1. Introduction

Abuse of antibiotics has induced worldwide antibiotic resistance, which may render routine surgery lethal due to the low efficacy of current antibiotics and seriously jeopardised public health [1]. The IDSA Emerging Infections Network conducted a national poll of infectious-disease specialists in 2011. >60 % of respondents had seen a pan-resistant, untreatable bacterial infection in the previous year [2]. Many public health groups have described the fast spread of resistant bacteria as a "crisis" [3]. Antibiotic usage is driving the evolution of resistance [4]. Epidemiological studies have found a direct link between antibiotic abuse and the establishment and spread of antibiotic-resistant bacterial strains [5]. Over the last three decades, the number of new antibiotics produced and licenced has continuously dropped (although four new medications were approved in 2014), leaving fewer alternatives for treating resistant bacteria [6]. Therefore, finding novel antibiotic agents that differ from conventional signal-target antibiotics is increasingly urgent [7].

Antimicrobial peptides (AMPs) are a promising new class of naturally-produced medicinal agents with broad antibacterial activity against a variety of bacteria and the potential to overcome the limitations of traditional antibiotics in the face of antibiotic-resistance [8–10]. Compared to conventional antibiotics, AMPs are also less likely to induce drug-resistance as their rapid membrane-disruption mechanism makes it extremely difficult for bacteria to develop membrane-sparing mutations [11,12]. Currently, the Antimicrobial Peptide Database (APD) lists >3100 AMPs, most of which have been found in Nature [13]. It is interesting to note that one-third of these originate from frogs [14]. As occupying a special link in breaching the aquatic-terrestrial gap in vertebrate evolution, frogs are generally found in complex environments which are rich in microorganisms, parasites, and predators, all of which exercise their excellent defensive systems. From their skin secretions, many AMPs have been isolated and proven to be of pharmacological value [15,16]. As a taxon with a large global distribution, Hylarana species can be found in Africa and Asia. In tropical and subtropical Asia only, there have been around 40 different Hylarana amphibian species reported to date [17]. Like in other
anurans, researchers have found many AMPs in the skin secretions of *Hylarana latouchii*, some of which show great research value [18].

Although AMPs show unparalleled advantages over conventional antibiotics, some bottlenecks, such as high synthesis costs, high toxicity, and low efficiency of modification, greatly restrict their development [19]. However, how to efficiently control the length of peptides to reduce the costs and preserve the crucial functional sites of AMPs, has so far remained elusive. Trypsin, as a typical serine protease found in the digestive system of many vertebrates, can specifically cleavage peptides or proteins at the carboxyl group of lysine and arginine and this specificity is also found in many other proteases [20,21]. Using trypsin as a model protease, long-chain peptides can be easily digested into shorter fragments if such residues are contained within their sequences [22]. In conjunction with predictive data from bioinformatic analyses, fragments with possible desired functionality can be located. Thus, in this study, we used a novel AMP, palustrin-2LTb, identified from *Hylarana latouchii*, as the template, and designed a set of truncated peptides by mimicking trypsin-cleavage processes and interrupting peptide fragments to demonstrate the feasibility of this approach. The antimicrobial activities, saline and serum sensitivity, and haemolytic activity, were then investigated to compare and verify the activity and safety of these fragments. Antimicrobial mechanism studies and in vivo antimicrobial activity studies were also performed to assess the principle of action and the potential of therapeutic worth.

2. Materials and methods

2.1. Acquisition of skin secretions from the frog, *Hylarana latouchii*

Adult frogs (*Hylarana latouchii*) were captured from different locations in Fujian Province, People’s Republic of China. After secretion harvesting, the frogs were released back into the wild. The skin secretions were obtained by mild transdermal electrical stimulation (2 V-5 V) or gently rubbing the frog’s back. Then the skin secretions were washed off the skin with de-ionised water, collected in a cold beaker, frozen using liquid nitrogen and lyophilised in a 1–2/LD freezer dryer (Alpha, Germany).

2.2. ’Shotgun’ cloning and sequencing of palustrin-2LTb precursor-encoding cDNA

’Shotgun cloning’ and sequencing were conducted to determine the nucleotide sequence of palustrin-2LTb. mRNA was separated from *Hylarana latouchii* skin secretion by use of a Dynabeads mRNA Direct Kit (Dynal Biotech, UK). Poly-A mRNA was attracted to the oligo (dT) beads. After elution, all the mRNAs were acquired. A SMART-RACE kit (Clontech, UK) was employed to obtain the DNA sequence of prepro-peptide nucleic acids using a NUP primer (supplied with the kit) and a degenerate sense primer (S1:50-GAWYYAYYHRAGCCYAAA DATGTTCA-30). After that, quick amplification of cDNA ends polymerase chain reaction (RACE-PCR) was utilised to amplify the cDNA; denaturation, annealing and extension were repeated for thermal cycling. The products were separated through gel electrophoresis and purified by a Hi-Bind DNA mini-column (Omega BIO-TEK, USA). Purified PCR was ligated to the vector using a pGEM-T easy vector kit (Promega, USA) and sequenced by an ABI 3100 automatic sequencer (Applied Biosystems, USA). The translated open reading frame was compared to similar sequences using the National Centre for Biotechnology Information (NCBI) BLAST programme. Sequences with high identities were then aligned with the novel peptide.

