Antimicrobial peptides with amphipathic β-hairpin-like structures have potent antimicrobial properties and low cytotoxicity. The effect of VR or RV motifs on β-hairpin-like antimicrobial peptides has not been investigated. In this study, a series of β-hairpin-like peptides, Ac-C(VR)$_n$PG (RV)$_n$C-NH$_2$ ($n = 1, 2, 3, 4,$ or $5$), were synthesized, and the effect of chain length on antimicrobial activity was evaluated. The antimicrobial activity of the peptides initially increased and then decreased with chain length. Longer peptides stimulated the toxicity to mammalian cells. VR3, a 16-mer peptide with seven amino acids in the strand, displayed the highest therapeautic index and represents the optimal chain length. VR3 reduced bacterial counts in the mouse peritoneum and increased the survival rate of mice at 7 days after *Salmonella enterica* serovar Typhimurium infection *in vivo*. The circular dichroism (CD) spectra demonstrated that the secondary structure of the peptides was a β-hairpin or β-sheet in the presence of an aqueous and membrane-mimicking environment. VR3 had the same degree of penetration into the outer and inner membranes as melittin. Experiments simulating the membrane environment showed that Trp-containing VRW3 (a VR3 analog) tends to interact preferentially with negatively charged vesicles in comparison to zwitterionic vesicles, which supports the biological activity data. Additionally, VR3 resulted in greater membrane damage than melittin as determined using a flow cytometry-based membrane integrity assay. Collectively, the data for synthetic lipid vesicles and whole bacteria demonstrated that the VR3 peptide killed bacteria via targeting the cell membrane. This assay could be an effective pathway to screen novel candidates for antibiotic development.
length on antimicrobial activity was evaluated \textit{in vitro}, and the peptide with the greatest cell selectivity was identified. The antimicrobial activity of the peptides was further investigated \textit{in vivo}. To elucidate the peptide-membrane interaction mechanism, the tryptophan (W) fluorescence spectra of W-containing peptides were measured in the presence of synthetic lipid vesicles. The peptide-membrane interactions were further evaluated using the outer membrane (OM) permeabilization assay, the membrane depolarization assay, and flow cytometry analysis.

**MATERIALS AND METHODS**

**Peptide design and synthesis.** We designed a series of cyclic amphiphatic \( \beta \)-hairpin-like antimicrobial peptides according to the sequence template Ac-C(VR)n[PG(RV)]C-NH\(_2\) (\( n = 1, 2, 3, 4, \) or 5) (Table 1; Fig. 1). The peptide VRW3 was derived from the peptide VR3 by adding W to its C terminus to monitor lipid-peptide interactions using tryptophan fluorescence. The peptides were synthesized by GL BioChem Shanghai (Shanghai, China) by solid-phase methods using \( N \)-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry. The C terminus was amine protected, and the N terminus was Ac protected. Electrospray mass spectrometry was used to identity the peptides. The purity of peptides was determined to be >95% using reverse-phase high-performance liquid chromatography.

**Antimicrobial assays.** The MICs of the peptides were measured using a modified version of the Clinical Laboratory and Standards Institute broth microdilution method to determine the \textit{in vitro} antimicrobial activities of the peptides (46). *Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Typhimurium C77-31, *Bacillus subtilis* CMCC 63501, and *Staphylococcus epidermidis* ATCC 12228 were obtained from the School of Veterinary Medicine, Northeast Agricultural University (Harbin, China). Bacteria were grown overnight at 37°C to mid-log phase and then diluted in Mueller-Hinton broth (MHB) (Sigma) to give a final concentration of 1.0 \( \times 10^5 \) CFU/ml. Peptides were dissolved and diluted in 0.01% acetic acid and 0.2% bovine serum albumin (BSA) (Sigma). Bacterial aliquots of 100 \( \mu \)l were incubated for 18 to 24 h at 37°C with 100 \( \mu \)l peptide in MHB. The tests were performed in triplicate. The MICs were calculated as the lowest concentration of peptide that prevented visible turbidity.

