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Targeting Methicillin-Resistant *Staphylococcus aureus* with Short Salt-Resistant Synthetic Peptides

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The seriousness of microbial resistance combined with the lack of new antimicrobials has increased interest in the development of antimicrobial peptides (AMPs) as novel therapeutics. In this study, we evaluated the antimicrobial activities of two short synthetic peptides, namely, RRIKA and RR. These peptides exhibited potent antimicrobial activity against *Staphylococcus aureus*, and their antimicrobial effects were significantly enhanced by addition of three amino acids in the C terminus, which consequently increased the amphipathicity, hydrophobicity, and net charge. Moreover, RRIKA and RR demonstrated a significant and rapid bactericidal effect against clinical and drug-resistant *Staphylococcus* isolates, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *S. aureus* (VRSA), linezolid-resistant *S. aureus*, and methicillin-resistant *Staphylococcus epidermidis*. In contrast to many natural AMPs, RRIKA and RR retained their activity in the presence of physiological concentrations of NaCl and $MgCl_2$. Both RRIKA and RR enhanced the killing of lysostaphin more than 1,000-fold and eradicated MRSA and VRSA isolates within 20 min. Furthermore, the peptides presented were superior in reducing adherent biofilms of *S. aureus* and *S. epidermidis* compared to results with conventional antibiotics. Our findings indicate that the staphylocidal effects of our peptides were through permeabilization of the bacterial membrane, leading to leakage of cytoplasmic contents and cell death. Furthermore, peptides were not toxic to HeLa cells at 4- to 8-fold their antimicrobial concentrations. The potent and salt-insensitive antimicrobial activities of these peptides present an attractive therapeutic candidate for treatment of multidrug-resistant *S. aureus* infections.

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have become a major public health concern in both hospital and community settings (1, 2). Since the late 1990s, community-associated MRSA (CA-MRSA) emerged as a principal cause of skin and soft tissue epidemics throughout the world (3). It has been estimated that mortality rates from MRSA infections in U.S. hospitals are higher than those from infections caused by HIV/AIDS and tuberculosis combined (4). Furthermore, there is a considerable medical challenge in treating MRSA infections due to the remarkable ability of *S. aureus* to develop resistance to multiple antibiotics, thus limiting the number of viable therapeutic options (2, 5). Therefore, there is an urgent need to develop novel antimicrobials with unique mechanisms of action to combat MRSA.

Antimicrobial peptides (AMPs) are key components of the host innate defense against infections in most creatures (6, 7). The potential therapeutic applications of AMPs are significant (8). They have rapid and broad-spectrum antibacterial activity. In addition, it is extremely difficult for bacteria to evolve resistance against AMPs that disrupt the microbial membrane, since it would necessitate fundamental alterations in the lipid composition of the bacterial membranes. Though AMPs have many important antibacterial properties, several studies have exposed their potential limitations as therapeutic agents.

A significant number of natural AMPs are large and have high host cytotoxicity and moderate antimicrobial activity. Moreover, their production cost is high (8, 9). In addition, many natural AMPs lose their antimicrobial activity in physiological salt concentrations (10, 11). These characteristics have substantially hindered their pharmaceutical development as new therapeutic agents. Thus, successful development of novel AMPs as future therapeutics requires identification of short AMPs demonstrating strong antimicrobial activity, salt tolerance, and minimal toxicity to host tissues.

Previously, our group developed short synthetic peptide templates for therapeutic applications against hyperplasia and inflammation (12–14). However, their antimicrobial activity was never explored. In the present study, we screened four synthetic peptides and identified two peptides, namely, RRIKA and RR, with potent antimicrobial activities against a panel of clinical and drug-resistant *Staphylococcus* strains. In addition, we investigated the synergistic activities of these peptides in combination with clinically relevant antimicrobials and examined the peptides' ability to disrupt staphylococcal biofilms. Moreover, we performed a series of experiments to explore the antibacterial mechanism of action of RRIKA and RR and examined the toxicity of these peptides toward mammalian cells.

MATERIALS AND METHODS

Bacterial strains and media. All *Staphylococcus* strains tested in this study are presented in Table 1. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were purchased from Sigma-Aldrich, while Trypticase soy broth (TSB) and Trypticase soy agar (TSA) were purchased from Becton, Dickinson (Cockeysville, MD).

Reagents, peptides, and antibiotics. Nisin (N5764; Sigma), melittin from honeybee venom (M2272; Sigma), magainin I (M7152; Sigma), van-

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TABLE 1 MICs of peptides and antibiotics against clinical and drug-resistant *Staphylococcus* strains[a]