2.3. Physicochemical properties, modification and prediction analysis of palustrin-2LTb

The structural properties of palustrin-2LTb were analysed using several online tools. Firstly, the potential secondary structure of palustrin-2LTb was predicted by PEP-FOLD3 (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) and visualised by PyMOL (version 4.5). The quality of the predicted model was then validated by Ramachandran plots using PROCHECK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/). The possible antimicrobial activities of palustrin-2LTb and its tryptic peptides were assessed using the online prediction server CAMPRI3-RF (http://www.camp.bicnirrh.res.in). PeptideCutter (https://web.expasy.org/peptide_cutter/) was then used to predict the trypsin cleavage sites and seven fragments were obtained. The physicochemical properties of palustrin-2LTb and its tryptic peptides were acquired via Heliquest (https://heliquest.ipmc.cnrs.fr/cgi-bin/ComputeParams.py).

2.4. Secondary structure determinations by circular-dichroism

The secondary structure of synthetic peptides was determined using the JASCO-815 circular dichroism (CD) spectrometer (Jasco, Essex, UK), as mentioned previously [23]. Briefly, the peptide samples (50 µM) were prepared in 10 mM NH₄Ac solution, 50 % TFE/NH₄Ac (v/v), and 3 mM DPPC/DPPE (88:12) small unilamellar liposomes/vesicles (SUVs), respectively. The wavelength of the test was set in the range of 190–260 nm. The scanning speed was 200 nm/min, and the bandwidth and data pitch were 1 nm and 0.5 nm, respectively.

2.5. Solid-phase peptide synthesis (SPPS)

A Tribute peptide synthesiser (Protein Technologies, Inc, USA) was utilised to synthesise the mature peptide. After synthesis, the peptide was transferred to a 50-ml round-bottomed flask. Twenty-five ml per gram of mixture cleavage solution (94 % Trifluoroacetic acid (TFA) (Sigma-Aldrich, USA), 2 % dd H₂O, 2 % thiouanisole (TIS) (Sigma-Aldrich, USA) and 2 % 1–2 ethanedithiol (EDT) (Sigma-Aldrich, USA)) were added into the round-bottomed flask. The round-bottomed flask was stirred with a rotor in the fume hood for 2 h. After cleavage, the mixture solution was filtered by a Buchner funnel, and 3 ml of dichloromethane (DCM) were used to wash (Sigma-Aldrich, USA) three times. Diethyl ether (Et₂O) (Aldrich, USA) was added to the mixture to 50 ml in a 50-ml tube. Moreover, the tube was placed at a temperature of −20 °C to precipitate the peptide. After drying in the fume hood, 10 ml of HPLC buffer A (0.5 ml TFA/999.5 ml H₂O) were used to dissolve the peptide, and then buffer B (0.5 ml TFA/199.5 ml H₂O/800 ml acetonitrile (Sigma-Aldrich, USA)) 5 ml. The tube was put in an Alpha 1–2 LD plus freeze dryer (CHRIST, Germany) for 48–50 h. The dry peptide was then stored at −20 °C, prior to further analyses.

2.6. Minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC) determinations

Antimicrobial assays were conducted to test antimicrobial activity using Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), MRSA (NCTC 12493), *Enterococcus faecium* (NCTC 12697), Gram-negative bacteria *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 43816), *Pseudomonas aeruginosa* (ATCC 9027), *Acinetobacter baumannii* (ATCC BAA 747), and yeast, *Candida albicans* (ATCC 10231). The pure peptide was first dissolved in...
dimethyl sulphoxide (DMSO, Sigma-Aldrich, USA) to prepare a 12,800 μM solution. Then two-fold dilution was utilised to acquire a series of peptide concentrations (12,800–100 μM). The overnight cultures of microorganisms were separately transferred to 20-ml McCartney bottles of corresponding media provided by ATCC and cultured in an orbital shaking incubator SI500 (Cole-Parmer, USA) until the microbes reached log phase growth. The positive control groups for Gram-positive and Gram-negative bacteria were 1 μl of norfloxacin (2 mg/ml) and 99 μl of microbial culture, while for C. albicans, the positive control was 1 μl of amphotericin B (1 mg/ml) and 99 μl of microbial culture. After incubating for 20–24 h, the growth of bacteria and yeast was measured by a Synergy HT plate reader (BioTek, USA) at a wavelength of 550 nm. The viability of bacteria was calculated as follows: Viability % = (sample group-blank)/(negative group-blank) × 100 %. Ten μl of solutions were aspirated from the wells with no apparent microbial growth and dispersed on agar plates. After overnight growth, the lowest concentration with no bacterial colonies growth was the minimum bactericidal concentration.

