherichia coli* (NCTC 10418) and *Pseudomonas aeruginosa* (ATCC 27853), the yeast *Candida albicans* (NCPF 1467), and MRSA (Methicillin-resistant *Staphylococcus aureus*) (ATCC 12493). The reference strains of the microorganisms were initially incubated in Mueller-Hinton Broth (MHB) for 16–20 h, then the bacterial cultures were diluted to obtain $1\times10^6$ cfu/ml for the bacterial and the yeast culture to $5\times10^5$ cfu/ml. The samples were added to obtain final concentrations from 1 to 512 mg/l. After 24 h incubation, the OD of each well was measured at 550 nm. The MIC value was measured as the minimal concentration of peptide with an OD identical with that of the negative controls [13]. Upon achieved the data from MIC assays, 10 µl of the medium from each well was taken and inoculated onto Mueller-Hinton agar (MHA) plates. After 24 h incubation, the minimum bactericidal concentrations (MBCs) and the minimum fungicidal
concentration (MFC) were obtained, which were defined as the lowest concentration of peptide from which no colonies could be subsequently grown.

The MBECs of the synthetic peptides were determined against *Staphylococcus aureus* and performed following a standard method as shown in manufacturer’s instruction (Innovotech, UK). The MBEC assay plate with specialised peg architecture that is designed for the formation of biofilm was used for anti-biofilm susceptibility tests. The procedure of inoculate and subculture were performed as described before. The inoculum plate was prepared by transferring 200 µl inoculum to the 96-well plate and kept in a 150-rpm moist orbital incubator for 72 h at 37 °C. After which, the lid with pegs of the inoculum plate was rinsed by PBS twice, seven replicates of a serial of two-fold diluted peptides (1–512 mg/l) along with the positive/negative controls were added to corresponding wells. After incubation at 37 °C for 24 h, the recovery plate was prepared by adding 200 µl recovery medium (MHB/neutralising agents 20/0.5 (v/v)) into each well. The lid from the inoculum plate was rinsed and the placed to the recovery plate. After sonication for 30 min, the recovery plate was measured at 550 nm. The minimal biofilm eradication concentration was determined as the lowest concentration with no microbiology growth was detected. Melittin (Sigma-Aldrich, UK), firstly isolated from honeybee (Apis mellifera) venom, was taken as positive controls in comparison [14,15]. Both antimicrobial and biofilm eradication assays were independently performed three times.

### 2.7 Kinetic time-killing assays

The kinetic time-killing assays were performed with different concentrations of peptides alone or with another checkerboard titration predicted synergistic agents. The concentration series of peptides alone or with the synergistic counterpart were added to 1.5 ml micro-centrifuge tubes, which were then inoculated with a log phase culture of the test organism as described in the previous section. During the incubation, 50 µl sample of each tube was removed from culture tubes at 0, 5, 10, 20, 30, 60 and 120 min intervals for single peptides or 0, 0.5, 1, 3, 6, and 24 h intervals for synergistic pairs. After diluted serially with PBS, 50 µl of diluted samples were inoculated on MHA plates and incubated at 37 °C for 24 h for colony counts. The synergistic effect was defined as equal or higher to $2 \cdot \log_{10}$ -
CFU/ml decrease in bacterial counts compared to the effect of the most active single constituent [16].

Curves were constructed by plotting the log_{10} of CFU/ml against time.

2.8 Haemolysis assay

Defibrinated horse erythrocytes (TCS Biosciences Ltd, Buckingham, UK) were prepared to produce a 4% (v/v) suspension of red blood cells in PBS by repeated washings with sterile PBS. A range of concentrations of synthetic peptides (1 to 512 mg/l) were incubated with red blood cell suspension samples (200 µl) at 37 °C for 120 min. After incubation, the suspensions of each sample were centrifuged to obtain the final lysis of red blood cells. OD measurements of supernatants were recorded at 550 nm. Negative controls were prepared by a 2% (v/v) suspension with PBS in equal volumes (0% haemolysis), while the positive controls employed consisting a 2% (v/v) suspension with 2% (v/v) of the non-ionic detergent, Triton X-100 (Sigma–Aldrich, UK) in PBS. HC_{50} was defined as the peptide concentration that caused 50 % haemolysis.

2.9 Cytotoxicity testing

The cytotoxicity of synthetic peptides on mammalian cells was examined using human microvessel endothelial cell (HMEC-1), which were cultured with MCDB 131 medium (Gibco, UK) supplemented with 10% FBS, 10 mM L-Glutamine, 10 ng/ml EGF and 1% penicillin streptomycin.

5×10^3 cells/well were seeded into 96-well plates. After 24 h incubation at 37 °C with 5% CO_{2}, 12 h serum-free starvation was performed, peptides with 10^{-9}–10^{-4} M concentrations were added for 24 h treatment prior to 10 µl MTT (5 mg/ml PBS) incubation for 4 h, the grow medium was removed followed by adding 100 µl of DMSO to dissolve the formazan crystals. The absorbance was measured at 570 nm. Data from this study were analysed by t-test using GraphPad Prism (version 5.01). A p value less than 0.05 was considered a significant difference. Negative and positive control treatments were carried out with culture medium and 1% Triton X-100, respectively. Data from this study were analysed by one-way ANOVA with Bonferroni’s post-test.
2.10 Evaluation of combination effects of antimicrobial peptides

A two-dimensional checkerboard with 2-fold dilutions of each AMP was used for examining the combination effects with \textit{S. aureus}. The dissolved samples of each peptide or antibiotic agent were diluted from 4×MIC to 1/16 ×MIC. The series of component A were added along the row of a 96-well plate, while the columns were filled with the diluted component B. Growth control wells containing only micro-organism medium and sterility control wells with only MHB medium were included. After the addition of a log-phase bacterial inoculum of 1×10^6 cfu/ml, plates were incubated at 37 °C for 24 h and then measured at λ 550 nm. The combination effects were examined by calculating the fractional inhibitory concentration index (FICI) of each combination as follows:

\[
\frac{\text{MIC of component A, tested in combination}}{\text{MIC of component A, tested alone}} + \frac{\text{MIC of component B, tested in combination}}{\text{MIC of component B, tested alone}}
\]

After the combination ratio of the two tested compounds was confirmed, lower concentration pairs were selected to determine the FICI with more accuracy. The profile of the combination was interpreted as synergistic for FICI \(\leq 0.5\), additive for \(0.5 < \text{FICI} \leq 4.0\), and antagonistic for \(\text{FICI} > 4.0\) [17,18].

