RV-23, a Melittin-Related Peptide with Cell-Selective Antibacterial Activity and High Hemocompatibility

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Introduction

Antimicrobial peptides have become an important research focus owing to their possible application as a new source of antibiotics [32]. Among them, melittin, which contains 26 amino acid residues, is one of the most widely studied [1, 7, 20, 21]. Melittin is isolated from honeybee (Apis mellifera) venom and exhibits strong lytic activity against both eukaryotic and prokaryotic cells [11, 20]. Melittin-mediated lysis is associated with the membrane permeabilization of target cells, which causes the leakage of cell contents and cell death [3, 8, 22, 24, 25]. However, the clinical utility of melittin is limited by its nonselective toxicity against human cells. Biological studies have shown that the strong hemolytic activity of antimicrobial peptides generally correlates with structural parameters such as hydrophobicity, amphipathicity, net positive charge, and helicity [4, 12, 16]. Even minor structural modifications can have a large impact on the cytolitic action of melittin. Hydrophobic [2, 30] or positively charged residues [19] introduced on different sides of the helical structure could gradually change the conformation of melittin, leading to changes in its hemolytic activity.

In previous studies, it was found that two frog skin-derived melittin-related peptides, RV-23 from Rana draytonii [5] and AR-23 from Rana tagoi [6], exhibit lower cytotoxicity than melittin [14, 26]. The two peptides are each composed of 23 amino acids. AR-23 shows high sequence identity (78% of amino acid identity) with melittin, whereas RV-23 has similar numbers of leucine and isoleucine residues as melittin and AR-23 (Table 1). Urban et al. [26] compared the bioactivities of four frog skin-derived peptides in the temporin family and melittin-related peptides, and indicated that AR-23 and RV-23 exhibited excellent antimicrobial activity against a series of bacterial strains. Our previous data also confirmed that RV-23 has higher selective antibacterial activity against both eukaryotic and prokaryotic cells.
activity and lower cytotoxicity than AR-23 and melittin. The aim of this study was to explore the selective antibacterial mechanism of RV-23 by comparing it with melittin and AR-23, especially the effect of RV-23 structural parameters on its binding and lytic activity toward the membranes of bacterial and human cells. For this purpose, structural parameters of RV-23 such as helicity, hydrophobicity, and amphipathicity were compared with those of melittin and AR-23. The interaction between the peptides and bacterial and mammalian cells, the antibacterial activity of the peptides, and their cytotoxicity were also determined. For biomedical applications, the in vivo safety of a peptide is the most important issue. Hence, the hemocompatibility of RV-23 and the interactions of the antimicrobial peptides with blood were investigated, specifically their effects on key blood factors such as platelet coagulation, albumin conformational change, and blood coagulation.

Materials and Methods

Materials

TAMRA-labeled and unlabeled peptides of melittin, AR-23, and RV-23 were synthesized (Table 1) using the standard Fmoc procedure, purified by reverse-phase high-performance liquid chromatography (RP-HPLC), and dissolved in H₂O at 5 mg/ml as stock solution for further use. The purity of the synthetic peptides was greater than 95%. Trifluoroethanol (TFE) and human serum albumin (HSA) were obtained from Sigma. Sodium dodecyl sulfate (SDS) was purchased from Amresco. Branched polyethyleneimine (BPEI) was purchased from Sigma-Aldrich (Germany).

The purity of the peptides was verified by analytical RP-HPLC and was further characterized by mass spectrometry in electrospray positive-ion detection mode on Agilent 1100 ESI/MS (Agilent Technologies, USA). The RP-HPLC peptide retention time (tR) was determined at room temperature on a Kromasil 100-5C18 column (4.6 mm × 250 mm) using a linear AB gradient and a flow rate of 1.0 ml/min, where solvent A was 0.05% trifluoroacetic acid in 100% water, and solvent B was 0.05% trifluoroacetic acid in 80% acetonitrile.

