Potential of Novel Antimicrobial Peptide P3 from Bovine Erythrocytes and Its Analogs To Disrupt Bacterial Membranes *In Vitro* and Display Activity against Drug-Resistant Bacteria in a Mouse Model

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With the emergence of many antibiotic-resistant strains worldwide, antimicrobial peptides (AMPs) are being evaluated as promising alternatives to conventional antibiotics. P3, a novel hemoglobin peptide derived from bovine erythrocytes, exhibited modest antimicrobial activity *in vitro*. We evaluated the antimicrobial activities of P3 and an analog, JH-3, both *in vitro* and *in vivo*. The MICs of P3 and JH-3 ranged from 3.125 µg/ml to 50 µg/ml when a wide spectrum of bacteria was tested, including multidrug-resistant strains. P3 killed bacteria within 30 min by disrupting the bacterial cytoplasmic membrane and disturbing the intracellular calcium balance. Circular dichroism (CD) spectrometry showed that P3 assumed an α -helical conformation in bacterial lipid membranes, which was indispensable for antimicrobial activity. Importantly, the 50% lethal dose (LD₅₀) of JH-3 was 180 mg/kg of mouse body weight after intraperitoneal (i.p.) injection, and no death was observed at any dose up to 240 mg/kg body weight following subcutaneous (s.c.) injection. Furthermore, JH-3 significantly decreased the bacterial count and rescued infected mice in a model of mouse bacteremia. In conclusion, P3 and an analog exhibited potent antimicrobial activities and relatively low toxicities in a mouse model, indicating that they may be useful for treating infections caused by drug-resistant bacteria.

The overuse of traditional antibiotics has triggered the frequent emergence of multidrug-resistant (MDR) bacteria, which pose significant threats to public health. Bacterial resistance is usually attributable to mutations and can spread, rendering the problem worse (1–3). Extensively mutated bacteria become resistant to many conventional chemotherapeutics. Since the early 1960s, many novel antibiotics have been prepared, but these are usually chemical variations of older and conventional antibiotics (4). There is an urgent need to find novel antimicrobial agents that control infections caused by MDR bacteria.

Antimicrobial peptides (AMPs) have recently attracted significant attention (5). They are primitive components of innate immune systems and play multiple roles in immune defense (6, 7). AMPs are widespread in unicellular organisms, plants, and animals, and more than 1,600 have been identified in a wide range of organisms (8–10). Most natural AMPs exhibit broad-spectrum activities against bacteria (including MDR bacteria), fungi, viruses (including HIV), and tumors (11–13). Although many potent AMPs have been identified, a lack of knowledge about the mechanisms of action and high-level AMP toxicities are major obstacles on the path to clinical use (14). Therefore, the design of novel potent AMPs exhibiting low toxicities *in vivo* and the identification of the mechanisms of AMP-membrane interactions are important for the development of novel antimicrobial agents.

Recently, we reported the first isolation of P3, a component of the bovine hemoglobin α -subunit (15). Its primary sequence is VNFKLLSHSLVTLASHL (1,992.401 Da). The bovine hemoglobin P3 sequence is very similar to those of humans, pigs, sheep, and deer. In the present study, we increased P3 antimicrobial activity by creating analogs. In addition, we explored the efficacies of P3 and its analogs against drug-resistant clinical isolates of various pathogens, both *in vitro* and in a mouse model of infection.

MATERIALS AND METHODS

Bacteria and reagents. Among the microorganisms used in this study, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Candida albicans* ATCC 90029 were purchased from the American Type Culture Collection (ATCC). The following clinical MDR bacterial strains were obtained from Xinxiang Medical University: (i) an MDR *E. coli* strain from an infant, (ii) an MDR *S. aureus* strain, (iii) a *P. aeruginosa* strain, and (iv) a *C. albicans* strain. All of the bacteria were cultured at 37°C in LB medium (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl [pH 7.2]), and fungi were cultured at 28°C in YPD broth (1% yeast extract, 2% peptone, 2% D-glucose [all wt/vol]). Red blood cells were from specific-pathogen-free (SPF) New Zealand rabbits. The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids. 1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester (Fura 2-AM), and Cell Counting kit 8 (CCK-8) were purchased from Dojindo. Propidium iodide (PI) was purchased from Sigma-Aldrich. Ampicillin sodium (Amp) and polymyxin B (PMB) were purchased from Solarbio. All of the

Received 3 December 2014 Returned for modification 16 December 2014
Accepted 16 February 2015

Accepted manuscript posted online 9 March 2015

Citation Zhang Q, Xu Y, Wang Q, Hang B, Sun Y, Wei X, Hu J. 2015. Potential of novel antimicrobial peptide P3 from bovine erythrocytes and its analogs to disrupt bacterial membranes *in vitro* and display activity against drug-resistant bacteria in a mouse model. *Antimicrob Agents Chemother* 59:2835–2841. doi:10.1128/AAC.04932-14.

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doi:10.1128/AAC.04932-14

other reagents were of analytical grade and were obtained from commercial sources.