For assessing the synergetic activity of bombinin and bombinin H against the growth of mammalian cell lines, both CalcuSyn software [19] and Jin’s formula [20] were employed. Combination index (CI) plots were generated by using CalcuSyn software. A value of CI < 1 represents synergy. The following formula in Jin’s formula was used: \(Q=E_a+b (E_a+E_b-E_a\times E_b)\). \(Q\) is the combination index; \(E_a+b\) represents the cell proliferative inhibition rate of two AMPs; \(E_a\) and \(E_b\) represents the cell proliferative inhibition rate for individual peptide. After calculation, the results \(Q > 1.15\) indicates synergy, and \(0.85 < Q < 1.15\) indicates an additive effect [19].

3. Results

3.1 Molecular cloning of skin secretion precursor cDNA encoding bombinin and bombinin H
The full-length biosynthetic precursor-encoding cDNAs were cloned from the skin secretion-derived cDNA libraries of *Bombina orientalis*. The nucleotide of full ORF of the cloned precursor transcripts and its translated sequences are shown in Figure 1, which contains 139 residues and encodes a novel bombinin (BHL-bombinin) and a novel bombinin H (bombinin HL). The data of the novel AMPs has been deposited in Genbank Nucleotide Sequence Database with the accession code LT615078.

The sequences of two novel AMPs were subjected to online BLAST program analysis with the NCBI on-line portal. The resulting typical primary structures were compared in Figure 2. The BHL-bombinin and bombinin HL, which exhibited as tandem mature peptides in biosynthetic precursor in Figure 1, revealed 96% and 82% sequence identity respectively, with other bombinins identified from *Bombinatoridae*. The main sequence difference was indicated in the last two or three residues in the C-terminus, where BHL-bombinin is -Ala-Asn- loss and bombinin HL is truncated of -Lys-Lys-Ile- with a typical Valine residue at 12th position from N-terminus (Figure 2).

### 3.2 Identification and structure characterisation of novel bombinin and bombinin H by rp-HPLC and MS/MS fragmentation

HPLC fractions with molecular masses coincident with predicted from molecular cloning for BHL-bombinin and bombinin HL were identified (Figure 3) following detected by the ion trap of the LCQ Fleet mass spectrometer with further tested by MS/MS fragmentation sequencing of doubly-charged ions derived from frog skin secretions (Figure 4 and 5).

### 3.3 Circular dichroism (CD) spectra and bioinformatic analysis

The secondary structures of synthetic replicates of AMPs were investigated in 10 mM of ammonium acetate (pH 7.0, mimicking aqueous environment) and 50% trifluoroethanol (TFE, mimicking the hydrophobic environment of the microbial membrane) by CD spectroscopy. As shown in Figure S2, all the peptides displayed random coil conformations in the aqueous environment. However, the spectrums of peptides were characteristic of α-helix conformations in the presence of 50% TFE, as indicated by the presence of double negative dichroic bands at approximately 208 and 222 nm. The
web server K2D3 calculation revealed that the helical content for BHL-bombinin is 87.59%, and for bombinin HD and bombinin HL are 77.73 % in 50 % TFE solution.

The physiochemical parameters of novel AMPs are listed in Table 1, which not only provides evidence of the possible interactions between peptides and bacterial membrane but also gives more information on their synergistic mechanisms. The molecular masses of synthetic peptides were determined by MALDI-TOF (Figure S1). Physiochemical parameters including charge, hydrophobic moment and hydrophobicity were determined using the Heliquest server [21]. The hydrophobic moment (µH) was determined by Eisenberg’s scale with a full window in Heliquest server. BHL-bombinin elicits higher cationic but much less hydrophobic properties than bombinin HL, which exhibit a highly structural and physiochemical differences between the two co-encoded AMPs. These findings suggest that these two peptides may possess distinguishing roles in the interaction with micro-organisms and exhibit potent antibacterial activities synergistically.

3.4 Antimicrobial and haemolytic activities

The antimicrobial effects of synthetic AMPs on the growth of the tested microorganisms, and the biofilm eradication effects on S. aureus are illustrated in table 2. The BHL-bombinin exhibited stronger antimicrobial activities on Gram-positive bacteria (MIC/MBC: 4mg/l/16mg/l) and yeast (MIC/MBC: 4mg/l/16mg/l) than Gram-negative bacteria (MIC/MBC: 16-64mg/l/64-128mg/l). In addition, BHL-bombinin was found to possess a relatively low level of haemolytic activity (0–12.6%) at the MIC determined against S. aureus and C. albicans (Figure S3). Interestingly, BHL-bombinin displayed potent inhibitory effects (MIC: 4-16mg/l) towards MRSA and biofilm. By contrast, the MIC values for bombinin HL and bombinin HD against S. aureus were 256mg/l and 128mg/l respectively with undetected MBC, which were significantly less effective compared to BHL-bombinin. The selectivity indices (SIs), which represent the degree of antibacterial selectivity, are showed in Table 2, higher SI value reflecting a better selectivity towards microbial over mammalian membranes [22]. As indicated, the BHL-bombinin had a higher SI compared to bombinin HL and bombinin HD, which is in agreement with previous studies that high level of hydrophobicity may decrease the antimicrobial selectivity of α-helical peptides [23]. Additionally, compared with the melittin peptide, all the AMPs
investigated in this study exhibited 32 to 128 times higher SI values, which emphasises that amphibian-derived AMPs are potential research targets for therapeutic alternatives to current antibiotics. The time-killing curves demonstrated the faster cell-killing effects of BHL-bombinin compared with the ampicillin, while the kill rates of bombinin HL and bombinin HD were relatively low (Figure S4).