Hydrophobic Moment Analysis

Peptide amphipathicity was determined by calculation of the hydrophobic moment, using http://emboss.bioinformatics.nl/cgi-bin/emboss. The default angle of 100 degrees was used for the α-helix and 160 degrees for the β-sheet. Helical wheel projections were performed online using Helical Wheel Projections (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi).

Blood Samples

Blood samples from healthy donors were obtained from Beijing Red Cross Blood Centre. All procedures performed in studies involving human samples were in accordance with the ethical standards of the Beijing Institute of Blood Transfusion committee, and the donors signed informed consent forms. A 5 ml syringe was loaded with 0.5 ml of 3.8% disodium citrate prepared in 1× PBS. The blood samples were prepared for hemolytic assays, platelet aggregation, and thromboelastograph (TEG) assays.

Cell Culture

HeLa (human cervix carcinoma) cells from ATCC (VA, USA) were maintained in our laboratory. The HeLa cells were cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin unless otherwise specified and maintained in a humidified incubator with 5% CO₂ at 37°C.

Circular Dichroism (CD) Spectrometry of the Peptides

CD spectra were recorded on a Jasco-J-715 spectra polarimeter (Jasco Inc., MD, USA) in 0.1 cm path length cells under nitrogen at 25°C. The spectra were recorded between 195 and 240 nm at a peptide concentration of 50 μM in water or water containing 50% TFE (v/v) or 30 mM SDS. The percentage of α-helix structure was calculated as:

$$\alpha-\text{helix}(\%) = \frac{[\theta]_{222} - [\theta]_{190}}{[\theta]_{190}}$$

where [\theta]_{222} is the experimentally observed absolute mean residue ellipticity at 222 nm. Values for [\theta]_{222} and [\theta]_{190}, corresponding to 0% and 100% α-helix content at 222 nm, were estimated to be -2,000 and -28,400 deg cm²/dmol, respectively [15].

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Table 1. Amino acid sequence, molecular weight, charge, and hydrophobicity value of each peptide used in this study.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence</th>
<th>Theoretical MW (^a)</th>
<th>Observed MW</th>
<th>tR (min) (^b)</th>
<th>μH (^c)</th>
<th>Net charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melittin</td>
<td>GIGAILKVLATGLPTLSIWIKKKRKQ.NH₂</td>
<td>2,844.75</td>
<td>2,845.4</td>
<td>19.14</td>
<td>0.499</td>
<td>5</td>
</tr>
<tr>
<td>AR-23</td>
<td>AIGSILGALAKGLPTLSIWIKNR.NH₂</td>
<td>2,390.45</td>
<td>2,391.3</td>
<td>16.60</td>
<td>0.548</td>
<td>3</td>
</tr>
<tr>
<td>RV-23</td>
<td>RIGVLLARLPKLSFLKLMGKKV.NH₂</td>
<td>2,625.68</td>
<td>2,626.4</td>
<td>11.05</td>
<td>0.618</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\)Molecular weight (MW) as measured by mass spectroscopy.

\(^b\)tR, retention time as measured by reverse-phase HPLC.

\(^c\)μH, mean relative hydrophobic moment determined at website: http://emboss.bioinformatics.nl/cgi-bin/emboss.
Antimicrobial Activity of the Peptides

The antibacterial activity of the peptides against *Escherichia coli* a gram-negative bacterium, and *Staphylococcus aureus* a gram-positive bacterium, was examined in sterile 96-well plates using the microdilution method [20, 34]. Briefly, bacteria were grown in liquid LB medium at 37°C to the mid-log phase, and then the bacteria were diluted to 2 × 10^6 colony-forming units (CFU)/ml. The peptides were serially diluted with PBS. Diluted peptide (50 µl) was added to 50 µl of the bacterial suspension in 96-well plates. After incubation at 37°C for 18–20 h, the absorbance of each well was recorded using a multiwell microplate reader (SpectraMaX M5; Molecular Devices, CA, USA) at 600 nm. The lowest concentration at which the peptide inhibited complete growth of the bacteria was taken as the minimal inhibitory concentration (MIC).