**Quantification of hemolytic activity.** The hemolytic activities of the peptides were determined using a previously described method (40). Briefly, fresh human red blood cells (HRBCs) were collected and then centrifuged at 1,000 \( \times g \) for 5 min at 4°C. The erythrocytes obtained were washed three times with phosphate-buffered saline (PBS) (pH 7.2), and resuspended in PBS. A 100-\( \mu \)l HRBC solution was incubated with 100 \( \mu \)l of the respective peptide dissolved in PBS for 1 h at 37°C. Intact erythrocytes were centrifuged at 1,000 \( \times g \) for 5 min at 4°C, and the supernatant was transferred to a 96-well microtiter plate. The release of hemoglobin was monitored by measuring the optical density at 570 nm (OD\(_{570}\)). As negative and positive controls, HRBCs in PBS and 0.1% Triton X-100 were employed, respectively. Minimum hemolytic concentrations (MHCs) are defined as the peptide concentrations resulting in 10% hemolysis. The therapeutic index (TI) is the ratio of the MHC to the geometric mean of the MIC (GM). Larger values indicate greater cell specificity.

**Tryptophan fluorescence and quenching.** Small unilamellar vesicles (SUVs) were prepared for tryptophan fluorescence experiments as described previously (20). SUVs, including egg yolk \( \alpha \)-phosphatidylcholine (PC), egg yolk \( \alpha \)-phosphatidyl-N-acetylglucosamine (PG), egg yolk \( \alpha \)-phosphatidylethanolamine (PE), and cholesterol, were obtained from Sigma-Aldrich Corporation (St. Louis, MO). Following chloroform evaporation, the PE-PG (7:3, wt/wt) or PC-cholesterol (10:1, wt/wt) lipids were resuspended in 10 mM Tris-HCl buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 0.1 mM EDTA) by vortexing. The lipid dispersions were sonicated in ice water for 20 min using an ultrasonic cleaner until the solutions clarified. Tryptophan fluorescence spectra were measured using an F-4500 fluorescence spectrophotometer (Hitachi, Japan). The procedure was performed for each peptide in 10 mM Tris-HCl buffer (pH 7.4) with 500 \( \mu \)M PE-PG or PC-cholesterol lipids. The peptide/lipid molar ratio was 1:50, and the peptide-liposome mixture was allowed to interact for 2 min at 25°C. An excitation wavelength of 280 nm was used, and emission was scanned at wavelengths ranging from 300 to 400 nm. Spectra were baseline corrected by subtracting blank spectra of the corresponding solutions without the peptide. Quenching of fluorescence was accomplished using acrylamide (Sigma). To reduce the absorbance of acrylamide, Trp was excited at 295 nm instead of 280 nm (47). The final concentration of acrylamide was 0.4 M as determined by titrating the 4 M stock solution.
TABLE 2 MICs, minimum hemolytic concentrations, and therapeutic indices of the peptides against Gram-negative and Gram-positive bacterial strains

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Gram-negative strains</th>
<th>Gram-positive strains</th>
<th>GM&lt;sup&gt;a&lt;/sup&gt; (µg/ml)</th>
<th>MHC&lt;sup&gt;b&lt;/sup&gt; (µg/ml)</th>
<th>TI&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR1</td>
<td>E. coli 256 256</td>
<td>S. Typhimurium 256 256</td>
<td>&gt;256 256</td>
<td>&gt;256 256</td>
<td>512.0</td>
</tr>
<tr>
<td>VR2</td>
<td>32 32</td>
<td>DR. subtilis 16 128</td>
<td>4 4</td>
<td>5.7</td>
<td>&gt;128</td>
</tr>
<tr>
<td>VR3</td>
<td>8 8</td>
<td>S. epidermidis 128 128</td>
<td>4 4</td>
<td>5.7</td>
<td>&gt;128</td>
</tr>
<tr>
<td>VR4</td>
<td>&gt;256 &gt;256</td>
<td>B. subtilis 8 16</td>
<td>4 4</td>
<td>5.7</td>
<td>&gt;128</td>
</tr>
<tr>
<td>VR5</td>
<td>&gt;256 &gt;256</td>
<td>S. epidermidis 64 &gt;256</td>
<td>64 &gt;256</td>
<td>304.4</td>
<td>&gt;256</td>
</tr>
<tr>
<td>VRW3</td>
<td>8 16</td>
<td>S. epidermidis 2 4</td>
<td>2 4</td>
<td>5.7</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Melittin</td>
<td>8 16</td>
<td>S. epidermidis 1 4</td>
<td>1 4</td>
<td>5.7</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> MICs were determined as the lowest concentration of peptide that prevented visible turbidity.