Strain type	Strain ID	Origin	Phenotypic property(ies)	MIC (μM)					
				Peptide RRIKA	Peptide RR	Lin	Van	Nisin	Amp
Methicillin-sensitive *S. aureus* (MSSA)	ATCC 6538		Quality control and biofilm-forming strain	2	16	2	0.5	1	≤0.25
	RN4220	United States	Resistant to mupirocin	4	8	1	0.5	ND	≤0.25
	NRS72	United Kingdom	Resistant to penicillin	4	32	4	0.5	2	>32
	NRS77	United Kingdom		4	16	2	0.5	2	≤0.25
	NRS846			4	16	4	0.5	2	≤0.25
	NRS860			4	16	1	0.5	2	≤0.25
Methicillin resistant *S. aureus* (MRSA)	USA100	United States (Ohio)	Resistant to ciprofloxacin, clindamycin, erythromycin	2	16	4	0.5	ND	>32
	USA200	United States (North Carolina)	Resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, methicillin	4	8	4	0.5	ND	>32
	USA300	United States (Mississippi)	Resistant to erythromycin, methicillin, tetracycline	4	16	4	0.5	1	>32
	USA400	United States (North Dakota)	Resistant to methicillin, tetracycline	2	8	4	0.5	ND	>32
	USA500	United States (Connecticut)	Resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, methicillin, tetracycline, trimethoprim	4	8	4	1	ND	>32
	USA700	United States (Louisiana)	Resistant to erythromycin, methicillin	2	8	4	1	ND	>32
	USA800	United States (Washington)	Resistant to methicillin	2	8	4	0.5	ND	>32
	USA1000	United States (Vermont)	Resistant to erythromycin, methicillin	4	8	4	0.5	ND	>32
	USA1100	United States (Alaska)	Resistant to methicillin	2	8	4	1	ND	>32
	NRS194	United States (North Dakota)	Resistant to methicillin	2	8	4	1	ND	>32
	NRS108	France	Resistant to gentamicin	4	8	4	0.5	ND	>32
	NRS119 (Lin^r)	United States (Massachusetts)	Resistant to linezolid	4	16	128	0.5	ND	>32
	ATCC 43300	United States (Kansas)	Resistant to methicillin	4	16	4	0.5	ND	>32
	ATCC BAA-44	Lisbon, Portugal	Multidrug-resistant strain	4	16	4	0.5	ND	>32
	NRS70	Japan	Resistant to erythromycin, spectinomycin	4	16	2	0.5	2	>32
	NRS71	United Kingdom	Resistant to tetracycline, methicillin	4	16	2	0.5	2	>32
	NRS100	United States	Resistant to tetracycline, methicillin	4	16	2	0.5	2	>32
	NRS123	United States (North Dakota)	Resistant to tetracycline, methicillin	4	16	2	0.5	1	>32
Vancomycin-intermediate *S. aureus* (VISA)	NRS1	Japan	Resistant to aminoglycosides and tetracycline; glycopeptide-intermediate *S. aureus*	2	16	2	8	ND	>32
	NRS19	United States (Illinois)	Glycopeptide-intermediate *S. aureus*	4	8	2	4	ND	>32
	NRS37	France	Glycopeptide-intermediate *S. aureus*	2	8	2	8	ND	>32
Vancomycin-resistant *S. aureus* (VRSA)	VRS1	United States	Resistant to vancomycin	4	32	1	>128	1	>32
	VRS2	United States	Resistant to vancomycin	4	16	1	16	1	>32
	VRS3a	United States	Resistant to vancomycin	4	16	1	128	2	>32
	VRS4	United States	Resistant to vancomycin	4	32	2	128	ND	>32
	VRS5	United States	Resistant to vancomycin	4	32	2	128	ND	>32
	VRS6	United States	Resistant to vancomycin	4	32	1	>128	ND	2
	VRS7	United States	Resistant to vancomycin	4	16	2	128	ND	>32
	VRS8	United States	Resistant to vancomycin	4	16	1	>128	ND	32
	VRS9	United States	Resistant to vancomycin	4	16	1	>128	ND	>32
	VRS10	United States	Resistant to vancomycin	4	16	4	>128	ND	>32
	VRS11b	United States	Resistant to vancomycin	4	32	1	>128	ND	>32
	VRS12	United States	Resistant to vancomycin	4	32	2	>128	ND	>32
	VRS13	United States	Resistant to vancomycin	4	16	1	>128	1	16
S. epidermidis	NRS101	United States	Prototype biofilm producer; resistant to methicillin, gentamicin	2	8	2	0.5	ND	>32

[a] Strain ID, strain designation; Lin, linezolid; Van, vancomycin; Amp, ampicillin; ND, not determined.

comycin hydrochloride (Gold Biotechnology), linezolid (Selleck Chemicals), ampicillin sodium salt (IBI Scientific), recombinant lysostaphin (3,000 U/mg) from *Staphylococcus simulans* (L9043; Sigma), and calcein AM (Molecular Probes, Life Technologies) were all purchased from commercial vendors.

Peptide synthesis. The peptides RRIKA, RR, KAF, and FAK were synthesized on Knorr-amide resin (Synbiosci Corp.) using standard 9-fluorenylmethoxy carbonyl (FMOC) chemistry. Two different chemistries were used to couple each amino acid. The first pair of coupling reagents was *N*-hydroxybenzotriazole (HoBt) and *N*,*N*′-diisopropylcarbodiimide (DIC), and the second pair of coupling reagents was 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and lutidine. For fluorescein isothiocyanate (FITC)-labeled YARA, an aminohexanoic acid spacer was added to the N terminus to serve as a spacer for

the addition of FITC isomer 1 (Molecular Probes). The FITC isomer was solubilized in 12:7:5 pyridine-dimethylformamide-dichloromethane and incubated with the deprotected peptide overnight. A ninhydrin test was used to check complete coupling of FITC to the peptide. Following synthesis, the peptide was cleaved from the resin using a trifluoroacetic acid-based cocktail, precipitated in ether, and recovered by centrifugation. The recovered peptide was dried *in vacuo*, resuspended in MilliQ purified water, and purified using a fast protein liquid chromatography (FPLC) instrument (Äkta Explorer; GE Healthcare) equipped with a 22/250 C_{18} prep-scale column (Grace Davidson). An acetonitrile gradient with a constant concentration of 0.1% trifluoroacetic acid was used to achieve purification. The desired molecular mass was confirmed by time-of-flight matrix-assisted laser desorption ionization (MALDI) mass spectrometry using a 4800 Plus MALDI time-of-flight (TOF)/TOF analyzer (Applied Biosystems).

Antibacterial assays. MICs of peptides and antibiotics were evaluated using the broth microdilution technique in MHB with an initial inoculum of 5×10^5 cells in nontreated Polystyrene microtiter plates CC7672-7596; (CytoOne) in accordance with the Clinical and Laboratory Standards Institute (CLSI) (15). The MIC was interpreted as the lowest concentration of peptide or antibiotic that completely inhibited the visible growth of bacteria after 16 h of incubation of the plates at 37°C. Each agent was tested in triplicate in at least two independent experiments. The highest MIC value was reported.

Antimicrobial activity in the presence of salts. To investigate the activities of peptides in the presence of high salt concentrations, the MIC was again identified as described above, except that fixed concentrations of NaCl or $MgCl_2$ were added to each well of the microtiter plate. For further analysis of the effect of salinity on the killing ability of peptides, logarithmic-phase MRSA USA300 and VRSA VRS13 at 5×10^5 CFU/ml were incubated with 2× MIC RRIKA and RR in MHB with different concentrations of NaCl (0, 50, 100, and 150 mM) or $MgCl_2$ (0, 1, 2, and 3 mM) for 4 h. Bacteria were serially diluted and plated in triplicate on TSA plates. CFU were counted after incubation of plates for 24 h at 37°C.