Hemolytic Activity of the Peptides

The hemolytic activity of the peptides was assayed by a standard procedure with slight modification [27]. Fresh human red blood cells (hRBCs) were washed three times and resuspended to 20% hematocrit in PBS. The PBS-diluted peptide solution (180 µl) was added to a V-bottom 96-well plates (Millipore, MA, USA), after which 20 µl of erythrocytes was added. After incubation at 37°C for 30 min, the samples were centrifuged and the absorbance of the supernatant was measured at 450 nm using a multiwell microplate reader (SpectraMaX M5) and compared with the 100% hemolysis caused by 0.1% Triton X-100. The percentage of hemolysis was calculated according to the following equation:

\[
\text{Hemolysis(%) } = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{Triton-X100}} - A_{\text{blank}}} \times 100
\]

Cytotoxicity of the Peptides

The toxicity of the peptides against HeLa cells was checked by using the standard MTT assay [18]. HeLa cells were seeded into 96-well plates (Corning Inc., MA, USA) at 5 × 10^3 cells/well and incubated overnight. Diluted peptides were added to the HeLa cells and incubated for 3 h. After the media was replaced with fresh media, the cells were incubated for 24 h. A total of 20 µl of MTT (5 mg/ml) solution was added and incubated for 4 h. The medium was then removed and 150 µl of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm on a microplate reader. The absorbance was calculated using the following equation:

\[
\text{Viability(%) } = \frac{A_{\text{treated}} - A_{\text{blank}}}{A_{\text{untreated}} - A_{\text{blank}}} \times 100
\]

HeLa Cell and Bacterial Cell Membrane Damage Induced by the Peptides

Peptide-induced membrane damage was examined by detection of propidium iodide (PI) influx [13, 29]. HeLa cells (1 × 10^4 cells) were treated with peptide (final concentration 20 µM) and incubated for 30 min at 37°C, and the cells were washed with PBS and resuspended in PI solution (final concentration 2 µg/ml). The bacteria (*E. coli* or *S. aureus*) were cultured at 37°C to an OD₆₀₀ = 0.5 (the number for *E. coli* was 1-2 × 10⁶ CFU/ml and for *S. aureus* it was 4-5 × 10⁶ CFU/ml), after which 20 µM of peptide was added and the culture was incubated for 30 min. The bacteria were collected and resuspended in PI solution (final concentration 2 µg/ml). The fluorescence signal in the treated cells was determined by flow cytometry (Cytomics FC 500; Beckman Coulter, USA).

Localization and Binding of the Peptides onto Mammalian and Bacterial Cells

Localization and binding of the peptides onto mammalian and bacterial cells were evaluated using TAMRA-labeled peptides [20]. HeLa cells (1 × 10⁵ cells/well) were seeded in a CVG 8-well chamber (NUNC Lab-Tek). 24 h later, 4 µM of TAMRA-labeled peptide was added to the HeLa cells. After incubation for 30 min, the cells were washed with PBS and fixed with 4% paraformaldehyde. Then, the cells were stained with the nuclear stain DAPI (300 nM) and observed under a confocal scanning laser (Radiance 2100TM; Bio-Rad, CA, USA) in an inverted fluorescence microscope (TE300; Nikon, NY, USA). Typical fluorescence images were taken at the same time. Mid-log phase (OD₆₀₀ = 0.5) suspensions of *E. coli* and *S. aureus* were also incubated with TAMRA-labeled peptide solution (final concentration 20 µM) for 30 min at 37°C. Then, localization of the peptides on the bacterial cells was observed under a confocal scanning laser (Radiance 2100TM) with an inverted fluorescence microscope (TE300).

HeLa cells (1 × 10⁵ cells) and mid-log phase (OD₆₀₀ = 0.5) suspensions of *E. coli* and *S. aureus* were also incubated with TAMRA-labeled peptide solution (final concentration 20 µM) for 30 min at 37°C. Then, to quantify the localization of the peptides on the bacterial cells, flow cytometry was performed.

Hemocompatibility of the Peptides

Effects of the peptides on platelet.