<sup>b</sup> The geometric mean of the peptide MICs (GM) against all four bacterial strains was calculated. When no detectable antimicrobial activity was observed at 256 µg/ml, a value of 512 µg/ml was used to calculate the therapeutic index.

<sup>c</sup> The MHC is the minimum hemolytic concentration that caused 10% hemolysis of human red blood cells (hRBCs). When no detectable hemolytic activity was observed at 128 µg/ml, a value of 256 µg/ml was used to calculate the therapeutic index.

<sup>d</sup> The therapeutic index (TI) is the ratio of the MHC to the geometric mean of the MIC (GM). Larger values indicate greater cell selectivity.

with liposomes at a lipid/peptide molar ratio of 50:1. The effects of the quenching reagent on peptide fluorescence intensities were assessed by the quenching constant (K<sub>SV</sub>), which was estimated using the Stern-Volmer equation, F/F<sub>0</sub> = 1 + K<sub>SV</sub>(Q), where F<sub>0</sub> and F are the fluorescence values of the peptide in the absence or the presence of acrylamide, respectively, K<sub>SV</sub> represents the Stern-Volmer quenching constant, and Q represents the concentration of acrylamide.

**Outer membrane permeabilization assay.** Outer membrane permeabilization activity was determined using the fluorescent dye N-phenyl-1-naphtylamine (NPN) as described by Lee et al. (19). E. coli UB1005 cells were obtained from the State Key Laboratory of Microbial Technology, Shandong University. Briefly, E. coli cells were suspended in 5 mM sodium HEPES buffer (pH 7.4) containing 5 mM glucose. NPN was added to 2 ml of cells in a quartz cuvette to give a final concentration of 10 µM, and the background fluorescence was recorded (excitation wavelength = 350 nm, emission wavelength = 420 nm). Changes in fluorescence were recorded with an F-4500 fluorescence spectrophotometer (Hitachi, Japan). Peptide aliquots were added to the cuvette, and fluorescence was recorded as a function of time until no further increase in fluorescence was observed. As the outer membrane permeability increased due to the addition of peptide, NPN incorporated into the membrane resulted in an increase in fluorescence. Values were converted to percent NPN uptake using the equation % NPN uptake = (F<sub>obs</sub> - F<sub>0</sub>)/(F<sub>100</sub> - F<sub>0</sub>) × 100, where F<sub>obs</sub> is the observed fluorescence at a given peptide concentration, F<sub>100</sub> is the initial fluorescence of NPN with E. coli cells in the absence of peptide, and F<sub>100</sub> is the fluorescence of NPN with E. coli cells upon addition of 10 µg/ml polymyxin B (Sigma). Polymyxin B is used as a positive control because of its strong outer membrane-permeabilizing properties.

**Membrane depolarization.** The membrane depolarization activity of the peptides was determined with intact E. coli UB1005 cells and the membrane potential-sensitive fluorescent dye diSC<sub>3</sub>-5 (Sigma), according to the method described by Friedrich et al. (10). Briefly, E. coli were grown at 37°C with agitation to the mid-log phase and harvested by centrifugation. Cells were washed twice with washing buffer (5 mM HEPES, 20 mM glucose, pH 7.4) and resuspended to an OD<sub>600</sub> of 0.05 in the same buffer. The cell suspension was incubated with 0.4 µM diSC<sub>3</sub>-5 until maximal dye uptake was reached. KCl was added to equilibrate the cytoplasm to a final concentration of 0.1 M and incubated at room temperature for 10 min.