2.7. Time-killing assay

The time-killing assays were carried out using the Gram-positive bacterium, S. aureus (ATCC 6538) and the Gram-negative bacterium, E. coli (ATCC 8739), to assess the antimicrobial kinetics of various peptides. In brief, bacteria were incubated with peptides at different concentrations (1*MIC, 2*MIC and 4*MIC). The peptide and bacteria mixtures were spotted onto the MHA plates at different time intervals (0, 5, 10, 20, 30, 60, 90 and 120 min). After being incubated overnight, the bacterial colonies were counted.

2.8. Outer membrane permeability assays

The effects of peptides on the bacterial outer membrane permeability were assessed using E. coli (ATCC 8739) in N-phenyl-1-naphthylamine (NPN) uptake assays. Peptides were incubated with 10 μM NPN (Solarbio) and 1 × 10^5 cfu/ml (OD600 = 0.5) bacterial culture at the logarithmic phase. The bacteria were centrifuged at 3000 g for 10 min and washed twice with 5 mM glucose and 5 mM HEPES buffer at pH = 7.2. Melittin (GIGAVKVLTTGLPALISWIKKKRQQ-NH2 from Apis mellifera) was used as the positive control. Each well contained 100 μl bacterial culture, 50 μl NPN and 50 μl of peptide solution (1*MIC, 2*MIC or 4*MIC). Then the peptide and bacteria were incubated for 2 h at 37 °C. The plate was analysed at 360 nm/460 nm using the Synergy HT plate reader. The membrane permeability rate was expressed as NPN uptake (%) = (F – F0)/(Fmax – F0) × 100 %, where F is the fluorescence intensity of the bacterial culture treated with peptides and F0 and Fmax are culture treated with HEPES buffer and melittin, respectively [24].

2.9. Kinetic SYTOX green permeability assays

SYTOX green was used to assess the membrane permeabilising ability of peptides. The bacteria were grown in TSB medium to the log phase and then centrifuged at 4 °C at 1000C for 10 min to assess the permeability of MRSA (NCTC 12493). After that, the bacteria were collected and washed twice with a 5 % TSB/0.85 % NaCl solution. The bacteria were then diluted to achieve an OD value at 590 nm of 0.7. Each well of the black 96-well plate was then filled with 50 μl of peptide solution, 10 μl of SYTOX green (50 μM, Life Technologies, UK), and 40 μl of bacterial culture. Using the Synergy HT plate reader, the plate was analysed at 485 nm and 528 nm excitation and emission wavelengths for 120 min (interval 5 min) at 37 °C.

2.10. Fluorescence microscopy

Propidium iodide (PI, Sigma, UK) and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, UK) were used to stain the bacteria cells of MRSA NCTC 12493 and E. coli ATCC 8739 as previously described with minor modification [25]. In brief, bacteria in the mid-logarithmic phase were treated with peptide sample (1, 4 × MIC) for 1 h at 37 °C. After 15 min centrifugation at 3000 g, separated bacterial cell pellets were incubated with PI (5 μg/ml) for 15 min. Then, unbound PI was washed away with PBS, and bacterial cells were followed stained with DAPI (10 μg/ml) for another 15 min. Each dye incubation was completed at 4 °C in the dark. The stained bacteria were observed under Leica DMi8 fluorescence microscopy (Leica, Wetzler, Hesse, Germany) using the 100× oil-immersion objective.

2.11. Haemolysis assays

Haemolysis assays were conducted using a 2 % (v/v) suspension of washed horse erythrocytes. A series of peptide concentrations were prepared using twofold dilutions (128 μM – 1 μM). Each concentration of peptide (100 μl) was incubated with 100 μl of horse erythrocyte suspension for 2 h at 37 °C. Then the mixtures were centrifuged at 930 × g for 5 min. The supernatants were added to wells of a 96-well plate, and the haemolytic activity was detected by measuring absorbance at 570 nm. 0.2 % (v/v) Triton X-100 (Sigma, USA) was utilised as a 100 % haemolysis positive control, and PBS acted as a negative control. The haemolysis % in each well was calculated through the following formula:

\[
\text{Haemolysis %} = \frac{\text{(sample group – negative group)}}{\text{(positive group – negative group)}} \times 100\.\%
\]

2.12. Condition sensitivity assays

Sensitivity of peptides under different saline and serum conditions was evaluated with a MIC assay [26]. S. aureus ATCC 6538 and E. coli ATCC 8739 were chosen as representative bacteria for the gram-positive bacteria and the gram-negative bacteria to compare the impacts of physiological conditions on antimicrobial performance of peptides. Briefly, different concentrations of salts (150 mM NaCl, 4.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, and 10 % fetal bovine serum (FBS) (Gibco, USA) were added to the corresponding medium that was used for bacterial suspension dilution. Then the diluted bacterial suspension was mixed with peptide solutions and incubated overnight at 37 °C.