Whole blood was centrifuged at 200 × g for 5 min to obtain platelet rich-plasma (PRP). The PRP (0.9 ml) was incubated with 0.1 ml of peptide solution (5 µM in PBS) for 10 min, and then the platelets were fixed with 4% paraformaldehyde for 60 min. Platelet aggregation was observed by fluorescence microscopy (Leica DMI4000B; Leica Microsystems, Germany). Platelets activated by ADP (final concentration 10 µM) were set as the positive control.

Effects of the peptides on blood coagulation.

The effects of the peptides on blood coagulation were assessed by the Thromboelastograph Hemostasis System 5000 (Haemoscope Corporation; USA) [33]. Briefly, citrated whole blood (900 µl) was mixed with 100 µl of peptide solution (5 µM in PBS) in a tube containing kaolin. Then, 340 µl of sample was added to the TEG cup. The TEG analysis was initiated by the addition of 20 µl of CaCl₂ solution (0.2 M). The main parameters recorded included clotting time (R), clot formation time (K), α-angle, and maximum clot thickness (MA). R is defined as the time from the start of the
Interaction between the peptides and human serum albumin.
The peptides (5 µM) were mixed with 0.33 mg/ml HSA solution.
The mixture was incubated at 37°C for 30 min. BPEI (final concentration 1 mg/ml) was used as the positive control [33]. The UV absorption of pure HSA and peptide-HSA mixtures was measured at room temperature by a microplate reader (SpectraMax M5). Quartz cuvettes of 1 cm were used and the absorption spectra were recorded from 200 to 400 nm. The fluorescence spectra of the pure HSA and peptide-HSA mixtures were also recorded at λexc 295 nm and λem from 300 to 500 nm on a microplate reader. The excitation and emission slit widths (each 1 nm) and scan rate were set for all the experiments.

Statistical Analysis
A statistical analysis was performed using Student’s t-test between two groups. Differences were considered significant at \( p < 0.05 \).

Results and Discussion
Characterization of the Peptides
The measured molecular weights of all the peptides were consistent with their theoretical values (Table 1), suggesting that the peptides had been successfully synthesized. Helical wheel projections indicated that all three peptides can attain well-defined amphipathic helical structures (Fig. 1). RP-HPLC results showed that the retention times of melittin, AR-23, and RV-23 were 19.14, 16.60, and 11.05 min, respectively. Because a short retention time is correlated with low hydrophobicity [4], the results indicated that RV-23 had the lowest hydrophobicity, whereas melittin was the most hydrophobic. In addition, the amphipathicity of the peptides was determined (Table 1) by calculation of their hydrophobic moment [10]. Increasing amphipathicity is the outcome of increasing the number of charged residues on the polar face, and RV-23 displayed higher amphipathicity (0.618 vs 0.499 and 0.548).

Secondary Structures of the Peptides
The secondary structures of the peptides were determined in two membrane-mimicking environments, 50% TFE or 30 mM SDS, as well as in water, by CD spectroscopy. Characteristic α-helical peaks were observed at 208 and 222 nm. According to the CD spectra, all the peptides formed random coil structures in water (Fig. 2A); however, they formed a well-defined α-helical structure in the presence of 50% TFE or 30 mM SDS (Figs. 2B and 2C). The helix content in 50% TFE was estimated to be 83.36% for melittin, 53.37% for AR-23, and 36.38% for RV-23. The helix content in 30 mM SDS was higher than in 50% TFE for all the peptides: 84.82% for melittin, 68.07% for AR-23, and 44.42% for RV-23.

Antimicrobial Activity of the Peptides
The peptides were incubated with gram-positive S. aureus and gram-negative E. coli and the surviving cell fraction was assessed by microdilution [20]. As shown in Table 2,
all the peptides exhibited significant inhibitory activity against *S. aureus* and *E. coli*. The MIC of melittin, AR-23, and RV-23 was 8.33, 22.67, and 3.33 µM, respectively, against *E. coli*, suggesting that RV-23 had the highest antibacterial potential against *E. coli*, whereas AR-23 had the lowest. Notably, all the peptides exhibited almost the same antibacterial activity against *S. aureus*.