![FIG 2](https://aac.asm.org) Correlation between antimicrobial activity and the chain lengths of Ac-C((VR)<sub>n</sub>)<sub>c</sub>PG (RV), C-NH<sub>2</sub> peptides. The data can be fit to a power law as log GM = a(log n)<sup>2</sup> - b(log n) + c (n = 1, 2, 3, 4, or 5), with a, b, and c equal to 11.97, 8.90, and 2.79 (R<sup>2</sup> = 0.82), respectively.
Two milliliters of the cell suspension was placed in a 1-cm cuvette, and the peptides were added. Changes in fluorescence were recorded using an F-4500 fluorescence spectrophotometer (Hitachi, Japan) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

**FACScan analysis.** The membrane integrities of the peptides were determined using a previously described method (28). In brief, *E. coli* was grown to log phase and harvested. The peptides were added and incubated for 30 min at 28°C with constant shaking at 140 rpm. The cells were harvested by centrifugation, washed three times, and incubated with propidium iodide (PI) (10-µg/ml final concentration, Sigma) at 4°C for 30 min, followed by removal of the unbound dye through washing with an excess of PBS. Flow cytometry was performed using a FACScan instrument (Becton Dickinson, San Jose, CA).

**CD analysis.** To investigate the secondary structures of the peptides in different environments, circular dichroism (CD) spectra were measured under inert conditions with 10 mM sodium phosphate buffer (pH 7.4) (mimicking an aqueous environment), 50% trifluoroethanol (TFE) (mimicking the hydrophobic environment of the microbial membrane) (Sigma), and 30 mM SDS micelles (giving an environment comparable to a negatively charged prokaryotic membrane) (Sigma). The circular dichroism spectra of peptides were measured at 25°C with a J-820 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a rectangular quartz cell with a path length of 0.1 cm. Spectra were recorded at a scanning speed of 10 nm/min over a wavelength range of 190 to 250 nm. An average of five to eight scans were collected for each peptide. The final concentration of peptides was 60 µM.

**In vivo assay.** Male KM mice (4 weeks of age, 20.2 ± 1.5 g) were purchased from the Animal Center, Harbin Medical University (Harbin, China) and acclimatized for 1 week. Subsequently, the mice were infected with *S. Typhimurium* C77-31. Exponential-phase bacteria were resuspended in sterile PBS to achieve a final concentration of ~1 × 10⁸ CFU/ml. Animals were infected by intraperitoneal (i.p.) injection with 0.2 ml of the bacterial suspensions. Ten mice per group received a 0.2-ml i.p. injection of vehicle (PBS) or peptides approximately 60 min after the bacterial challenge. Peptides without bacteria were injected alone into each mouse as a control. The mice were monitored for 7 days. Two milliliters of sterile PBS was injected intraperitoneally into each mouse at 24 h, and peritoneal fluids (approximately 2 ml) were serially diluted. The colony counts of viable bacteria were determined by plating the fluids on Mueller-Hinton agar plates.

**RESULTS**

**Antimicrobial activity.** The MICs of synthetic peptides against Gram-negative and Gram-positive bacteria are presented in Table 2. The potency of antimicrobial activity was ranked according to the geometric means of the MICs as follows: VR3 > VR2 > VR4 > VR5 > VR1. The MICs of VR3 ranged from 4 to 8 µg/ml. VR4

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fluorescence emission maximum (nm)</th>
<th>KSV (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer PE-PG PC-cholesterol Buffer PE-PG PC-cholesterol</td>
<td></td>
</tr>
<tr>
<td>VRW3</td>
<td>351 334 (17) 347 (4) 6.12 0.51 2.88</td>
<td></td>
</tr>
<tr>
<td>Melittin</td>
<td>351 335 (18) 335 (16) 11.8 0.9 1.7</td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses are the blue shift of the emission maximum compared to Tris buffer.

The Stern-Vollmer constant KSV was calculated by the Stern-Vollmer equation, F/F₀ = 1 + KSV(Q), where Q is the concentration of the quencher (acrylamide). Concentrations of the quencher were increased from 0.01 to 0.40 M. A smaller KSV value reflects a more protected Trp residue.
showed antimicrobial activity against Gram-positive bacteria, with MICs ranging from 8 to 16 μg/ml, but showed no antibacterial activity against Gram-negative bacteria at the tested concentrations. VR2 displayed moderate antibacterial activity, with MICs ranging from 16 to 128 μg/ml. The strand lengths of the peptides were quadratically correlated with antibacterial activity (Fig. 2). The 16-residue-long VR3 had the strongest antimicrobial activity, which was similar to those of VRW3 and the well-known peptide melittin.