2.13. Determination of in vivo antimicrobial activity of peptides

The larvae of Galleria mellonella were used to evaluate the in vivo antibacterial activity of palustrin-2L Tb and its tryptic peptides as mentioned previously [27]. By injecting 10 μl of MRSA (NCTC 12493) bacterial suspension (1 × 10^7 cfu/ml), the infection model was created. Each infected larva received a further injection of 10 μl of peptide solution at varied doses of 5 mg/kg, 10 mg/kg, and 15 mg/kg after 1 h. Negative control was infected larvae given 10 μl of PBS, and positive control was the infected larvae given 10 mg/kg of vancomycin. Ten larvae made up each group, and for five days, all larvae were examined every 24 h.

2.14. Statistical analysis

Prism software was used for the data analysis (Version 6.0; GraphPad Software Inc., San Diego, CA, USA). The standard error of the mean (SEM) for each set of data in the three replicates from
three experiments was shown by the error bars surrounding the mean data points in the graphs.

3. Results

3.1. ‘Shotgun’ cloning of palustrin-2LTb precursor-encoding cDNA from the skin secretion of Hylarana latouchii

Using the ‘shotgun’ cloning strategy, precursor-encoding cDNA was cloned from the cDNA library of defensive skin secretion of *Hylarana latouchii*. The results of the precursor-encoding cDNA and the open reading frame (ORF) are shown in Fig. 1. The peptide precursor consists of a putative signal peptide with 22 residues, and the following parts were acidic amino acid residue-rich region with 19 amino acid residues and a mature peptide with 31 amino acid residues. The multiple-sequence alignment results indicated that this peptide shares high similarity with known palustrin-2 family peptides and should thus be a novel palustrin-2 type peptide. The identical amino acids and amino acids with small and large similarities are marked in Fig. 1b. Since the novel peptide shared 96 % identity with palustrin-2LTa and was also from *Hylarana latouchii*, it was named in accordance with accepted nomenclature as palustrin-2LTb. The nucleotide sequence of peptide palustrin-2LTb was deposited in the GenBank database under the accession number OP157221.

3.2. Predicted secondary structure and physicochemical properties of palustrin-2LTb

The secondary structure prediction result generated by PEP-FOLD3 showed that palustrin-2LTb may adopt a partial alpha-helical structure in its N-terminus region (from Ser-1 to Lys-11) and to a smaller extent in the middle of the sequence (from Phe-13 to Lys-20). The remaining of palustrin-2LTb probably forms a random-coil structure (Fig. 2a and b). The Ramachandran plot indicated that most of the amino acids in the predicted model were plotted in the most favourable region, showing that the quality of the predicted model is high (Fig. S1).

3.3. Peptide design, prediction analysis and secondary structure viability of peptides in different environments

As one of the major digestive enzymes secreted in the human body, trypsin works specifically to hydrolyse the peptide bonds on the carboxyl side of arginine or lysine residues. To optimise the peptide length, preserve its antimicrobial efficacy, and improve selectivity, the trypsin cleavage-mimicking process was used to truncate palustrin-2LTb and look for valid active fragments. As shown in Fig. 3, there are seven possible cleavage sites within the palustrin-2LTb sequence. Given the potential protective effects of the disulphide bridge formed between Cys-23 and Cys-29, the cleavage site at Arg-24 was omitted. According to the predicted cleavage sites, seven truncated fragments were obtained as listed in Table 1. The potential binding affinity of these fragments with the built bacterial membrane model was also calculated (Table S1). After synthesis, all peptides were purified by RP-HPLC and characterised using MALDI-TOF mass spectrometry (Fig. S2). The purities of the peptides were >95 % (Fig. S3).

Prediction results for secondary structure indicated that these peptides may adopt an alpha-helical structure (Fig. 4). The actual secondary structure of synthetic peptides was measured with CD spectroscopy (Fig. 5). In water-mimicking environments, palustrin-2LTb and its fragments exhibited typical random coil structures. The random coil CD spectra of palustrin-2LTb and fragments were shifted to α-helical CD characteristics with a negative minimum at 208 and 222 nm in membrane-mimicking environments.

The sequences of palustrin-2LTb and its tryptic derivatives were also analysed using the online tool CAMP3-RF (http://www.camp.bincirrh.res.in). According to M. Gabere and W. Noble, this tool offers the best performance among the ten public AMP prediction servers. It can calculate the probability of a peptide as an antimicrobial peptide [28,29]. As shown in Fig. 6, the possibility of peptides 2 and 3 has not changed much, indicating that these peptides may still retain the antimicrobial activity of the parent peptides. For peptides 4 and 8, compared to peptides 1, 2, and 3, the probability is slightly decreased, indicating that the truncation may affect its antimicrobial functions. For the rest fragments, the possibility was reduced to a different extent (generally below 0.5), manifesting that these peptides may be less likely to maintain antimicrobial activities.