The combined administration of BHL-bombinin with either bombinin HL or bombinin HD revealed a synergistic antimicrobial effect against S. aureus (FICI 0.375). In addition, BHL-bombinin showed additive property with classic antibiotics ampicillin (FICI 0.75), while the novel bombinin H, either D- or L-amino isoforms, displayed synergistic activities with β-lactam and ampicillin (FICI 0.5). The synergistic effects were further confirmed by the outcomes of time-killing assays. Figure 6 (a) (b) exhibits that the isolated S. aureus had a 6.16(±0.76) log10 decrease in cfu/ml at 24 h when incubated with BHL-bombinin (0.75 mg/l) and bombinin HL (48 mg/l), compared to the single peptide effect. The time-killing value was 5.83(±0.67) log10 for combined effects of BHL-bombinin (0.75mg/l) and bombinin HD (24 mg/l). The synergistic effects were also observed when co-administrated bombinin HL (64 mg/l) with ampicillin (0.016 mg/l), or bombinin HD (32 mg/l) with ampicillin (0.016 mg/l), which demonstrated a 7.51 (±0.97) log10 and 6.57 (±0.77) log10 decrease in CFU/ml at 24 h respectively.

3.5 Cytotoxicity assessment of novel bombinin, bombinin H and their synergistic effect on HMECs (human microvessel endothelial cells)

The anti-proliferative effect data obtained from MTT cell viability assays of each peptide on HMEC-1 cells are represented in Figure 7(a)(b), and their IC50 values are calculated. All the AMPs tested in this study exhibited low cytotoxicity with cell viabilities exceeding 90% up to the concentration 10^{-5}M against HMEC-1. For BHL-bombinin, at MIC concentrations (1.6–26.2 µM), 83.5–100.0% HMEC-1 cells remained viable. For bombinin HL and bombinin HD, they displayed relatively lower selectivity and higher cytotoxicity on HMEC-1 cells compared with BHL-bombinin. To identify the possible synergistic cytotoxicity between BHL-Bombinin and bombinin HL or bombinin HD, the cells were cultured with combinations of these two peptides at different doses but in a constant ratio (BHL-
bombinin to bombinin HL or bombinin HD: 5 μM to 10 μM, 10 μM to 20 μM, and 20 μM to 40 μM, respectively) for 24 h. The combination of 20 μM BHL-bombinin with 40 μM bombinin HL inhibited cell growth of 52.21%, compared with mono-administration of BHL-bombinin (43.93%) or bombinin HL (9.63%), indicating an additive effect (CI = 1.03; Q = 1.06). The values for combination of BHL-bombinin and bombinin HD were CI = 0.98; Q = 1.10. The results revealed that the synergistic relationship was abolished with 0.85 < Q < 1.15 and CI ≥ 1 with regards to their cytotoxicity on normal mammalian cells.

4. Discussion

Different from the well-studied bioactive peptides from the amphibian Pipidae, Hylidae, Ranidae, and Pseudidae families, skin secretions from Bombina species, remain to be investigated fully and may yield valuable promotion for drug development. The best-known constituent identified from Bombina skin secretions is bombesin, which led to the subsequent identification of the mammalian homologues, gastrin-releasing peptide (GRP) and neuromedin B (NMB) as neuropeptides [24]. Among all the molecules secreted from Bombina species, no counterparts of the novel BHL-bombinin and bombinin HL, which are encoded by single coding region precursor, have been identified in other amphibian genera or in mammals [25].

The present study describes the molecular cloning, primary structure identification, chemical synthesis, and bioactive examinations of two tandem-coded novel bombinin peptides. Since the D-isomer exists in some of the bombinin H-type molecular at the 2nd position of their sequences, the analogue bombinin HD was designed by substituting the L-leucine at such position. CD studies revealed that the helical content of BHL-bombinin was only approximately 10% higher than bombinin HL and bombinin HD, while the hydrophobicity of BHL-bombinin is significant lower than that of bombinin HL and bombinin HD. Therefore, all the tested AMPs in this study were found to adapt an amphipathic α-helical conformation in a membrane mimetic environment, a feature that is essential for allowing AMPs to exert their bioactivities [26]. However, due to the diversities of their primary...
structures and physiochemical parameters, the functional mechanisms that they employed can be significantly different.