**Hemolytic activity.** The ability of these peptides to induce hemolysis of human erythrocytes was examined as a measure of their toxicity to mammalian cells (Table 2). The VR1, VR2, and VR3 peptides had no hemolytic activity at concentrations of as high as 128 μg/ml. The longer peptides VR4 and VR5 had 10% hemolytic activity at 65.1 μg/ml and 27.4 μg/ml, respectively. The MHCs of VR3 and its analog VRW3 were greater than 128 μg/ml, which is approximately 45 times greater than the MICs.

The therapeutic index is defined as the ratio of the MHC to the
geometric mean of the MIC (GM) (Table 2) and was used to evaluate cell selectivity. VR3 had the highest TI at 44.9, whereas VR4 and VR5 had lower TIs of 0.9 and 0.1, respectively. VR2 had a slightly higher TI than VR4 and VR5.

Cytotoxicity. The five peptides were tested for cytotoxic activity on MRC-5 cells, and the results are depicted in Fig. 3. The cytotoxic activities of the peptides were determined by the colorimetric MTT viability assay. Dose-response studies revealed that longer peptides (VR4 and VR5) displayed significantly higher cytotoxic activities on MRC-5 cells than the shorter peptides (VR1, VR2, and VR3). At the highest concentration of 128 μg/ml, the cell survival rates of VR1, VR2, and VR3 were 100%, 87%, and 82%, respectively, while VR4 and VR5 had greater cytotoxic activity, with cell viability being 36% and 15%, respectively.

Binding of peptides to model membranes. The fluorescence emission spectra for the tryptophan-containing peptides are shown in Table 3. VRW3 was used to evaluate the VR3 peptide because of their similar biological activity. VRW3 had maximal fluorescence emission at approximately 350 nm. The blue shifts observed with PE-PG phospholipid vesicles were larger than those observed with PC-cholesterol SUVs, which was consistent with their antimicrobial and hemolytic activities. In particular, a very

FIG 6 Flow cytometric analysis. Exponential-phase E. coli cells were treated with VR3, and cellular fluorescence was analyzed by flow cytometry. The increments of the log fluorescence signal represent PI uptake resulting from peptide treatment. (A) No peptide, negative control; (B) VR3 (1× MIC, 8 μg/ml); (C) VR3 (2× MIC, 16 μg/ml); (D) VR3 (5× MIC, 40 μg/ml); (E) melittin (1× MIC, 8 μg/ml); (F) melittin (2× MIC, 8 μg/ml); (G) melittin (5× MIC, 40 μg/ml).
small blue shift was observed in PC-cholesterol phospholipid vesicles in the presence of the VRW3 peptide, which was consistent with its low hemolytic activity. As a reference, melittin induced larger blue shifts in both PE-PG and PC-cholesterol vesicles than VRW3, which was consistent with the stronger biological activity of melittin.

Quenching of tryptophan fluorescence with acrylamide. The relative extent to which the tryptophan residues of the peptides were buried into the phospholipid layer was examined by tryptophan fluorescence quenching (Table 3). The $K_{sv}$ value of VRW3 in PE-PG vesicles was less than that in PC-cholesterol, suggesting that the peptides were more protected in bacterium-mimicking cell membranes. This was in agreement with the observation that the antimicrobial activities of the peptides were stronger than their hemolytic activities.

Permeabilization of OMs. Outer membranes (OMs) play an important role as a drug barrier in Gram-negative bacteria (14). NPN has been used to examine the permeabilization of the outer membranes of Gram-negative bacteria (34). The addition of VR3 to E. coli suspensions in the presence of NPN caused a rapid increase in fluorescence (Fig. 4). One hundred percent NPN uptake was produced at 2 µg/ml for VR3 and 0.5 µg/ml for melittin. The concentration of VR3 causing 100% NPN uptake was 4 times less than its MIC.