### Cytotoxic Activity of the Peptides

To test the cytotoxicity of the peptides, their hemolytic activity against hRBCs and their growth inhibition against HeLa cells were investigated. Among them, melittin exhibited the highest hemolytic activity, whereas RV-23 exhibited the lowest hemolytic activity. For example, the hemolytic rate of 6 µM melittin was 90.73 ± 7.08%, whereas that of RV-23 was only 2.56 ± 0.41% at the same concentration (Fig. 3A).

The MIT assay data were consistent with the hemolytic assay. That is, melittin exhibited the highest cytotoxicity. For example, the viability of 6 µM melittin-treated cells was 1.61 ± 0.88%, whereas that of RV-23-treated cells was 83.71 ± 5.47% at the same concentration (Fig. 3B). It is worth noting that RV-23 was not toxic to either hRBCs or HeLa cells at its MIC or even somewhat higher.

### Membrane Damage Induced by the Peptides

PI was employed as a fluorescent probe to visualize peptide-induced membrane damage. As shown in Fig. 4, untreated bacterial cells showed no PI fluorescence. PI staining of HeLa cells following the treatment of the peptides is presented in Fig. 4A. The peptide-induced damage of HeLa cell membranes showed a similar trend to the hemolytic activity and cytotoxicity of the peptides. However, 64.7%, 57.6%, and 85% of melittin-, AR-23-, and RV-23-treated *E. coli* cells were stained with PI, respectively (Fig. 4B). With regard to *S. aureus*, all the peptides exhibited significant membrane disruption activity, and almost all the *S. aureus* cells were stained with PI (Fig. 4C). This result indicated that RV-23 had stronger antibacterial activity than the other two peptides.

### Membrane Penetrating Activity of the Peptides

The interaction of TAMRA-labeled peptides with the membrane of HeLa and bacteria cells was investigated by confocal microscopy and FACS. The FACS results indicated that 100% of TAMRA-labeled peptide-treated HeLa cells exhibited fluorescence (Fig. 5A). Regarding bacterial cells, approximately 68.2%, 47%, and 93.3% of TAMRA-labeled melittin-, AR-23-, and RV-23-treated *E. coli* cells showed fluorescence, respectively (Fig. 5A). The results were
consistent with the antibacterial studies, indicating that RV-23 had stronger binding and penetrating activities against the *E. coli* cell membrane than either melittin or AR-23. However, red fluorescence was detected in almost 100% of *S. aureus* cells treated with any of the TAMRA-labeled peptides. Some melittin- and AR-23-treated cells showed red fluorescence, and most of the signal was focused in the cytoplasm, and the red fluorescence in melittin-treated cells was the strongest (Fig. 5B). However, only a weak red fluorescence signal could be observed on the surface of RV-23-treated cells. That is, unlike RV-23, melittin and AR-23 at 4 µM could penetrate into HeLa cells. Confocal microscopy also indicated that the TAMRA-labeled peptide could penetrate the *E. coli* cell membrane, as well as the membrane of *S. aureus* cells. TAMRA-labeled AR-23 exhibited the lowest ability of the three peptides to bind and penetrate the *E. coli* cell membrane (Fig. 5C), and the three peptides showed similar ability to bind and penetrate the *S. aureus* cells membrane (Fig. 5D).

The results indicated that all the peptides had the same inhibitory activity against *S. aureus* but differing activities against *E. coli*. Notably, RV-23 could efficiently penetrate *E. coli* cells with low hemolytic activity and low cytotoxicity. The data further indicated that the selective lytic activity of RV-23 against bacteria is due to its discriminating penetration activity against the membranes of bacterial and human cells.

**Hemocompatibility**

**Effects of the peptides on the activation of platelets.** The effects of the peptides on platelets were studied by observing platelet aggregation with microscopy. As shown in Fig. 6A, compared with the positive control (ADP), melittin, AR-23, and RV-23 did not cause platelet aggregation,
which indicated that these peptides would not affect the aggregation of platelets.