Synthetic BHL-bombinin were found to possess potent antimicrobial activities against *S. aureus* and *C. albicans*, but relatively lower activity against *E. coli* and *P. aeruginosa*. The MBCs for all the four tested micro-organisms were approximately equal to or over 4-fold of their respect MICs. Clinically, the formation of biofilm and conventional antibiotic resistant strain MRSA are two major causes of antibiotic crisis. BHL-bombinin showed potent effects for eliminating *S. aureus* biofilm and inhibiting the growth of isolated MRSA. However, the MICs observed for wild-type bombinin HL and analogue bombinin HD, were moderately effective against *S. aureus* with undetected MBC. The antimicrobial properties of the peptides reported in this study were further compared against melittin, which is a well-studied bee venom derived AMP [15]. BHL-bombinin exhibited similar antimicrobial activity to melittin, but weaker haemolytic activity, which indicates a better selectivity. Both bombinin H peptides possessed mild antibacterial property but higher selective antimicrobial activity compared to melittin. Following on from this, the BHL-bombinin and bombinin HL are tandem-encoded in single ORF, which prompt us to speculate that their combination effect might be vital for frogs to survive in pathogens-rich environments. Of note, the combination effect between novel components and conventional antibiotics has also been proven as a promising solution to amplify the potency of antibiotics, a good example is Co-amoxiclav, which enormously enhances the Amoxicillin potency after combined use of clavulanic acid [27]. As expected, the combination interaction of BHL-bombinin with either bombinin HL or bombinin HD showed synergistic inhibition activities against *Staphylococcus aureus* (FICI 0.375). Moreover, BHL-bombinin showed additive effect with classic antibiotics ampicillin (FICI 0.75), while the bombinin HL and HD, displayed synergistic activities with β-lactam, ampicillin (FICI 0.5). The results were further confirmed by time-killing assays, that the BHL-bombinin exerted higher bactericidal rate compared with ampicillin. However, the killing rates for bombinin HL and bombinin HD were diminished. The mechanism of the positive outcomes between peptides and conventional antibiotics (ampicillin in this study) appears to be complex. The fractional inhibitory concentration (FIC) index as a measure of synergy employed in this study is the
best known and a very basic method for evaluating the inhibitory effects of paired agents comparing
the sum of their effects alone. We calculate their FICI according to the protocol for investigating their
synergistic relationships at preliminary level in this study [17,18]. For addressing the more
complicated natural environment, the detailed concentration and structural relationship between
peptides in this work needs a further and more systematic mechanism evaluation, which depends on
the physiochemical parameters and their combination results of BHL-bombinin and bombinin HL,
BHL-bombinin may have direct and selective membrane permeabilizing-activity, which increases the
uptake of other antibacterial agents that initiate the process to interfere with intracellular targets or
enhance the effect of highly hydrophobic molecules like bombinin HL. On the other hand, either
bombinin HL or bombinin HD may cause degradation of the peptidoglycan by triggering the activity
of bacterial murein hydrolases, which can enhance the activity of the β-lactams [28-30].

Safety evaluations via haemolytic assay demonstrated the relatively lower SIs of both bombinin HL
and bombinin HD on horse erythrocytes compared with BHL-bombinin, as a consequence, when
treated HMEC-1 cells with the peptides using their MIC concentrations for MTT-based viability
assessment, bombinin HL and HD exhibited higher cytotoxicity. The typical theory is that the
increased hydrophobicity of the AMPs is associated with higher antimicrobial activity, but in contrast,
the high hydrophobic peptides are more associated with stronger self-assembly, which can result in
the formation of dimers or oligomers. This spatial character may in turn decrease their potential for
passing through the target cell wall and bacterial membrane [31]. The high toxicity of BHL-bombinin
in this study might be mainly due to its innate character of highly hydrophobicity. Additionally, the
abolishment of combined effect of BHL-bombinin and bombinin H against HMEC-1 revealed their
high functional selectivity. The application of combined antimicrobial agents, either with AMPs or
with conventional antibiotics, is a prospective strategy to improve clinical therapy caused by multi-
drug resistant pathogens and decrease the side-effect [32].

5. Conclusion
In this project, the novel BHL-bombinin, bombinin HL and analogue bombinin HD are reported from less-studied frog species Bombina orientalis. They revealed comparable antimicrobial property individually and enhanced synergistic effect and selectivity jointly, all these inherent and robust characteristics hold significant potential to alleviate the current antibiotics crisis.

Author contribution

Jie Xiang, Yuxin Wu and Tianbao Chen conceived and designed the experiments. Jie Xiang performed the experiments. Jie Xiang, Yuxin Wu and Mei Zhou analysed the data. Chris Shaw and Lei Wang contributed reagents/materials/analysis tools. Jie Xiang and Yuxin Wu wrote the paper. Yuxin Wu and Tianbao Chen edited the paper.

Declaration of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding Source:

This work was supported by internal funding of School of Pharmacy, Queen’s University Belfast

References

1. Csordas, A.; Michl, H. Isolation and structure of an hemolytic polypeptide from defensive secretion of european bombina species. *Monatsh Chem* 1970, 101, 182-6.
2. Mignogna, G.; Simmaco, M.; Kreil, G.; Barra, D. Antibacterial and haemolytic peptides containing d-alloisoleucine from the skin of bombina variegata. *The EMBO journal* 1993, 12, 4829.
3. König, E.; Bininda-Emonds, O.R.; Shaw, C. The diversity and evolution of anuran skin peptides. *Peptides* 2015, 63, 96-117.
4. Coccia, C.; Rinaldi, A.C.; Luca, V.; Barra, D.; Bozzi, A.; Di Giulio, A.; Veerman, E.C.; Mangoni, M.L. Membrane interaction and antibacterial properties of two mildly cationic peptide
diastereomers, bombinins h2 and h4, isolated from bombina skin. *European biophysics journal* **2011**, *40*, 577-588.

5. Mangoni, M.L.; Grovale, N.; Giorgi, A.; Mignogna, G.; Simmaco, M.; Barra, D. Structure-function relationships in bombinins h, antimicrobial peptides from bombina skin secretions. *Peptides* **2000**, *21*, 1673-1679.

6. Bozzi, A.; Mangoni, M.L.; Rinaldi, A.C.; Mignogna, G.; Aschi, M. Folding propensity and biological activity of peptides: The effect of a single stereochemical isomerization on the conformational properties of bombinins in aqueous solution. *Biopolymers* **2008**, *89*, 769-778.