3.4. Screening antimicrobial activity of palustrin-2LTb and its fragments

Antimicrobial activity was further evaluated by use of MIC/MBC assays. As shown in Table 2, palustrin-2LTb showed apparent antibacterial activity against tested bacteria, especially for *E. coli* ATCC 8739 and *K. pneumoniae* ATCC 43816. With the truncation
of peptide length, the antibacterial ability of fragments rose first and then decreased. The antibacterial activity of fragments reached a peak after removing the C-terminal ‘Rana Box’. The fragments lost all antimicrobial efficacy as the peptide length was reduced to and beyond 12 amino acids. For peptides with antimicrobial activities, fragment 3 showed the strongest antimicrobial activities against all test microorganisms. Their geometric means (GMs) ranged from 4 µM to 24.7 µM. Of interest, unlike the parent peptide, peptide 3 showed relatively better antibacterial activities against tested Gram-positive bacteria and also exhibited antifungal activity against *C. albicans* ATCC 10,231 with an MIC of 32 µM.

3.5. Time-killing kinetic studies on palustrin-2LTb and its fragments

Time-killing assays were used to determine the bactericidal activity of peptides with highest antimicrobial activity (<256 µM MICs/MBCs, see Table 2). The Gram-positive bacterium, *S. aureus* ATCC 6538 and the Gram-negative bacterium, *E. coli* ATCC 8739, were chosen to compare the bactericidal kinetics of peptides 1, 2, 3, and 4 against different types of bacteria. The *S. aureus* killing kinetic results showed that the four tested peptides could not completely kill the bacteria at MIC concentrations within 120 min. However, for concentrations of 2*MIC and 4*MIC, all tested peptides eradicated bacteria in 20 min (Fig. 7a). For the Gram-negative bacterium, *E. coli* (ATCC 8739), the killing kinetics results demonstrated a different trend in which the parent peptide kills the bacteria rapidly for 30 min at 1*MIC, while peptide 3 and 4 failed to eradicate bacteria in 120 min at their MIC concentrations (Fig. 7b).

3.6. Preliminary mechanistic study of palustrin-2LTb and its fragments

To study the antibacterial mechanism of palustrin-2LTb and its fragments, the impact of these peptides on membrane integrity was evaluated using *E. coli* ATCC 8739 for the outer membrane and MRSA NCTC 12493 for the cell plasma membrane. The outer membrane permeability was assessed using the NPN assay. As shown in Fig. 8a, the outer membrane permeability of all test peptides occurred in a concentration-dependent manner. Peptide 2 showed similar permeability to the parent peptide at their MIC and 2*MIC, but at the concentration of 4*MIC, the permeability of peptide 2 was increased and nearly reached 100 %. Peptides 3 and 4 showed relatively weaker permeability than the parent peptide. To study the cell plasma membrane permeability of palustrin-2LTb and its fragments, the SYTOX green permeability test was performed.

### Table 1

| Peptide        | Sequence                  | Theoretical molecular Weight (Da) | Net charge | Hydrophobicity <H> |
|---------------|---------------------------|-----------------------------------|------------|--------------------|
| 1(Palustrin-2LTb) | SLWENFKNAGKKFLINILDKIRCVRAGCRT | 3550.2                            | +5         | 0.364              |
| 2             | SLWENFKNAGKKFLINILDKIRCVRAGCR-NH₂ | 3448.1                            | +6         | 0.367              |
| 3             | SLWENFKNAGKKFLINILDKIR-NH₂ | 2647.1                            | +4         | 0.383              |
| 4             | SLWENFKNAGKKFLINILDK-NH₂ | 2377.8                            | +3         | 0.381              |
| 5             | SLWENFKNAGK-NH₂ | 1420.6                            | +3         | 0.1                |
| 6             | SLWENFKNAGK-NH₂ | 1292.4                            | +2         | 0.199              |
| 7             | SLWENFK-NH₂ | 972.0                             | +1         | 0.214              |
| 8             | FILNILDK-NH₂ | 974.2                             | +1         | 0.271              |
conducted. The experimental results showed that peptide 1 and 2 had the fastest membrane permeability rates. Similar to the outer membrane permeability results, not all peptides achieved a 100% membrane permeability rate after two hours. The DAPI/PI staining results confirmed that the presence of palustrin-2LTb can lead to a high degree of membrane permeabilisation at the
concentrations of 1- and 4-fold MICs against test bacteria, while peptide 3 only damaged the cell membrane of partial MRSA and E. coli at test concentrations (Fig. 9).

3.7. Haemolytic activity of palustrin-2LTb and its fragments

All synthetic peptides were evaluated for their in vitro haemolytic activity using horse erythrocytes. The haemolytic activity is shown in Table 3. Compared to the parent peptide, the removal of the C-terminal threonine increased haemolytic activity, while for the following residue removals, its haemolysis was decreased. The therapeutic index (TI) was calculated to evaluate the selectivity of peptides and potential clinic value. Among test peptides, peptide 3 showed the highest TI values (TI overall = 64), approximately sixteen times that of the parent peptide (TI overall = 4.2) (Table 3).