Coagulation assays (thromboelastometry). TEG is considered to be one of the most useful tools for evaluating

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Fig. 5. Binding and localization of the TAMRA-labeled peptides to HeLa cells, E. coli and S. aureus, as measured by FACS and confocal microscopy.

(A) Binding of the TAMRA-labeled peptides to HeLa cells, E. coli, and S. aureus as measured by FACS. (B) Localization of the TAMRA-labeled peptides to HeLa cells. (C) Localization of the TAMRA-labeled peptides to E. coli. (D) Localization of the TAMRA-labeled peptides to S. aureus.
the safety of blood-contacting biomaterials for different applications. As shown in Fig. 6B, there was no difference in the four parameters of coagulation between the saline control group and the peptide-treated groups \((n = 3, \ p > 0.05)\). The data indicated that the three peptides had no effect on blood coagulation.

**Interaction between the peptides and human serum albumin.** Intrinsic fluorescence is often used to assess the structural changes of proteins during interactions with drugs or other materials. The fluorescence spectra of pure HSA and HSA in the presence of the peptides are shown in Figs. 7A and 7B. The fluorescence peaks at λem 342 nm of tryptophan (Trp) did not shift with the presence of the peptides, indicating that the peptides do not affect the polarity of tryptophan molecules. However, the fluorescence intensity of HSA increased with the presence of melittin and AR-23, but it decreased somewhat after RV-23 was added. Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample, and it can occur via a variety of different mechanisms such as excited-state reactions, energy transfer, complex formation, and collisional quenching. According to this, HSA and RV-23 may interact, most likely by their electrostatic attraction, leading to a change of the microenvironment around Trp residues and decreasing the solubility of Trp in solution. This influence has also been reported with other materials such as BSA and BPEI (Fig. 7A).

The characteristic absorption band at 200–240 nm is the α-helix structure of HSA. As shown in Figs. 7C and 7D, the absorption peak of HSA at 200–240 nm did not shift in the presence of any of the peptides, which indicated that none of the peptides affected the structure of HSA.

**Discussion**

Obtaining the maximum possible antimicrobial activity with minimum toxicity toward the host is an attractive direction for antimicrobial peptides research and development. To this end, investigation of the mechanisms of the membrane-selective activity of antimicrobial
peptides may be most important. Membrane selectivity varies among different antimicrobial peptides [8, 15, 23]. According to the selectivity of their interaction with eukaryotic versus prokaryotic cell membranes [13, 29], antimicrobial peptides are classified as either prokaryotic-active peptides, eukaryotic-active peptides, or non-membrane-selective peptides. Melittin is one of the most widely studied amphipathic α-helical antibacterial peptides, and it is known to have non-cell-selective lytic activity against both bacterial and human cells. Cationic antimicrobial peptides of the α-helical class have two unique features: a net positive charge of at least +2 and an amphipathic character, with a nonpolar face and a polar/charged face. In the present study, the results indicated that the three peptides are all cationic antimicrobial peptides with α-helical structure.

In this study, both the antibacterial activity assay and peptide-induced membrane damage experiments revealed that all three peptides had almost the same antibacterial and membrane penetrating activities against \textit{S. aureus} (Fig. 3B), whereas RV-23 showed the highest antibacterial and penetrating activities against \textit{E. coli} cells (Fig. 3A). The results suggest that the high inhibitory activity of RV-23 against \textit{E. coli} is due to its high bacterial membrane penetrating activity. By contrast, the hemolysis and MTT results (Figs. 4A and 4B) both indicated that RV-23 has low cytotoxicity, which may due to its low lytic activity against the membrane of human cells. The results suggest that RV-23 may be a membrane-selective antibacterial peptide that exerts its cell selectivity by discriminatory membrane disrupting ability and lytic potential against bacterial versus human cells.