7. Simmaco, M.; Kreil, G.; Barra, D. Bombinins, antimicrobial peptides from bombina species. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **2009**, *1788*, 1551-1555.

8. Xiang, J.; Wang, H.; Ma, C.; Zhou, M.; Wu, Y.; Wang, L.; Guo, S.; Chen, T.; Shaw, C. Ex vivo smooth muscle pharmacological effects of a novel bradykinin-related peptide, and its analogue, from chinese large odorous frog, odorrana livida skin secretions. *Toxins (Basel)* **2016**, *8*.

9. Bai, B.; Hou, X.; Wang, L.; Ge, L.; Luo, Y.; Ma, C.; Zhou, M.; Duan, J.; Chen, T.; Shaw, C. Feleucins: Novel bombinin precursor-encoded nonapeptide amides from the skin secretion of bombina variegata. *Biomed Res Int* **2014**, *2014*, 671362.

10. Hou, X.; Du, Q.; Li, R.; Zhou, M.; Wang, H.; Wang, L.; Guo, C.; Chen, T.; Shaw, C. Feleucin-bo1: A novel antimicrobial non-apptide amide from the skin secretion of the toad, bombina orientalis, and design of a potent broad-spectrum synthetic analogue, feleucin-k3. *Chem Biol Drug Des* **2015**, *85*, 259-267.

11. Wu, Y.; Long, Q.; Xu, Y.; Guo, S.; Chen, T.; Wang, L.; Zhou, M.; Zhang, Y.; Shaw, C.; Walker, B. A structural and functional analogue of a bowman-birk-type protease inhibitor from odorrana schmackeri. *Biosci Rep* **2017**, *37*.

12. Louis – Jeune, C.; Andrade – Navarro, M.A.; Perez – Iratxeta, C. Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. *Proteins: Structure, Function, and Bioinformatics* **2012**, *80*, 374-381.

13. Mouton, J.W.; Brown, D.; Apfalter, P.; Canton, R.; Giske, C.; Ivanova, M.; MacGowan, A.; Rodloff, A.; Soussy, C.J.; Steinbakk, M. The role of pharmacokinetics/pharmacodynamics in setting clinical mic breakpoints: The eucast approach. *Clinical Microbiology and Infection* **2012**, *18*.

14. Lubke, L.L.; Garon, C.F. The antimicrobial agent melittin exhibits powerful in vitro inhibitory effects on the lyme disease spirochete. *Clin Infect Dis* **1997**, *25 Suppl 1*, S48-51.

15. Raghuraman, H.; Chattopadhyay, A. Melittin: A membrane-active peptide with diverse functions. *Biosci Rep* **2007**, *27*, 189-223.

16. Hindler, J.A.; Wong-Beringer, A.; Charlton, C.L.; Miller, S.A.; Kelesidis, T.; Carvalho, M.; Sakoulas, G.; Nonejutie, P.; Pogliano, J.; Nizet, V. In vitro activity of daptomycin in combination with β-lactams, gentamicin, rifampin, and tigecycline against daptomycin-nonsusceptible enterococci. *Antimicrob Agents Ch* **2015**, *59*, 4279-4288.

17. Odds, F.C. Synergy, antagonism, and what the chequerboard puts between them. *Br Soc Antimicrob Chemo* 2003.

18. Hall, M.; Middleton, R.; Westmacott, D. The fractional inhibitory concentration (fic) index as a measure of synergy. *J Antimicrob Chemoth* **1983**, *11*, 427-433.

19. Chou, T.C. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* **2006**, *58*, 621-681.

20. Jin, Z.J. [addition in drug combination (author’s transl)]. *Zhongguo Yao Li Xue Bao* **1980**, *1*, 70-76.

21. Gautier, R.; Douguet, D.; Antonny, B.; Drin, G. Heliquest: A web server to screen sequences with specific α-helical properties. *Bioinformatics* **2008**, *24*, 2101-2102.

22. Khara, J.S.; Lim, F.K.; Wang, Y.; Ke, X.-Y.; Voo, Z.X.; Yang, Y.Y.; Lakshminarayan, R.; Ee, P.L.R. Designing α-helical peptides with enhanced synergism and selectivity against
mycobacterium smegmatis: Discerning the role of hydrophobicity and helicity. *Acta Biomater* **2015**, *28*, 99-108.

23. Jiang, Z.; P Higgins, M.; Whitehurst, J.; O Kisich, K.; I Voskuil, M.; S Hodges, R. Anti-tuberculosis activity of α-helical antimicrobial peptides: De novo designed l-and d-enantiomers versus l-and d-ll37. *Protein and peptide letters* **2011**, *18*, 241-252.

24. Gonzalez, N.; Moody, T.W.; Igarashi, H.; Ito, T.; Jensen, R.T. Bombesin-related peptides and their receptors: Recent advances in their role in physiology and disease states. *Current opinion in endocrinology, diabetes, and obesity* **2008**, *15*, 58.

25. Luca, V.; Barra, D.; Mangoni, M.L. In *Handbook of biologically active peptides*, Elsevier Inc., 2013.

26. Corbier, C.; Krier, F.; Mulliert, G.; Vitoux, B.; Revol-Junelles, A.-M. Biological activities and structural properties of the atypical bacteriocins mesenterocin 52b and leucocin b-ta33a. *Appl Environ Microb* **2001**, *67*, 1418-1422.

27. Yu, G.; Baeder, D.Y.; Regoes, R.R.; Rolff, J. Combination effects of antimicrobial peptides. *Antimicrob Agents Ch* **2016**, *60*, 1717-1724.