3.8. Antimicrobial activity sensitivity of palustrin-2LTb and peptide 3 in salts and serum conditions

To explore the antimicrobial activity of peptides under physiological environments, sensitivities of palustrin-2LTb and peptide 3 were evaluated. For palustrin-2LTb, the treatment of different ions produced slight impacts on its antimicrobial performance against test bacteria. The MIC against E. coli increased fourfold in the presence of 10 % FBS. However, incubation with ions caused two to fourfold increase of peptide 3 MICs. Especially, FBS induced four to sixteenfold increase of peptide 3 MICs against S. aureus ATCC 6538 and E. coli ATCC 8739 (see Tables 4 and 5).

3.9. In vivo antibacterial activity of peptide 2 and 3 in the treatment of MRSA-infected larvae model

To better compare the antibacterial effect on MRSA (NCTC 12493) in vivo, an infected insect larvae model was used to evaluate the therapeutic effect of these two peptides. Among test peptides, peptide 2, 3, and 4 showed relatively higher potent anti-MRSA activity. Given the higher MIC value of peptide 4, peptide 2 and 3 were chosen. Healthy larvae were first incubated with peptide 2 and 3 alone (15 mg/ml) to verify their biocompatibility. As shown in Fig. 10, the presence of peptide did not affect the viability of the larvae after five days' incubation. Compared to peptide 2, peptide 3 had a better therapeutic effect on larvae that had been infected with MRSA. At the maximum dose of the peptide (15 mg/kg), the five-day survival rate with peptide 3 reached 60 %, while that of peptide 2 could merely reach 30 %. At the concentration of 10 mg/kg, the peptide 2 treated group died after 96 h. At a peptide concentration of 5 mg/kg, peptide 3 had a survival rate of 0 % at 108 h, while larvae treated with peptide 2, were all dead after 72 h.

4. Discussion

As promising antibiotic agents against multi-drug resistant pathogens, AMPs have unparalleled advantages over conventional antibiotics due to their membrane-active properties and low probability of resistance [30,31]. However, several factors, such as high synthesis costs, low selectivity and low efficiency of modification, greatly hinder the application of AMPs [32,33]. Therefore, shorter peptides with potent antimicrobial efficacy are more favourable than the longer ones [34]. In previous studies, the truncation-modification of AMPs was generally performed by capturing fragments in sequence randomly, and the truncated fragments usually failed to preserve their original antimicrobial activities or showed increased toxicity to human erythrocytes or other cell lines [35]. In this study, we introduced a modification approach by combining
the trypsin-cleavage mimicking modification strategy with bioinformatics tool analysis to quickly search the valid fragments of a long-chain AMP.

By using “shotgun” cloning, we discovered a novel AMP, named palustrin-2LTb, from *Hylarana latouchii* skin secretion. This novel peptide contained 31 amino acid residues. According to the multi-sequence alignment results, the homology comparison between the newly-discovered peptide and other palustrin-2 peptides revealed high levels of identity and thus it obviously belonged to the palustrin-2 family. Palustrin-2LTb was then used as a template to generate a set of truncated fragments by using the PeptideCutter programme. CAMPR3-RF was subsequently employed to the probability of tryptic peptides as antimicrobial peptides. According to the predicted results, fragments 2 and 3...
showed a high likelihood of being antimicrobial peptides, indicating that they may preserve the antimicrobial functions of the parent peptide. The probability of fragments 4 and 8 was lower than 2 and 3, marking the antimicrobial functions of this peptide may be decreased. For fragments 5, 6, and 7, their probabilities were generally lower than 0.5, manifesting they are less likely to be antimicrobial peptides, and their antimicrobial activities may be affected by the truncation.

To prove the feasibility of this modification approach and verify the activity of generated fragments, all fragments were synthesised and employed in a set of antimicrobial experiments. Antimicrobial screening results demonstrated that palustrin-2LTb showed potent antibacterial activities against test bacteria, among which the Gram-negative bacteria seem to be more susceptible with a lower GM value of 13.5 μM. For truncated peptides, their antimicrobial results were generally consistent with the calculated results of CAMPR3-RF. Peptides 2, 3, and 4 not only preserved the functions of the parent peptides but also showed improved antimicrobial capacity. The GM (overall) values of peptides 2, 3, and 4 were decreased from 24.7 μM (the parent peptide) to 14.7 μM, 4 μM, and 13.5 μM, respectively. However, peptides 5, 6, 7 and 8, did not show obvious antimicrobial activities within test concentrations. These results indicated that the ‘active-site’ of palustrin-2LTb may indeed exist outside the C-terminal ‘Rana-Box’ since removing the ‘Rana-Box’ did not affect the antimicrobial activity of peptide 3 and truncating of N-terminal amino acids also decrease the antibacterial activity of peptides 5, 6, 7 and 8, and probability calculated by CAMPR3-RF tool could provide a useful reference for the predictions of AMPs.