Membrane depolarization. To assess bacterial membrane depolarization, the membrane potential-sensitive dye diSC$_3$-5 was used. The chemical potential of K$^+$ inside and outside the cells was balanced by adding 0.1 M KCl to the buffer. Depolarization by different concentrations of VR3 was monitored over a period of 1,200 s (Fig. 5). VR3 depolarized the bacterial cytoplasmic membrane in a dose- and time-dependent manner. Melittin caused depolarization of the cytoplasmic membrane faster than VR3, but VR3 and melittin displayed similar peak fluorescence values at 8 µg/ml after 1,200 s.

FACScan analysis. Propidium iodide staining of nucleic acids in cells is indicative of compromised cell membrane permeability and cell death (5). Therefore, flow cytometric analysis was used to determine membrane integrity (Fig. 6). The analysis demonstrated that the control (no peptide) resulted in only 4.6% PI-positive cells. The percentage of PI-positive cells with membranes damaged by VR3 was 27.4% (MIC), 67.6% (2× MIC), and 78.0% (5× MIC). Melittin treatment resulted in positive nucleic acid staining of 31.8% (MIC), 48.3% (2× MIC), and 56.8% (5× MIC). Both peptides damaged the cell membrane in a dose-dependent manner, but VR3 caused a higher accumulation of PI than melittin at concentrations greater than 2× the MIC.

CD analysis. Circular dichroism (CD) spectroscopy was performed for all five peptides in sodium phosphate buffer, 50% TFE, and 30 mM SDS (Fig. 7). The VR1 spectra demonstrated significantly more β-turns in 30 mM SDS micelles, with minima between 200 and 205 nm and maxima between 225 and 230 nm (11). In TFE, VR1 and VR2 exhibited a population having a β-hairpin conformation characterized by a negative ellipticity near 205 nm and a crossover at 200 nm (44). In sodium phosphate buffer, VR1, VR2, and VR3 showed a range of secondary structures, but the spectra suggested a population with β-sheet structure. The spectra of VR3 showed an increase in β-sheet content in TFE and SDS. VR4 and VR5 displayed a maximum near 200 nm and a minimum just below 220 nm, suggesting a β-sheet structure (17).

In vivo activity. The ability of VR3 to protect mice from a lethal challenge of bacteria from infections with S. Typhimurium C77-31 was determined (Fig. 8). By the i.p. infection model, we compared the efficacies of VR1 and VR3. At i.p. doses of 1.25 mg/kg, 2.5 mg/kg, and 5 mg/kg, the survival rates were 14%, 57%, and 71% with VR3, respectively (Fig. 8B). However, mice were not protected from bacterial challenge when VR1 was administered i.p. (Fig. 8A). As shown in Fig. 8A and B, 100% survival after 7 days was found in the groups that had been injected alone with 5 mg/kg of VR3 or VR1. Similar results were observed with exponentially growing cultures of S. Typhimurium from peritoneal fluids. VR3 decreased the bacterial load by 10$^3$-fold at 24 h postinjection in treated mice compared to control mice ($P < 0.05$). In contrast, VR1 was relatively ineffective at reducing the CFU of S. Typhimurium (Fig. 8C).

DISCUSSION

The relationship between the chain length and the antibacterial activities of peptides has been widely studied; however, poor
agreement between these studies has been observed (7, 22, 25). Niidome et al. (25) designed a series of peptides with simple repeats, H-(LARL)$_n$-(LRAL)$_n$-NH$_2$ (n = 0, 1, 2, or 3). Antibacterial activity decreased while hemolytic activity increased with increasing chain length of the peptides (25). However, another study indicated that longer-chain linear peptides had effective antibacterial activity and stimulated hemolytic activity as well (22). In the current study, the relationship between the lengths of five β-hairpin-like antimicrobial peptides and antibacterial activity was described by a quadratic function. We determined that the VR3 peptide containing 3 VR units in the strand showed the highest antibacterial activity, suggesting that optimal strand length improved the antibacterial activity of the peptides. The results from the analysis of secondary structure by CD provided more information regarding the quantitative structure-activity relationships (QSARs) of the peptides. Although VR1 and VR2 displayed greater β-hairpin content in a membrane-mimetic environment, they are also prone to fold into β-structures in aqueous environments. We used a $^{13}$Pro-Gly segment to achieve cyclization of the backbone because the $^{12}$Pro-Gly segment was a very strong inducer of β-sheet formation (3, 39). Furthermore, disulfide cyclization induced the formation of a superimposable β-turn in aqueous solution (41). The peptides form a β-hairpin conformation when the strand is not enough long; however, chain lengths longer than 12 amino acids may result in increased β-sheet content. VR3 underwent conformational transitions from an aqueous environment to a membrane-mimicking environment, which suggests that VR3 has an increase in β-sheet content upon binding to membranes.