Time-kill assay. An overnight culture of MRSA USA300 was diluted in fresh MHB and incubated until an optical density at 600 nm (OD_{600}) of ≈1 was reached and then washed twice with phosphate-buffered saline (PBS) diluted to $\sim 5 \times 10^5$ CFU/ml in MHB. Peptides, vancomycin, and linezolid were added at concentrations equivalent to 5× MIC. Peptide diluent (sterile water) served as a negative control. Bacterial cell viability was monitored up to 24 h. Samples were removed at specific intervals, serially diluted in PBS, and plated in triplicate on TSA plates. CFU were counted after 24 h of incubation of plates at 37°C. To study the effect of serum and salinity on the killing kinetics of the peptides, a time-kill assay was performed as described above with the exception that bacteria and peptides at 5× MIC were incubated in MHB alone or with 10% fetal bovine serum (FBS) or incubated in PBS (containing 137 mM NaCl).

Synergy with antimicrobials. The fractional inhibitory concentration (FIC) index was utilized to determine the relationship between antimicrobial agents. Peptide MICs against test microorganisms were determined in triplicate samples. Two-fold serial dilutions of antimicrobials (lysostaphin, vancomycin, linezolid, and nisin) were tested in the presence of a fixed concentration of peptide, equal to ¼× peptide MIC. It is worth noting that none of the peptides killed the test microorganisms at their quarter-MICs. The FIC index was calculated as follows: FIC = 0.25 + MIC(antibiotic in combination)/MIC(antibiotic alone); 0.25 is equal to MIC(peptide in combination)/MIC(peptide alone). An FIC index of ≤0.5 is considered to demonstrate synergy. Additive effect was defined as an FIC index of 1. Antagonism was defined as an FIC index of >4 (16).

To further investigate the synergism of peptides with lysostaphin, the time-kill method of determining synergy was utilized as described elsewhere (17). MRSA USA300 and VRSA VRS13 were incubated with ½× MIC of RRIKA or ½× MIC of RR alone or in combination with ¼× MIC of lysostaphin (MICs of lysostaphin against MRSA USA300 and VRSA VRS13 were 0.04 and 0.08 μM, respectively). Samples were obtained at different time points and then diluted and plated on TSA plates. The plates were incubated for 24 h at 37°C before CFU were determined. Synergy was defined as a 100-fold or 2-$\log_{10}$ decrease in colony count by the combination of two agents together compared to each agent tested alone. Additivity or indifference was defined as a 10-fold change in CFU, and antagonism was defined as a 100-fold increase in CFU by the combined peptide treatment in comparison to results with the single treatment.

Bacterial membrane disruption activity (bacteriolysis). Cell lysis, as indicated by a decrease in the OD_{595}, was determined as described before (18). Briefly, MHB was inoculated with an overnight culture of MRSA USA300 and incubated aerobically at 37°C until an OD_{595} of ≈0.6 was reached and then diluted to an OD_{595} of ≈0.2 in MHB (equivalent to $\approx 10^8$ CFU/ml). Two hundred microliters of diluted bacteria were added to all wells of a 96-well plate. RRIKA and RR were added in concentrations equivalent to 4× MIC. Nisin at 4× MIC was used as a positive control and untreated bacteria served as a negative control. MHB with the same concentrations of drugs served as blanks. Turbidity was monitored at defined intervals up to 10 h in a Molecular Devices Vmax microplate reader at 595-nm absorption. The assay was carried out in triplicate samples for each treatment regimen.

Calcein leakage assay. Membrane permeabilization of *S. aureus* by peptides was monitored and quantified by the leakage of the preloaded fluorescent dye, calcein, as described before (19). MRSA USA300 was grown in MHB to logarithmic phase at 37°C. Cells were then harvested by centrifugation, washed twice with PBS, and then adjusted spectrophotometrically to an OD_{600} of 1.0 ($\approx 10^9$ CFU/ml) in PBS containing 10% (vol/vol) MHB. Then, MRSA cells were incubated with 3 μM calcein AM for 1 h at 37°C. Calcein-loaded cells were harvested by centrifugation (3,000 × *g*, 10 min), suspended in PBS, and diluted to achieve a final inoculum of 10^7 CFU/ml. Aliquots of 100 μl (each) were then added to a sterile black-wall 96-well plate. RRIKA and RR were added in concentrations equivalent to 5× and 10× MIC. Bacteria treated with peptide diluent (sterile water) and nisin (10 μg/ml) served as negative and positive controls, respectively. Calcein leakage was measured for 120 min using a fluorescence plate reader (FLx800 model; BioTek Instruments, Inc., Winooski, VT). Membrane permeabilization (%) was calculated as the absolute percent calcein leakage by peptides with respect to calcein-loaded-with-no-peptide-treated cells. Experiments were done in triplicate and repeated independently twice.

DNA binding assay. The ability of RRIKA and RR to bind DNA was investigated by using the electrophoretic mobility shift assay, as has been previously described (20). In brief, increasing concentrations of RRIKA, RR, and magainin were incubated with 250 ng of plasmid DNA (pUC19) in 30 μl binding buffer (10 mM Tris-HCl and 1 mM EDTA buffer, pH 8.0) at room temperature for 30 min. After incubation, the DNA was analyzed by 1% gel electrophoresis. DNA migration was visualized by ethidium bromide staining.

Quantification of activity against biofilms. Biofilm-forming clinical isolates of *S. aureus* (ATCC 6538) and *Staphylococcus epidermidis* (ATCC 35984) were grown overnight in TSB. After incubation, cultures were diluted 1:100 in TSB supplemented with 1% glucose. Diluted bacteria were inoculated into either 24- or 96-well flat-bottom cell culture plates (polystyrene) and incubated at 37°C for 24 h. The culture medium was subsequently removed, and wells were carefully washed with PBS three times to remove planktonic bacteria before refilling wells with fresh MHB. Peptides and antibiotics were added at different concentrations, and plates were incubated at 37°C for 24 h. After the removal of medium at the end of incubation, wells were rinsed by submerging the entire plate in a tub containing tap water. Biofilms were stained with 0.1% (wt/vol) crystal violet for 30 min. After staining, the dye was removed and the wells were washed with water. The plates were dried for at least 1 h prior to addition of ethanol (95%) to solubilize the dye bound to the biofilm. The OD of biofilms were measured at 595-nm absorbance by using a microplate reader (Bio-Tek Instruments Inc.). An inverted microscope (Vista Vision,

TABLE 2 Amino acid sequence and physicochemical properties of peptides used in this study

Peptide	Amino acid sequence	Length	Molecular wt	Charge	% of amino acids that are hydrophobic
RR	WLRRIKAWLRR	10	1,553.9	+5	54
RRIKA	WLRRIKAWLRRIKA	13	1,866.3	+6	57
KAF	KAFAKLAARKA	11	1,174.4	+4	63
FAK	FAKLAARLYRKA	12	1,407.7	+4	58

VWR) with an attached camera and 25× objective was used to photograph the biofilm in 24-well plates.