28. Giacometti, A.; Cirioni, O.; Del Prete, M.; Barchiesi, F.; Fortuna, M.; Drenaggi, D.; Scalise, G. In vitro activities of membrane-active peptides alone and in combination with clinically used antimicrobial agents against stenotrophomonas maltophilia. *Antimicrob Agents Ch* **2000**, *44*, 1716-1719.

29. LeBel, G.; Piché, F.; Frenette, M.; Gottschalk, M.; Grenier, D. Antimicrobial activity of nisin against the swine pathogen streptococcus suis and its synergistic interaction with antibiotics. *Peptides* **2013**, *50*, 19-23.

30. Giacometti, A.; Cirioni, O.; Barchiesi, F.; Scalise, G. In-vitro activity and killing effect of polycationic peptides on methicillin-resistant staphylococcus aureus and interactions with clinically used antibiotics. *Diagn Micr Infec Dis* **2000**, *38*, 115-118.

31. Chen, Y.; Guarnieri, M.T.; Vasil, A.I.; Vasil, M.L.; Mant, C.T.; Hodges, R.S. Role of peptide hydrophobicity in the mechanism of action of α-helical antimicrobial peptides. *Antimicrob Agents Ch* **2007**, *51*, 1398-1406.

32. Zhu, X.; Shan, A.; Ma, Z.; Xu, W.; Wang, J.; Chou, S.; Cheng, B. Bactericidal efficiency and modes of action of the novel antimicrobial peptide t9w against pseudomonas aeruginosa. *Antimicrob Agents Ch* **2015**, *59*, 3008-3017.

---

**Figure legends and Tables**

**Figure 1** The nucleotide sequence and open-reading frame amino acid sequence of full length preprobombinin and preprobombinin H peptides encoding cDNA from the oriental fire-bellied toad, *Bombina orientalis*. The putative signal peptide was double-underlined. The mature peptide was single-underlined for bombinin and dash-underlined for bombinin H. The stop codon was indicated by an asterisk.
Figure 2 Alignment of the primary structure of the novel peptides BHL-Bombinin (a) and bombinin HL (b) with other bombinin family peptides. An * (asterisk) indicates positions that have a single, fully conserved residue. Substitutions are highlighted in grey. D-amino acids are boldfaced and italic. Gap residues are represented by dashes. * Sequences deduced from BLP-3 and BLP-7 genes, GH means gene-derived bombinin H-like peptide.

Figure 3 Region of reverse phase HPLC chromatogram of *Bombina orientalis* skin secretion with arrow indicating the retention times of the novel BHL-Bombinin (1) and Bombinin HL (2). The detection wavelength was 214 nm with a flow rate of 1 ml/min in 240 min.

Figure 4 Thermoquest LCQ™ fragment scan spectrum derived from ions corresponding to BHL-Bombinin (a) and electrospray ion-trap MS/MS fragmentation dataset (b). Expected single- and double-charged b- ions and y-ions arising from MS/MS fragmentation were predicted using the MS Product programme through Protein Prospector on-line. Truly observed ions are indicated in bold typeface and underlined.

Figure 5 Thermoquest LCQ™ fragment scan spectrum derived from ions corresponding to Bombinin HL (a) and electrospray ion-trap MS/MS fragmentation dataset (b). Expected single- and double-charged b- ions and y-ions arising from MS/MS fragmentation were predicted using the MS Product programme through Protein Prospector on-line. Truly observed ions are indicated in bold typeface and underlined.

Figure 6 Time-killing curves for combinational treatment of peptides and antimicrobial agents against *S. aureus*. Control (red circle/line), bombinin HL and bombinin HD (green square/line), ampicillin (brown triangle/line), BHL-Bombinin (purple triangle/line), combination pairs (black inverted triangle/line), The detection limit is indicated as a dashed line. The graphs were derived from values of three independent trials.

Figure 7 Cytotoxicity assessment of AMPs. Dose-dependent antiproliferative effects of BHL-Bombinin (a), bombinin HL and bombinin HD (b) against HMEC-1 after 24 h of incubation (V, N and P represent vehicle control, negative control and positive control respectively). The levels of significance are: * p < 0.05; ** p <
0.01; *** p < 0.001 versus vehicle. The growth inhibition rate graphs of combination effects among a series concentration between BHL-bombinin with bombinin HL(c) or bombinin HD (d) against HMEC-1 after incubation for 24 h. (e) Combination index Q of the combination treatment of synergistic pairs, where Q < 0.85, Q > 1.15 and 0.85 < Q < 1.15 represent antagonism, synergy, and additive effect, respectively. (f) CI-effect plots were generated using CalcuSyn software. The points represent CI values for the combinations 5, 10, 20 µM BHL-bombinin with 10, 20, 40 µM bombinin HL or bombinin HD in a constant ratio against HMEC-1.

Table 1 Physico-chemical characteristics of the novel AMPs BHL-Bombinin and Bombinin HL.

Table 2 The susceptibility of novel AMPs and melittin peptide against microbial strains, bacterial biofilm and their selectivity indices (SIs) against *S. aureus*. a NA: not active; no inhibition or bactericidal activity was observed using peptide concentrations up to and including 512mg/L. b SI (selectivity index) defined as the ratio of HC50 to MIC against *S. aureus* value (HC50/MIC). When no or mild haemolysis was observed at the highest concentration employed (512 mg/l), a value of 1024 mg/l was used for calculation.