In previous studies, many AMPs were found with better activity against Gram-positive bacteria. Researchers generally contributed this to the thick layer of lipopolysaccharides (LPS), which may impede actions or capture the cationic AMPs [36,37]. In this study, peptides 1, 2, 3, and 4 exhibited stronger antibacterial activity against the Gram-negative bacteria with respect to the Gram-positive bacteria. Given the fact that many AMPs are reported to have outer-membrane permeating activity recently, we hypothesise that the lower activity of palustrin-2LTb and its tryptic peptides against the test Gram-positive bacteria may be provoked by the thicker layers of peptidoglycans of the Gram-positive bacteria.
These peptides may easily interact with the outer membrane of the Gram-negative bacteria while they cannot penetrate peptidoglycans readily. Compared to the Gram-negative bacteria, the membranes of the Gram-positive bacteria contain more layers of peptidoglycans, and this thick ‘barrier’ may impede peptide access to the bacterial plasma membrane, thereby inducing weaker activity [38].

Meanwhile, the time-killing kinetics results showed that peptides 1, 2, 3, and 4 merely exerted a bacteriostatic function against S. aureus at 1*MIC in 120 min, but could eradicate the bacteria quickly at concentrations of 2*MIC and 4*MIC. For peptides 3 and 4, 2*MIC and 4*MIC could eliminate the bacteria in 10 min. Peptides 2, 3, and 4 showed slightly lower kinetics in killing E. coli at concentrations of 2*MIC and 4*MIC, while 100 % killing was yielded within 120 min of incubation. For S. aureus, the truncation of peptides seems to produce no apparent impact on the killing kinetics of peptides. However, the modifications greatly affected the killing speed of peptides against E. coli. Especially, after removing of the ‘Rana-Box’, the kill kinetics of peptides 3 and 4 greatly decreased, and they could merely exert bactericidal function at concentrations of 2*MIC and 4*MIC. These results indicated that the existence of the ‘Rana-Box’, even though it may not produce obvious effects on the final antibacterial concentrations of peptides, may play a role in outer-membrane interactions since the removal of this loop structure renders peptides 3 and 4 with a lower permeability in the NPN assay and slower killing rates in time-killing kinetic studies. The presence of the ‘Rana-Box’ may help palustrin-2LTb bind to the LPS and make the interactions easier and faster.

Membrane-permeabilising activity is considered one of the most representative working modes of AMPs [34]. In the initial step, the coulomb force pushes cationic AMPs to approach the anionic bacterial membrane via electrostatic interactions. The hydrophobic core of peptides subsequently interacts with the phospholipid bilayer and disrupts the integrity of the bacterial membrane, thereby resulting in membrane depolarisation and leading to cell death [39]. Our preliminary antibacterial mechanism studies demonstrated the treatment of palustrin-2LTb and its fragments (peptides 2, 3, and 4) can result in the membrane-disruption of E. coli ATCC 8739 and MRSA NCTC 12493 in a concentration-dependent manner. Therefore, palustrin-2LTb and

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**Fig. 9.** Fluorescence graphs and merged graphs of DAPI/PI staining of (a) MRSA NCTC 12493 and (b) E. coli ATCC 8739 treated with 1-fold and 4-fold MICs of palustrin-2LTb and peptide 3.
### Table 3
HC\textsubscript{50} and TI values of palustrin-2LTb and its fragments.

| Peptide      | HC\textsubscript{50} (\textmu M) | TI (MRSA NCTC 12493) | TI ('G +') | TI ('G -') | TI ('Fungi') | TI ('overall') |
|--------------|----------------------------------|-----------------------|------------|------------|--------------|----------------|
| 1 (Palustrin-2LTb) | 102.4                            | 3.2                   | 4.1        | 7.6        | 0.4          | 4.2            |
| 2             | 58.9                             | 7.5                   | 5.8        | 6.2        | 0.2          | 4.0            |
| 3             | >128                             | 128                   | 128        | 64         | 8            | 64             |
| 4             | >128                             | 8                     | 12.7       | 32         | 8            | 19             |
| 5             | >128                             | 1                     | 1          | 1          | 1            | 1              |
| 6             | >128                             | 1                     | 1          | 1          | 1            | 1              |
| 7             | >128                             | 1                     | 1          | 1          | 1            | 1              |
| 8             | >128                             | 1                     | 1          | 1          | 1            | 1              |

*Therapeutic index (TI) was calculated as HC\textsubscript{50}/GM. When no detectable haemolysis activity was observed at 128 \textmu M, a value of 256 \textmu M was used to calculate the TI.*