Hemolysis assay. Human red blood cells (RBCs) (Innovative Research, Novi, MI) were pelleted by centrifugation at 2,000 rpm for 5 min, followed by washing three times with PBS. An 8% (vol/vol) suspension of RBCs was prepared in PBS, and 50 μl of the solution was transferred to a 96-well plate. Then, 50 μl of different concentrations of peptides in PBS was added to give a final suspension of 4% (vol/vol) of RBCs. PBS served as a negative control. Triton X-100(0.1%) and 5 μM melittin served as a positive control. The plate was incubated at 37°C for 1 h. The plate was subsequently centrifuged at 1,000 rpm for 5 min at 4°C. Seventy-five-microliter aliquots of the supernatants in each well were carefully transferred to a new sterile 96-well plate. Finally, the hemolytic activity was evaluated by measuring the optical absorbance at OD_{405} with a microplate reader (Vmax; Molecular Devices). The hemolysis percentage was calculated based on the 100% release with 0.1% Triton X-100 or 5 μM melittin. Experiments were done in triplicate.

Cytotoxicity assay. The toxicities of RRIKA, RR, and melittin against HeLa cells were determined using the CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay kit from Promega (21). Briefly, ~2×10^4 HeLa cells suspended in 200 μl of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS were seeded in 96-well plates and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. The HeLa cells were further incubated with different concentrations of peptides for 24 h. At the end of the incubation period, the culture medium was discarded and the cells in each well were washed with PBS. One hundred microliters of cell culture medium and 20 μl CellTiter 96 AQ_{ueous} assay reagent were added next {the solution reagent contains a tetrazolium compound, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt [MTS]}. The plate was returned to the incubator for 2 h to allow color development. The intensity of color was quantified at 490 nm using a 96-well microplate reader (Vmax; Molecular Devices). Results were expressed as percentages of mean absorbance by cells upon incubation with peptide with respect to results with incubation with the control (untreated wells).

Statistical analyses. The mean absorbances of crystal violet in biofilm reduction were compared between the groups using the two-tailed Student *t* test ($P < 0.05$). All statistical analyses were performed with Microsoft Excel's statistical software program.

RESULTS

Antimicrobial activity. The amino acid sequences and characteristics of the synthetic peptides used in this study are shown in Table 2. We screened the synthetic peptides against a panel of clinical and drug-resistant *Staphylococcus* strains (Table 1). RRIKA inhibited the growth of methicillin-sensitive *S. aureus* (MSSA), MRSA, vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *S. aureus* (VRSA), linezolid-resistant *S. aureus*, methicillin-resistant *S. epidermidis*, and multidrug-resistant strains from concentrations ranging from 2 to 4 μM. RR inhibited the same isolates, with MICs ranging from 8 to 32 μM. In contrast, the KAF and FAK peptides were inactive against all strains tested up to 64 μM (data not shown).

Bacterial killing kinetics. Figure 1 presents the rates of microbial killing by RR, RRIKA, vancomycin, and linezolid when MRSA USA300 was exposed to 5× MIC of each treatment over a 24-h incubation period at 37°C. Both RRIKA and RR exhibited a rapid bactericidal effect, with a 3-$\log_{10}$ reduction (99.9% clearance) within 60 and 90 min, respectively. In addition, both peptides completely eliminated the inoculum (5×10^5 CFU/ml) within 120 and 180 min, respectively. In comparison, vancomycin achieved a 3-$\log_{10}$ bacterial reduction only after 24 h, while linezolid (a protein synthesis inhibitor) exhibited a bacteriostatic effect, producing only a single log bacterial reduction over the 24-h incubation. The killing kinetics of RRIKA and RR in PBS (containing 137 mM NaCl) did not significantly change from that in MHB (Fig. 2). In the presence of 10% FBS, RRIKA retained its bactericidal activity, though it required longer to kill the bacteria completely (6 h), while RR lost its antibacterial activity under the same condition.

Antimicrobial activity in the presence of salts. The MICs of RRIKA and RR for MRSA USA300 were determined in the presence of NaCl or $MgCl_2$. There was no increase in the MICs of RRIKA, and only a 1-fold increase in case of RR was observed in the presence of 100 mM NaCl. Moreover, the antimicrobial activities of RRIKA and RR were not impeded in the presence of $MgCl_2$ at a concentration equivalent to reported physiological conditions (2 mM). To study the effect of salinity on the killing abilities of the peptides, CFU were counted after incubation of the MRSA USA300 and VRSA VRS13 strains with RRIKA and RR (2× MIC) in increasing concentrations of NaCl or $MgCl_2$. There was no significant difference in the bactericidal activity of RRIKA in the presence of NaCl up to 150 mM (data not shown). Similar patterns were observed for RR with the exception of a 1-$\log_{10}$ increase in CFU observed at 150 mM NaCl; however, this peptide retained its bactericidal activity at this concentration (producing a 3-$\log_{10}$ reduction). Furthermore, physiological concentrations of $MgCl_2$ (1, 2, and 3 mM) had no effect on the killing ability of both RRIKA and RR.

Synergy with antimicrobial agents. Both RRIKA and RR acted synergistically with lysostaphin against MSSA, MRSA, and VRSA strains, with a FIC index equal to 0.26 (Table 3). When the two peptides were combined, only a 1-fold decrease in each peptide's MIC was observed (FIC index = 0.75). However, an additive effect was observed when each peptide was combined with vancomycin, linezolid, or nisin against tested strains (data not shown). There

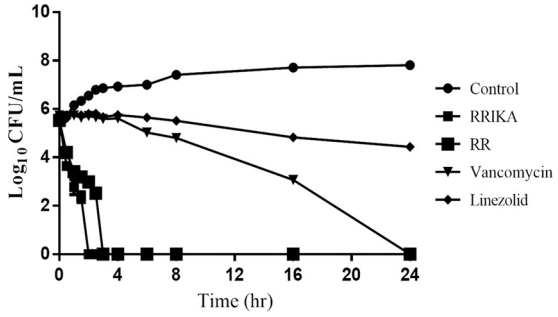

FIG 1 Bacterial killing kinetics of RRIKA, RR, vancomycin, and linezolid at 5× MIC against MRSA USA300 in MHB (Mueller-Hinton broth). Samples treated with peptide diluent (sterile water) were used as a control. The results are given as means ± SD ($n = 3$; data without error bars indicate that the SD is too small to be seen).