Table 3 The combinational effects of the novel AMPs with ampicillin against *S. aureus* using checkerboard titration method. * The MIC values of Ampicillin is 0.0625 mg/l against *S. aureus*. 
Figure 1

| M | N | F | K | Y | I | V | A | V | S | F | L | I | A | S | A | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ATGAAATTTTA AGTACATAGT TGCAGTGTCC TTTTTAATAG CATCTGCATA |
| 51 | TGGACAGAAGT GTACAATGATG ATGAACAGTC TCTGAGTCAG AGGGATGTTT |
| 101 | TAAAGCATAGTAAGTACATAGT TGCAGTGTCC TTTTTAATAG CATCTGCATA |
| 151 | AGTTTGGTAA AATCAGCTTT AAAAGGCTTG GCTAAAGGAT TGGCTGAGCA |
| 201 | TTTTGGGAAG ATCACTGAGG GAAATCAGAG GTATAGGAGG AGCCCTCCTA |
| 251 | CCAGAATGCG TGATCTAGAT TCCTTGGATT ATCCAGAGGA AGCTTCTGAA |
| 301 | AGGGAAACCA GAAGCTTCAA TCAAGAGGAG ATTGCAAATC TTTTTACTAA |
| 351 | AAAAGAGAAA CGCCTTTTGG GGCCAGTATT AGGTTTGGTT TCTAATGTAC |
| 401 | AAAAGAGAAA CGCCTTTTGG GGCCAGTATT AGGTTTGGTT TCTAATGTAC |
| 451 | AAAAGAGAAA CGCCTTTTGG GGCCAGTATT AGGTTTGGTT TCTAATGTAC |
| 501 | AAAAGAGAAA CGCCTTTTGG GGCCAGTATT AGGTTTGGTT TCTAATGTAC |
| 551 | AAAAGAGAAA CGCCTTTTGG GGCCAGTATT AGGTTTGGTT TCTAATGTAC |

---

Figure 2

BHL-Bombinin
Bombinin-like peptides BLP-3
Bombinin-like peptides BLP-7
FPA-bombinin-BO
FPA-bombinin-BV
Maximin-4

**

Bombinin HL
Bombinin HVD
Bombinin H6
Bombinin H7
Bombinin H2
Bombinin H4
Bombinin GH-1L
Bombinin GH-1D

---

(a)
Figure 3
Figure 4

(a)

GIGGAILSLSFQGKSAIKGLAKGLAEHF-NH₂
| #  | b(1+) | b(2+) | Seq. | y(1+)  | y(2+)  | #2  |
|----|-------|-------|------|--------|--------|-----|
| 1  | 58.03 | 29.52 | G    | 2384.38| 1192.69| 25  |
| 2  | 171.11| 86.06 | I    | 2271.30| 1136.15| 24  |
| 3  | 228.13| 114.57| G    | 2214.28| 1107.64| 23  |
| 4  | 285.16| 143.08| G    | 2157.25| 1079.13| 22  |
| 5  | 356.19| 178.60| A    | 1973.13| 987.07 | 21  |
| 6  | 469.28| 235.14| L    | 1860.05| 930.53 | 20  |
| 7  | 582.36| 291.68| L    | 1773.02| 887.01 | 19  |
| 8  | 669.39| 335.20| S    | 1625.95| 813.48 | 18  |
| 9  | 816.46| 408.73| F    | 1568.93| 784.97 | 17  |
| 10 | 873.48| 437.25| G    | 1440.83| 720.92 | 16  |
| 11 | 1001.58| 501.29| K    | 1353.80| 677.40 | 15  |
| 12 | 1088.61| 544.81| S    | 1282.76| 641.89 | 13  |
| 13 | 1159.65| 580.33| A    | 1169.68| 585.34 | 12  |
| 14 | 1272.73| 636.87| L    | 1041.58| 521.30 | 11  |
| 15 | 1400.83| 700.92| K    | 984.56 | 492.78 | 10  |
| 16 | 1457.85| 729.43| G    | 871.48 | 436.24 | 9   |
| 17 | 1570.93| 785.97| L    | 800.44 | 400.72 | 8   |
| 18 | 1641.97| 821.49| A    | 707.35 | 336.68 | 7   |
| 19 | 1770.06| 885.54| K    | 615.32 | 308.17 | 6   |
| 20 | 1827.09| 914.05| G    | 502.24 | 251.62 | 5   |
| 21 | 1940.17| 970.59| L    | 431.20 | 216.11 | 4   |
| 22 | 2011.21| 1006.11| A    | 302.16 | 151.58 | 3   |
| 23 | 2140.25| 1070.63| E    | 227.31 | 1139.16| 2   |
| 24 | 2277.31| 1139.16| H    | 165.10 | 83.05  | 1   |

(b) Amidated
Figure 5
| #  | b(1+) | b(2+) | Seq. | y(1+)  | y(2+)  | #2  |
|----|-------|-------|------|--------|--------|-----|
| 1  | 114.09| 57.55 | L    | 1519.96| 760.48 | 17  |
| 2  | 227.18| 114.09| L    | 1519.96| 760.48 | 16  |
| 3  | 284.20| 142.60| G    | 1406.87| 703.94 | 15  |
| 4  | 381.25| 191.13| P    | 1349.85| 675.43 | 14  |
| 5  | 480.32| 240.66| V    | 1252.80| 626.90 | 13  |
| 6  | 593.40| 297.20| L    | 1153.73| 577.37 | 12  |
| 7  | 650.42| 325.72| G    | 1040.65| 520.83 | 11  |
| 8  | 763.51| 382.26| L    | 983.62 | 492.32 | 10  |
| 9  | 862.58| 431.79| V    | 870.54 | 435.77 | 9   |
| 10 | 949.61| 475.31| S    | 771.47 | 386.24 | 8   |
| 11 | 1063.65| 532.33| N    | 684.44 | 342.72 | 7   |
| 12 | 1162.72| 581.86| V    | 570.40 | 285.70 | 6   |
| 13 | 1275.80| 638.41| L    | 471.33 | 236.17 | 5   |
| 14 | 1332.83| 666.92| G    | 358.24 | 179.63 | 4   |
| 15 | 1389.85| 695.43| G    | 301.22 | 151.12 | 3   |
| 16 | 1502.93| 751.97| L    | 244.20 | 122.60 | 2   |
| 17 | Amidated | 131.12| 66.06| 1       |