To evaluate the antimicrobial mechanism of VR3, VRW3 was designed and synthesized to implement the tryptophan fluorescent chromophore. PC-cholesterol (10:1, wt/wt) or PE-PG (7:3, wt/wt) SUVs were prepared to mimic zwitterionic or negatively charged membranes. The blue shift induced by VRW3 was greater in the negatively charged PE/PG vesicles than in the zwitterionic PC-cholesterol vesicles. This result suggests that the Trp of VRW3 penetrates bacterium-mimicking membranes more deeply than eukaryote-mimicking membranes. Peptide-membrane interactions are usually the result of electrostatic interactions, which may be essential for peptides to interact tightly with the membrane interface. Subsequently, peptides integrate into cell membranes, resulting in depolarization and microbial death (36). The peptides with a net positive charge of 7, such as VRW3 and its analog VR3, have electrostatic interactions between the positively charged residues and the negatively charged membrane surfaces (33). Our results suggest that VR3 preferentially bound to model membranes containing a negatively charged head group rather than those containing a zwitterionic head group.

Furthermore, the tryptophan quenching assay performed with the neutral fluorescence quencher acrylamide was used to examine the relative extent of peptide penetration into model membranes. The Trp residue in VRW3 penetrated more efficiently into negatively charged PE-PG vesicles than into zwitterionic vesicles. This observation suggests that the selective antibacterial activity of VR3 and VRW3 correlated with the targeted cell membrane components.

To further investigate the interaction of peptides with membranes, membrane permeability was assayed to detect the target site of the VR3 peptide. VR3 permeabilized the outer membrane at

**FIG 8** Evaluation of VR3 against *S. Typhimurium* in a bacterial infection model. *S. Typhimurium* was administered to mice by i.p. injection. (A and B) Survival analysis comparing various doses of VR1 (A) and VR3 (B) versus vehicle or peptide alone as control groups. The numbers of surviving mice were determined daily for 7 days. (C) Peritoneal bacterial counts were determined at 24 h for the VR1- and VR3-treated groups.
a low concentration. This process involves the displacement of divalent cations that stabilize adjacent lipopolysaccharide (LPS) molecules (8, 29). Antimicrobial peptides with amphipathic β-hairpin-like structures have binding affinities to LPS and lipid A (L.A) in the low micromolar range (9). This suggests that increased outer membrane permeability is due to the high binding affinity of the β-hairpin-like peptide with LPS in E. coli. VR3 induced a modest membrane depolarization in a dose- and time-dependent manner, indicating that the antibacterial target of VR3 was at the cytoplasmic membrane. To assess whether VR3 damaged the bacterial cell membrane, we determined PI staining of nucleic acids as an indicator of cell death. The results indicate that VR3 caused a higher accumulation of PI than melittin, suggesting that VR3 killed bacterial cells by disrupting their membranes. Initially, the peptides aggregate on the surface of the membrane. When a threshold is reached, they insert into the membrane bilayer to form pores or informal aqueous channels that allow the passage of ions and possibly larger molecules.

In summary, the 16-residue-long peptide VR3 had optimal cell selectivity and effectively protected mice against microbial infection. The relationship between antimicrobial activity and the chain lengths of the peptides was described by a quadratic function. The CD spectra demonstrated that the secondary structure of the peptides was a β-hairpin and/or β-sheet in aqueous and membrane-mimicking environments. The peptide killed bacteria by a membrane-active mechanism, which was evaluated by employing synthetic lipid vesicles and whole bacteria. These novel antimicrobial peptides can be designed by linking two-stranded antiparallel β-sheets with a short loop segment (5PG) and a disulide bridge. The VR3 peptide could be developed as a promising antibiotic candidate.

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