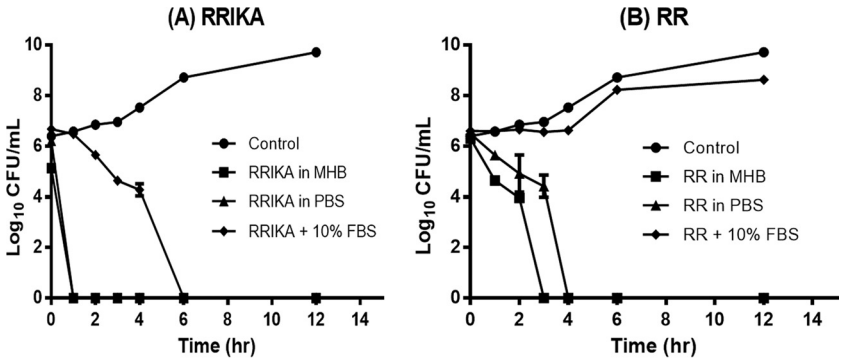

FIG 2 Effect of culture conditions on killing kinetics of RRIKA (A) or RR (B) at 5× MIC against MRSA USA300. Abbreviations: MHB, Mueller-Hinton broth; PBS, phosphate-buffered saline; FBS, fetal bovine serum. Untreated samples were used as a control. The killing curves were identical (overlapping in the figure) for RRIKA in MHB and in PBS. Each experiment was done in triplicate, and the values represent means ± SD (data without error bars indicate that the SD is too small to be seen).

were no antagonistic effects observed between the peptides and antimicrobials against all strains tested.

Time-kill synergy studies revealed that RRIKA and RR, at only ½× MIC, enhanced the killing of lysostaphin (at ¼× MIC) more than 1,000-fold and completely eradicated MRSA and VRSA isolates within 20 min (Fig. 3).

Bacterial membrane disruption activity (bacteriolysis). Since evidence of rapid cell lysis of treated cultures is indicative of membrane-damaging activity of antimicrobial peptides (22, 23), we assessed whether the bactericidal activities of our peptides were associated with lysis of bacterial cells by measuring culture turbidity over time after treatment. We monitored the turbidity of MRSA cultures treated with 4× MIC of peptides over a 10-h period at OD_{595} via a microplate reader. As shown in Fig. 4, both RRIKA and RR caused a rapid decrease in the OD_{595} even with a high inoculum concentration of 10^8 CFU/ml. Peptide RRIKA produced greater than 50% and 86% reductions in turbidity after 2.5 and 6 h, respectively. RR produced a percentage of turbidity similar to that with RRIKA, albeit at a lower rate (after 4.5 and 10 h). Nisin at 4× MIC, a polycyclic antibacterial peptide which is known as a membrane-perturbing agent (24), lysed cells at a slightly higher rate than RRIKA, with a more than 70% reduction in turbidity observed after 2.5 h. In contrast, wells treated with 4× MIC of vancomycin (a cell wall biosynthesis inhibitor) had no effect on culture turbidity within the same time frame.

Permeabilization of *S. aureus* membrane (calcein leakage assay). We monitored the effects of the peptides on MRSA membrane integrity by using the calcein leakage assay as described previously (19, 25). Damage to membrane integrity is indicated by calcein leakage from bacterial cells, leading to a reduction in fluorescence intensity. Figure 5 demonstrates that both peptides perturb the *S. aureus* cell membrane, leading to leakage of preloaded calcein in both a concentration- and time-dependent manner. RRIKA was faster and more potent than RR in membrane perturbation. At 5× MIC, RRIKA and RR caused more than 60% and 30% leakage, respectively, within 60 min. When the concentration of peptide was increased to 10× MIC, a significant change in membrane damage was observed for both peptides. There were more than 75% and 60% reductions in fluorescence intensity measured for RRIKA and RR, respectively, in the same time frame. Nisin at 5× MIC resulted in more than 70% calcein leakage from cells, which is comparable to results for RRIKA at 10× MIC. Vancomycin, as expected, had no effect on the bacterial cell membrane integrity even at a high concentration (10× MIC).

TABLE 3 FIC index for the combination of peptides together or with lysostaphin

	FIC index[a]					
	MSSA (RN4220)		MRSA (USA300)		VRSA (VRS13)	
Compound	RRIKA	RR	RRIKA	RR	RRIKA	RR
RRIKA		0.75		0.75		0.75
RR	0.75		0.75		0.75	
Lysostaphin	0.26	0.26	0.26	0.26	0.26	0.26

[a] The fractional inhibitory concentration (FIC) index was determined in the presence of a fixed concentration of peptide, equivalent to 0.25× MIC.

DNA binding properties. Since there was no obvious evidence that the antibacterial effect of AMPs was restricted to perforation of membranes, we explored the feasibility of other intracellular targets, such as DNA, by assessing the DNA-binding properties of peptides (26, 27). As shown in Fig. 6, both peptides were able to bind the plasmid DNA and delay its electrophoretic migration into agarose gel in a dose-dependent manner. At an 8 μM concentration of the RRIKA peptide, a fraction of the plasmid DNA was still able to migrate into the gel as noncomplexed DNA; however, at 16 μM, the majority of DNA remained in the gel's wells. At higher concentrations, complete retardation of DNA migration was exhibited, implying that the DNA was aggregated by peptide. Similar patterns of migration were observed with the RR peptide, except that free DNA was still seen at a 16 μM concentration. In contrast, magainin, a cationic antimicrobial peptide of amphibian skin, lacks this ability to bind DNA even at concentrations up to 80 μM.