In addition, the cell wall structure of gram-positive bacteria differs from that of gram-negative, which causes great differences in toxicity and sensitivity to certain drugs. Furthermore, lipopolysaccharide (LPS), as the main component of gram-negative outer membranes, is important to gram-negative bacterial activity [28]. In this study, melittin, AR-23, and RV-23 exhibited almost the same antibacterial activity against \textit{S. aureus} (MIC of approximately 3.5 µM) and different activity against \textit{E. coli}. However, RV-23 had the highest antibacterial potential against \textit{E. coli} (Table 2). The mechanism of the interaction between RV-23 and LPS is unclear and requires further investigation.

To investigate the membrane selectivity of RV-23, the structural parameters of the three peptides were compared. CD spectroscopy indicated that RV-23 has α-helical structural conformation similar to that of melittin and AR-23 (Figs. 2A, 2B, and 2C), which is the premise important for their antibacterial activity. Reduced helicity of the peptides leads to decreased hemolytic activity, as confirmed in this study in which RV-23 showed the lowest α-helix.
content and the lowest hemolytic activity (Fig. 4A).

Peptides with shorter retention times (low hydrophobicity) usually show lower hemolytic activity. In this study, the hemolytic activities of melittin, AR-23, and RV-23 (Fig. 4A) were exactly correlated with their retention times (Table 1), which suggests that the lower hemolytic activity of RV-23 may be due to its lower hydrophobicity. Furthermore, the hydrophobic moment may also be used to predict the membrane activity of peptide helices. However, a complete correlation of the hydrophobic moment and activity is rendered more difficult because of two factors [7]. First, unlike RV-23, the hydrophobic and hydrophilic residues of melittin and AR-23 are not regularly distributed within the chain. Second, the hydrophobic moment is calculated on the basis of an ideal helix, but the helicity of membrane-bound peptides is often considerably lower than 100%. Moreover, the number of positively charged residues on the polar face and net charge are both important for antimicrobial activity and hemolytic activity [12]. In the present study, the number of positively charged residues on the polar face is +2, +3, and +6 for melittin, AR-23, and RV-23, respectively (Fig. 1). These results further explain why RV-23 had the lowest cytotoxicity toward mammalian cells and retained the highest antibacterial activity.

Therefore, our results suggest that the highly selective antibacterial activity and reduced cytotoxicity of RV-23 may be partly due to its conformation. Specifically, the positively charged residues on its polar face, lower hydrophobicity, and reduced helicity lead to low binding affinity and membrane penetrating activity against human cells but not bacterial cells. It is therefore believed that RV-23 is a membrane-selective peptide, and it is classified as a prokaryotic-active peptide that exhibits selective lytic activity towards bacteria versus human cells.

In addition, hemocompatibility is a very important issue for the clinical application of antibacterial peptides, hence the effects of the peptides on key blood components and coagulation function were investigated. Platelets play a critical role during the processing of hemostasis. Platelet aggregation in vitro is often used as a marker for measuring thrombogenic properties, and the interaction of biomaterials with platelets is an important parameter for evaluating their hemocompatibility [9]. In addition, the activity of the coagulation system is of great importance in both thrombosis and bleeding. The effect of peptides on the coagulation system is another important parameter in evaluating hemocompatibility. Hence, the thromboelastograph was applied to evaluate the effects of both blood-soluble and -insoluble biomaterials on whole blood coagulation from the beginning of coagulation through clot formation to the ending with fibrinolysis [27]. Our results revealed that none of the three peptides at their MIC activated platelets or affected blood coagulation. On the other hand, serum albumin is important for maintaining the stability of the circulatory system, and therefore the interaction of peptides with serum albumin may affect its biological functions. Human serum albumin was used to evaluate the effects of the peptides on blood proteins. Intrinsic fluorescence and UV absorption are often used to assess the structural changes of HSA during its interaction with drugs or materials [17, 18, 31, 33]. The data suggest that the peptides do not affect the structure of HSA (Fig. 7). RV-23 is therefore a cell-selective antibacterial peptide with high hemocompatibility due to its unique structure.

In conclusions RV-23 is a membrane-selective peptide and classified to prokaryotic-active peptides. Moreover, it has better hemocompatibility than melittin and AR-23. Therefore, RV-23 represents a candidate for antibacterial drug development.

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