(b)
Figure 6

(a) Control
- 48mg/L Bombinin HL
- 0.75mg/L BHL-Bombinin
- 46mg/L Bombinin HL + 0.75mg/L BHL-Bombinin

(b) Control
- 24mg/L Bombinin HD
- 0.75mg/L BHL-Bombinin
- 24mg/L Bombinin HD + 0.75mg/L BHL-Bombinin

(c) Control
- 64mg/L Bombinin HL
- 0.016mg/L Ampicillin
- 64mg/L Bombinin HL + 0.016mg/L Ampicillin

(d) Control
- 32mg/L Bombinin HD
- 0.016mg/L Ampicillin
- 32mg/L Bombinin HD + 0.016mg/L Ampicillin
Figure 7

HMEC-1

Cell viability (%) vs. Log[BHL-Bombin] (M)

- V
- N
- P
- -4
- -5
- -6
- -7
- -8
- -9

* ***
(a) BHL-Bombinin
(b) Bombinin HL
(c) Bombinin HD

|       | BHL-Bombinin | Bombinin HL | Bombinin HD |
|-------|--------------|-------------|-------------|
| IC_{50} (µM) | 94.32 ± 0.72 | 103.51 ± 0.43 | 104.23 ± 0.24 |
Table 1

| Peptides            | Theoretical Mw | Measured Mw | Hydrophobicity (H) | Hydrophobic moment (µH) | Net charge | %Helicity |
|---------------------|----------------|-------------|--------------------|-------------------------|------------|-----------|
| BHL-Bombinin        | 2441.87        | 2441.50     | 0.462              | 0.404                   | +3         | 87.59     |
| Bombinin HL         | 1633.03        | 1633.02     | 0.920              | 0.501                   | +1         | 77.73     |
### Table 2

| Peptides    | MIC / MBC [mg/l(µM)] | MIC / MFC [mg/l(µM)] | MBEC [mg/L(µM)] | HC₅₀ [mg/l(µM)] | SI² |
|-------------|----------------------|----------------------|-----------------|-----------------|-----|
|             | Gram-positive bacteria | Gram-negative bacteria | Fungi           | S. aureus       |     |
|             | S. aureus | MRSA | E. coli | P. aeruginosa | C. albicans | S. aureus |
| BHL-Bombinin | 4(1.6)/ 16(6.6) | 16(6.6)/ 64(26.2) | 16(6.6)/ 64(26.2) | 64(26.2)/ 128(52.4) | 4(1.6)/ 16(6.6) | 4(1.6) | 64(26.2) | 16 |
| Bombinin HL | 256(156.8)/ NA² | NA/ NA | NA/ NA | NA/ NA | NA/ NA | NA | >512(313.5) | 4 |
| Bombinin HD | 128(78.4)/ NA | NA/ NA | NA/ NA | NA/ NA | NA/ NA | NA | >512(313.5) | 8 |
| Melittin    | 8(2.8)/16(5.6) | 32(11.2)/ 64(22.4) | 16(5.6)/ 32(11.2) | 16(5.6)/ 64(22.4) | 8(2.8)/ 16(5.6) | 8(2.8) | 1(0.4) | 0.125 |

### Table 3

| The antimicrobial treatment settings | Lowest FIC index ([A] / [B] in mg/l) | Results  |
|-------------------------------------|--------------------------------------|----------|
| A                                   | B                                    |          |
| BHL-Bombinin                        | Bombinin HL                          | 0.375(0.75/48) | Synergistic |
|                                     | Bombinin HD                          | 0.375(0.75/24) | Synergistic |
|                                     | Ampicillin                           | 0.75(2/ 0.016) | Additive    |
| Bombinin HL                         | Ampicillin                           | 0.5(64/0.016) | Synergistic |
| Bombinin HD | Ampicillin | 0.5(32/0.016) | Synergistic |
|-------------|------------|---------------|-------------|

Supplementary data
Figure S1 MALDI-TOF (Perceptive Biosystem, Bedford, MA, USA) mass spectrum of synthetic peptide (a) bombinin HL and (b) bombinin HD and (c) BHL-bombinin. In (b), the initial neutral molecule bombinin HD [M] and metal ion adducts ([M+Na]+: 1654.35Da and [M+K]+: 1669.70Da) were observed.
**Figure S2**  CD spectra of the peptides in 10 mM ammonium acetate buffer (triangles) and 50% TFE (circles). The mean residue ellipticity was plotted against wavelength. The values from three scans were calculated as average per sample. The peptide concentrations were fixed at 100 μM.
Figure S3  Haemolytic activities of BHL-bombinin (diamond), bombinin HL (square), bombinin HD (triangle) and melittin (cross) following incubation with horse erythrocytes for 2 h.
**Figure S4** Time-killing curves of peptides and ampicillin at a series of concentrations: control (red filled circle), 0.25×MIC (red unfilled circle), 0.5×MIC (green diamond), 1×MIC (blue inverted triangle), 2×MIC (purple triangle) and 4×MIC (black square) against A: *S.aureus* [(a) BHL-bombinin (b) bombinin HL (c) bombinin HD (d) ampicillin]; B: *E.coli* [(e) BHL-bombinin (f) ampicillin]; C: *C.albicans* [(g) BHL-bombinin]. The detection limit was indicated as dashed line and the graphs were derived value of three independent trials.