Quantification of activity against biofilms. In order to evaluate the efficacies of RR and RRIKA against established biofilms, we measured the biofilm mass posttreatment by crystal violet staining of biofilms formed by clinical isolates of *S. aureus* and *S. epidermidis*. As observed in Fig. 7A, both peptides significantly reduced *S. aureus* biofilms compared to results with the antibiotics of choice. RRIKA and RR, at only 2× MIC, dispersed more than 65%

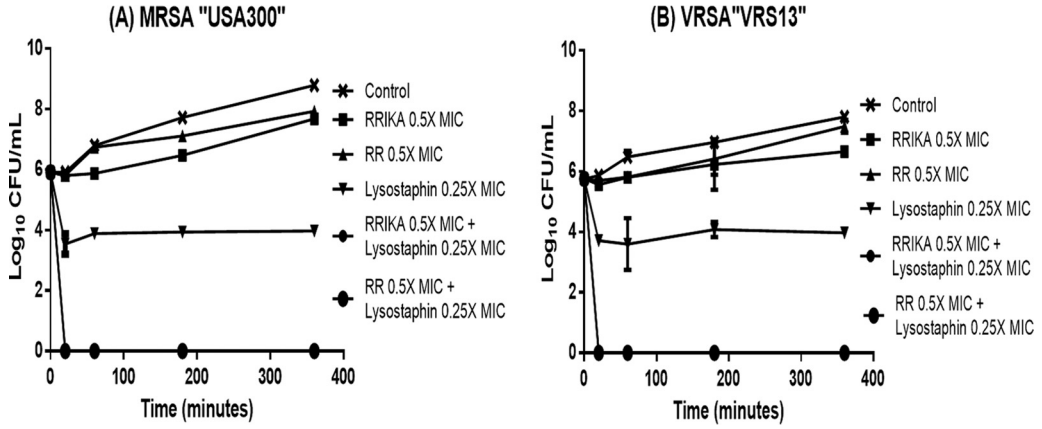

FIG 3 Synergistic killing of RRIKA and RR with lysostaphin by time-kill assay. MRSA USA300 (A) or VRSA VRS13 (B) was incubated with 0.5× MIC of RRIKA or RR alone or in combination with 0.25× MIC of lysostaphin. Samples were obtained at different time points and then plated and counted. The control was untreated samples. The killing curves were identical (overlapping in the figure) for RRIKA and RR in combination with lysostaphin. The results are given as means ± SD ($n = 3$; data without error bars indicate that the SD is too small to be seen).

of mature biofilms ($P < 0.01$), while vancomycin and linezolid, at 16× MIC, were capable of reducing only 40% of biofilm mass ($P < 0.01$). There was a significant difference between peptide- and antibiotic-treated biofilms ($P < 0.01$).

With regard to *S. epidermidis*, both RR and RRIKA, at 32× and 64× MIC, were able to reduce more than 50% of biofilm mass, respectively ($P < 0.01$) (Fig. 7B). Vancomycin and linezolid, on the other hand, reduced only 9.5 and 10.7% of biofilm mass, respectively ($P < 0.01$) at concentrations equivalent to 256× MIC. Also we found statistical significance between results for peptide- and antibiotic-treated biofilms ($P < 0.01$).

Hemolysis assay and cell toxicity. We assessed the release of hemoglobin from human erythrocytes exposed to different concentrations of peptides. As depicted in Fig. 8, even at concentrations as high as 300 μM, RRIKA and RR exhibited minimal hemolysis against RBCs (maximum of 10% hemolysis observed). In contrast, melittin completely lysed RBCs (100% hemolysis) at a significantly lower concentration of 5 μM.

The cytotoxic effect of the peptides on HeLa cells was evaluated by the MTS assay. As depicted in Fig. 9, neither RRIKA nor RR was toxic to the mammalian cells tested up to concentrations of 32 μM and 64 μM, respectively. These values correlate to 8× and 4× MIC for RRIKA and RR, respectively. The half-maximal effective concentrations (EC_{50}) of RRIKA and RR against macrophage cell lines were 64 and 128 μM, respectively. On the other hand, melittin showed high toxicity even at a bacteriostatic concentration, with an EC_{50} less than 5 μM.

DISCUSSION

In this study, we evaluated the antimicrobial activities of two short synthetic peptides and revealed their potential mechanisms of action. We observed that RRIKA and RR exhibited potent antibacterial activities against all tested *S. aureus* isolates. Moreover, our peptides demonstrated activities against multiple clinical isolates of MRSA, particularly MRSA USA300, a community-associated strain which is responsible for outbreaks of staphylococcal skin and soft tissue infections (SSTI) in the United States (28). Similarly, potent bactericidal activity was observed for other clinical MRSA isolates (USA100, USA200, and USA500) that are resistant to various antibiotic classes, including macrolides, aminoglycosides, lincosamides, and fluoroquinolones. Additionally, we found that these peptides retained their activities against strains of *S. aureus* which have resistance to drugs of choice in treating MRSA infections, namely, linezolid (MRSA NRS119) and vancomycin (VRSA strains). It is worth noting that the C terminus

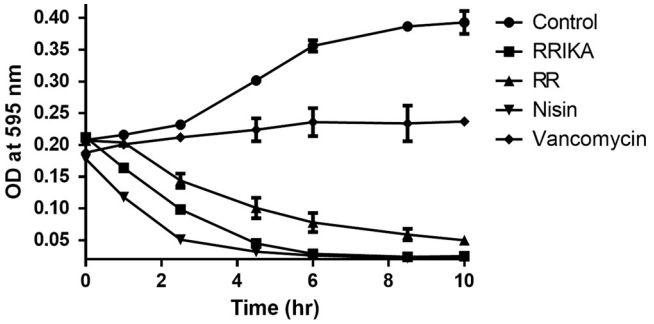

FIG 4 Killing kinetics of MRSA USA300 exposed to 4× MIC of RRIKA, RR, nisin, vancomycin, and sterile water (control) as observed by measuring the OD_{595} by microplate reader over time. Results are representative of two separate experiments, each done in triplicate. Error bars represent standard deviation values (data without error bars indicate that the SD is too small to be seen).

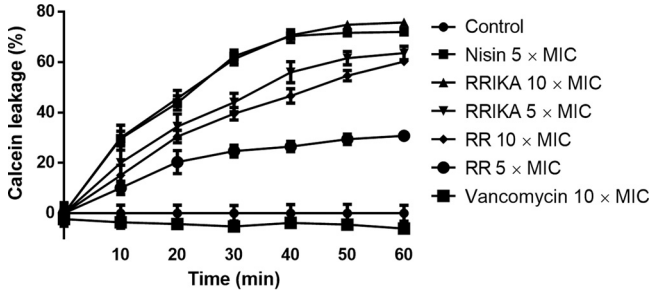

FIG 5 Permeabilization of the cytoplasmic membrane of MRSA USA300 as a function of peptide concentration, indicated by the percentage of calcein leakage for a 60-min exposure. The results are given as means ± SD ($n = 3$; data without error bars indicate that the SD is too small to be seen).