### Table 4
MICs (\textmu M) of peptide 1 and 3 against S. aureus (ATCC 6538) in salts and serum.

| Peptide | Control | NaCl | KCl | CaCl\textsubscript{2} | MgCl\textsubscript{2} | 10 % FBS |
|---------|---------|------|-----|-----------------------|-----------------------|----------|
| 1       | 32      | 32   | 32  | 32                    | 32                    | 32       |
| 3       | 2       | 4    | 2   | 2                     | 2                     | 8        |

*Therapeutic index (TI) was calculated as HC\textsubscript{50}/GM. When no detectable haemolysis activity was observed at 128 \textmu M, a value of 256 \textmu M was used to calculate the TI.*
its tryptic peptides may also exert their antibacterial functions via a membrane-breaking mechanism, similar to many reported AMPs. Interestingly, for MRSA NCTC 12493, peptide 3 showed quite a low permeable rate with respect to other test peptides, indicating its anti-MRSA mechanism may involve some intracellular targets, such as nucleic acids or nascent proteins, thereby affecting the downstream cell process such as DNA replication, protein synthesis, and cell wall construction [40–42].

In addition to testing the antimicrobial activities of peptides under a non-physiological environment, the sensitivity of palustrin-2LTb and peptide 3 in the presence of different ions and serum conditions was also conducted since previous studies indicated that the direct antimicrobial activities of many AMPs might be compromised by ions or host lipids and proteins [42]. The results indicated that palustrin-2LTb showed relatively more stable antimicrobial activities under different saline and serum environments, and peptide 3 was less tolerant to ions and serum, which may be related to the deletion of ‘Rana-Box’ since previous studies demonstrated that the existence of ‘Rana-Box’ may help stabilise some peptides’ alpha-helical structure and complement their stable activities [43,44]. Therefore, the ‘Rana-Box’ may also play an essential role in the structural stability of palustrin-2LTb, and the removal of such structure may destabilise peptide 3. Although peptide 3 exhibited weaker saline and serum tolerance, its average MICs were still lower than the parent peptide under physiological conditions. With further modification, such as introducing bulky amino acids or D-amino acids to improve its ion tolerance or enzymatic stability, peptide 3 is still a promising antibiotic candidate [26,45].

Overall, compared to palustrin-2LTb and other fragments, peptide 3 showed the greatest therapeutic efficacy in both in vitro and in vivo tests. It can not only efficiently inhibit and eradicate test ‘ESKAPE’ strains and even their biofilms but also significantly improve the survival rate of MRSA-infected insect larvae. The successful design of peptide 3 proves that our modification approach is feasible and able to help to explore the valid motif of a long peptide expeditiously and may provide new ideas for later AMP modifications. Moreover, the decreasing haemolysis of peptide 3

Table 5

| Peptide | Control | NaCl | KCl | CaCl₂ | MgCl₂ | 10 % FBS |
|---------|---------|------|-----|-------|-------|----------|
| 1       | 8       | 16   | 8   | 8     | 2     | 32       |
| 3       | 2       | 8    | 2   | 2     | 2     | 32       |

Fig. 10. The Kaplan-Meier-curves of (a) peptide 2 and (b) peptide 3 in wax moth larvae infected with MRSA NCTC 12493. The sterile PBS and vancomycin (10 mg/kg) were set as the negative and positive controls. The growth control was performed with MRSA only. The data were analysed by the survival analysis and curve comparison test comparing the survival rate of different concentrations of peptides with that of the growth control group. The significance is indicated by **** (p < 0.0001), *** (p < 0.001), ** (p < 0.01), and * (p < 0.05).
represents that our method may also be an alternative way to control the toxicity of AMPs while maintain (even improve) the antimicrobial activities of peptide. Peptide 3, with improved therapeutic potential, may be an excellent antibacterial candidate to address problems induced by resistant pathogens.

5. Conclusion

In this work, we demonstrated the feasibility and efficiency of a trypsin-cleavage mimicking modification strategy in AMP design via \textit{in silico} calculations and reality testing. A novel palustri-type AMP, named palustri-2LTb, was successfully identified in the skin secretion of the Kuutun frog, \textit{Hylarana laotouchi}, by use of molecular technology and utilised as the template to generate a set of fragments. \textit{In vitro} and \textit{in vivo} test results indicated that truncated fragment 3 not only preserved the antimicrobial activity of the template peptide but also showed improved therapeutic potential against test 'ESKAPE' strains and elongated survival times in an MRSA-infected insect larvae model. The design of fragment 3 demonstrates the feasibility of our modification strategy and also opens the door to optimisation of AMPs in later research.

CRediT authorship contribution statement

Wanchen Zou: Formal analysis, Investigation, Writing – original draft. Yingyi Zhang: Methodology, Data curation, Software. Mei Zhou: Conceptualization, Supervision, Resources. Xiaolong Chen: Methodology, Visualization. Chenghang Ma: Project administration. Tao Wang: Methodology, Writing – review & editing. Yangyang Jiang: Methodology, Supervision, Writing – review & editing. Tianbao Chen: Conceptualization, Supervision. Chris Shaw: Writing – review & editing. Lei Wang: Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.11.016.

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