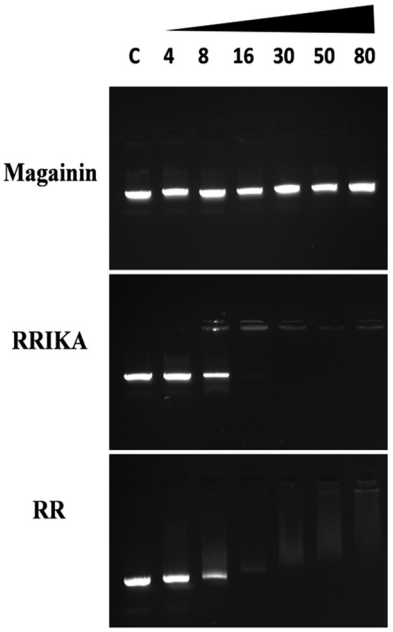

FIG 6 Interaction of peptides with plasmid DNA. Binding was assayed by measuring inhibition of migration by plasmid DNA. Different concentrations of peptides were incubated with 250 ng for 1 h at room temperature prior to electrophoresis on a 1.0% agarose gel. The numbers above the lanes represent the concentration (in μM) of magainin, RRIKA or RR. Lane C, control consisting of plasmid DNA only.

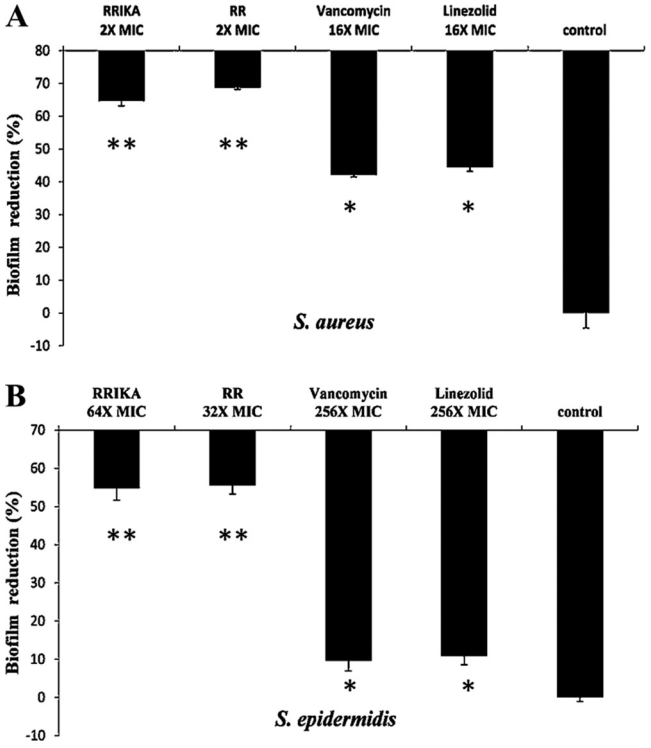

FIG 7 The effects of peptides (RRIKA and RR) and antibiotics (vancomycin and linezolid) on established biofilms of *S. aureus* (a) or *S. epidermidis* (b). The adherent biofilm was stained by crystal violet, and then the dye was extracted with ethanol, measured at a 595-nm absorbance, and presented as percentage of biofilm reduction compared to untreated wells ("control"). All experiments were done in triplicate for statistical significance. One asterisk, statistically different from the positive control ($P < 0.01$); two asterisks, statistically different from the antibiotic-treated wells ($P < 0.01$).

of the RR peptide was modified to contain three additional amino acids (isoleucine, lysine, and alanine), which made the RRIKA peptide more amphipathic and hydrophobic and also increased its positive net charge. These modifications augmented the antibacterial activity of RRIKA, which exhibited MICs 2- to 8-fold lower than those of RR. The enhanced efficacy of RRIKA may reveal a functional structure-activity relationship and provide a template for future peptide synthesis.

Time-kill kinetics revealed a major advantage of the synthetic peptides over tested antibiotics. While RRIKA and RR eliminated MRSA within 1 to 2 h after treatment, vancomycin required 24 h to reduce the bacterial CFU by 3 $\log_{10}$. Linezolid, on the other hand, exhibited only a bacteriostatic effect.

Growth kinetic measurements of MRSA exposed to peptides clearly demonstrated that the RRIKA and RR peptides exhibited kinetic behavior similar to that of membrane-lytic peptides, such as the lantibiotic nisin (24) and amphibian magainin (23), but different from that of nonlytic peptides, such as fungal plectasin (29) and amphibian buforin II (23). These data suggest that our peptides act by disrupting the integrity of the bacterial membrane. To validate this hypothesis, we studied the effect of peptides on MRSA membranes using a calcein leakage assay. Similar to findings for the well-known membrane-damaging peptide, nisin, both RRIKA and RR permeabilized *S. aureus* cells in a concentration- and time-dependent manner. These observations clearly validate that the mechanism of bacterial killing by our peptides is mediated by pore formation and disruption of the bacterial cell membrane, leading to leakage of cytoplasmic contents and ultimately cell lysis. However, recent evidence suggests that lysis of bacterial cells by pore formation is not the only mechanism of microbial killing of some AMPs and there are other possible intracellular targets, such as aggregation of DNA (26). To further explore this possibility, we examined the bacterial DNA-binding ability of the peptides by electrophoretic gel retardation. Our data indicated that both RRIKA and RR bind with bacterial DNA and alter its electrophoretic mobility in agarose gels. Although a detailed study of the

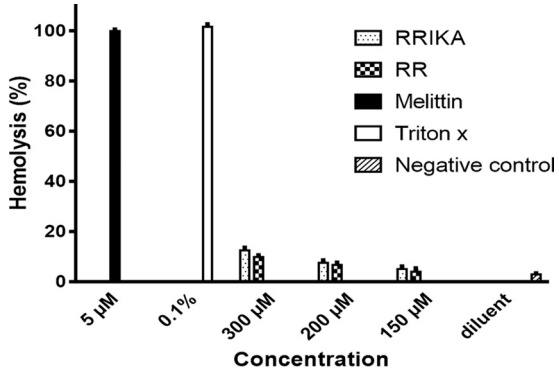

FIG 8 The release of hemoglobin in the supernatant of human erythrocytes after treatment with increasing amounts of RRIKA and RR was measured at 415 nm. Data collected after 1 h of incubation are presented. Melittin (5 μM) and 0.1% of Triton X-100 served as positive controls. Phosphate-buffered saline (PBS) served as a negative control.

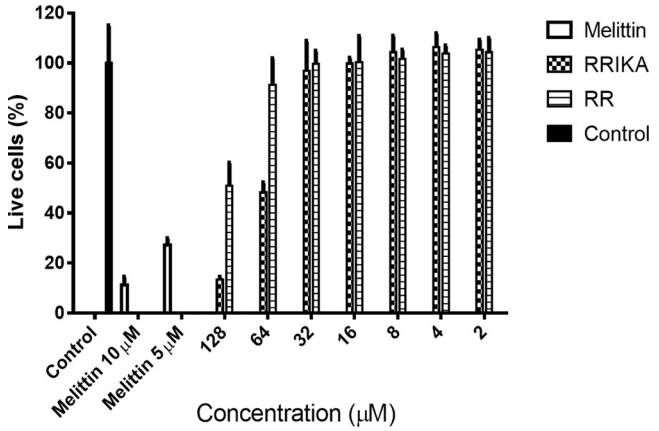

FIG 9 Cytotoxicity assay showing the percent mean absorbance at 490 nm after incubating HeLa cells with RRIKA, RR, and melittin at different concentrations. Diluent was used as a control. Cell viability was measured by MTS assay. Results are expressed as means from three measurements ± standard deviations.

interaction between peptides and nucleic acids *in vivo* is needed, the current results lead us to suggest that RRIKA and RR might inhibit bacterial functions by binding to bacterial DNA after perturbing the cell membranes, resulting in an augmented effect by two different mechanisms. While RRIKA and RR are not toxic to mammalian cells at the levels used here, possible crossing of the peptides to the nuclear membrane and binding to eukaryotic DNA must be examined further.

Major limitations with the use of AMPs for systemic applications are their possible inactivation by serum or physiological concentrations of salts. Serum inactivates peptides either through cleavage by proteases or through binding with proteins or lipids (30) We observed that RRIKA but not RR retained its bactericidal activity, although at a lower rate, in the presence of 10% FBS. Furthermore, both RRIKA and RR retained their antibacterial activity when tested in increasing concentration of NaCl and $MgCl_2$, in contrast to many well-studied AMPs (such as LL-37, human β-defensin-1, gramicidins, bactenecins, and magainins) which showed substantially reduced antibacterial activities under the same conditions (11). Previously, Turner et al. reported that LL-37 and human neutrophil peptide-1 (HNP-1) demonstrated 12-fold and 100-fold increases in the MIC of MRSA, respectively, when 100 mM NaCl was added to the test medium (10). The ability to resist the effects of salt and serum provide a selective advantage for our peptides for potential therapeutics in physiological solutions.

After identifying that our peptides were capable of inhibiting bacterial growth alone, we wanted to explore their ability to be used in combination with other antimicrobials, such as lysostaphin. Lysostaphin is a zinc metalloenzyme that specifically cleaves the abundant pentaglycine cross-bridges of the staphylococcal cell wall. Several studies have reported the potential applications of lysostaphin in the treatment of staphylococcal infections; however, *S. aureus* has developed resistance against lysostaphin via different mechanisms (31). When we tested both RR and RRIKA in combination with lysostaphin, we observed a synergistic relationship demonstrated by complete eradication of MRSA and VRSA within 20 min (at very low concentrations). The observed synergistic effect may be due to the cleaving of the cell wall peptidoglycan by lysostaphin, which allows more access of peptides to the bacterial membrane. The synergistic relationship observed between our peptides and lysostaphin is important because it has the advantage of reducing the emergence of bacterial resistance to both agents while also minimizing drug-associated toxicity (by lowering the therapeutic dose needed for each antimicrobial agent to effectively treat MRSA infection).

One of the difficult challenges facing current antibiotics is bacterial biofilms, which are vital in the pathogenesis of staphylococcal infections. Biofilms hinder the penetration of antimicrobials to access bacteria, leading to failure of treatments (32). Our data showed that both RR and RRIKA are capable of disrupting *S. aureus* and *S. epidermidis* biofilms more efficiently than drugs of choice (vancomycin and linezolid). Most bacteria living in biofilms are either slow-growing or nongrowing dormant cells that are difficult to treat with antibiotics that normally inhibit macromolecular synthesis in growing cells (33). However, our peptides target mainly the microbial cell membrane, a characteristic that is present not only in dividing organisms but also in quiescent cells or stationary-phase bacteria (34).

Cell toxicity is one of the major limitations in the development of antibacterials, particularly if the target of action is the cell membrane (7). We observed minimal hemolysis (less than 10%) with our peptides even with high concentrations (300 μM). On the other hand, 5 μM melittin completely lysed human RBCs (100% hemolysis). Furthermore, RRIKA and RR were not toxic to HeLa cells at 8× and 4× MIC, respectively.

In conclusion, our findings reveal the potent bactericidal action of peptides RRIKA and RR against MRSA. The mechanism of action of the peptides is due mainly to bacterial lysis as a consequence of bacterial membrane disruption and possibly of binding of the peptides to bacterial DNA, which interferes with necessary cellular functions vital for microbial survival. Such effects are extremely challenging for pathogens to overcome by developing resistance, unlike the case with current antibiotics, which usually inhibit metabolic pathways that can lead to bacterial resistance (8). To date, issues such as poor pharmacokinetics have limited the potential systemic applications of therapeutic AMPs (7, 8, 30). Therefore, AMPs which have advanced into preclinical or clinical trials are indicated for topical treatment of bacterial skin infections (7, 8, 30), such as pexiganan (Access Pharmaceuticals, Inc.) for curing diabetic foot ulcers (35). *S. aureus* is the most frequently isolated microorganism in diabetic foot infections, and those caused by MRSA are associated with worse outcomes and more frequent amputations (36–41). Additionally, bacterial biofilms appear to play an important role in increasing the difficulty of treating these ulcers (40). Furthermore, it has been proven recently that the clinical severity of *S. aureus* skin infection is driven by the inflammatory response to the bacteria rather than the bacterial burden (42–44). Taken together, the characteristics of the presented peptides with combined bactericidal, antibiofilm, and anti-inflammatory effect may offer an effective way for treating staphylococcal skin infection. Therefore, these results support the potential for further study and development of RRIKA and RR as topical therapeutics, particularly in an era of emerging drug resistance.

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