Membrane preparation. POPG and POPE are the principal components of bacterial lipid membranes, and we prepared artificial membranes using these materials. POPG and POPE were mixed at a 1:3 molar ratio in chloroform, dried under nitrogen, redissolved in cyclohexane, and lyophilized overnight. The dry powder mixture was suspended in 2 ml phosphate buffer (10 mM [pH 7.0]) and freeze-thawed six times to obtain a lipidic vesicle solution.

Peptide synthesis. P3 and its analogs were prepared via solid-phase synthesis using 9-fluorenylmethoxycarbonyl (F-moc) chemistry, according to the manufacturer's protocol (16). Peptides were purified with the aid of reverse-phase high-performance liquid chromatography (RP-HPLC). All purities were >98%. Atomic masses were confirmed using a triple quadrupole (TQD) mass spectrometer (Waters Corp.) equipped with an electrospray ionization source.

Antimicrobial activity. The MICs of P3 and its analogs were determined using a microdilution assay, as previously described, with minor modifications (17). Bacteria were cultured in LB (*E. coli* and *S. aureus*) or YPD (*C. albicans*) at 37°C, harvested during exponential growth (optical density at 600 nm [OD₆₀₀] of 0.6 to 0.8), centrifuged ($6 \times 10^3 \times g$ for 15 min at 25°C), resuspended in Muller-Hinton (MH) broth at approximately 1×10^6 CFU/ml, and plated into 96-well plates (50 µl/well) in triplicate. Peptides were dissolved in sterile distilled water at concentrations of 4 to 500 µg/ml and added to each well (50 µl/well). After 24 h of incubation at 37°C, the minimal peptide concentration affording optically clear (100% inhibition) wells was defined as the MIC.

Antimicrobial activity kinetics were determined by measuring changes in viable microbial counts after treatment with P3 or JH-3 (18, 19). Peptides were added at 2×MIC to bacteria growing exponentially (8×10^5 CFU/ml) in LB or YPD medium at 37°C. Samples were taken at 0, 5, 10, 20, 30, 40, 50, and 60 min, diluted, plated on LB or YPD agar, and incubated for 12 h at 37°C, after which microbial colonies were counted. Peptide-free cultures served as controls.

Hemolytic assay. The hemolytic activities of P3 and its analogs were evaluated using New Zealand rabbit red blood cells. Blood was collected from the ears of rabbits, treated with an anticoagulant, centrifuged at $8 \times 10^2 \times g$ for 5 min, and washed three times in phosphate buffer (10 mM [pH 7.3]). Peptides were mixed with 5% (vol/vol) erythrocyte suspensions and incubated for 1 h at 37°C. A 1% (vol/vol) Triton X-100 solution (100% hemolysis) served as a positive control. After incubation, the cells were centrifuged at $8 \times 10^2 \times g$ for 5 min. Aliquots (100 µl) of supernatants were transferred to 96-well microplates in triplicate, and hemoglobin content was measured at 540 nm (OD₅₄₀) using a microplate reader. The assay was performed in triplicate.

Cytotoxic activity. Cytotoxic activity was determined using the CCK-8 assay (20). The human keratinocyte cell line HaCaT was obtained from the ATCC. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum (Gibco) at 37°C. Cells at approximately 5×10^4 cells/ml (100 µl/well) were seeded into 96-well culture plates. Peptides were added to various concentrations, followed by incubation for 24 h. Next, 10 µl CCK-8 solution was added, the incubation continued for 2 h at 37°C, and the absorbance at 450 nm was read with the aid of a multifunctional microplate reader. The viability of control cells was assumed to be 100%. The viabilities of cells exposed to peptides were expressed as percentages of the control. All of the experiments were performed in triplicate.

Bacterial membrane permeability assay. Membrane permeability was determined by measuring bacterial uptake of PI. *E. coli* cells were incubated with 50 µg/ml P3 at 37°C for 30 min, whereas the control was incubated with PBS alone. Next, PI (to 100 µg/ml) was added to all of the samples, and the suspensions were incubated at 37°C for 10 min. Finally, samples were examined under a fluorescence microscope (Eclipse 80i; Nikon, Japan).

TABLE 1 Amino acid sequences, physicochemical properties of P3 and its analogs

Peptide	Sequence ^a	Mass (Da)	Net charge
P3	VNFKLLSHSLVTLASHL	1,992.35	8
JH-0	KLLSHSLVTLA	1,295.10	8
JH-1	KLLRHRLVTLA	1,432.81	12
JH-2	VRFKLLSHSLVTLASHL	2,034.43	11
JH-3	RR FKLLSHSLVTLASHL	2,091.51	12

^a Boldface indicates the substituted amino acid.

SEM. Bacteria were incubated with P3 in 0.01 M phosphate buffer for 30 min, washed, fixed in 5% (vol/vol) glutaraldehyde for 5 h at 4°C, dehydrated in a rising ethanol gradient (from 30 to 100% [vol/vol] at 15 min per bath), placed on circular coverslips, freeze-dried, sputter coated with a fine layer of gold, and examined by scanning electron microscopy (SEM) under a Quanta 200 FEG scanning electron microscope (FEI, Ltd.).

Measurement of released [Ca²⁺]_i (intracellular free calcium ion) in bacteria. Fura 2-AM, a fluorescent probe, the emission characteristics of which change in the presence of free calcium, was diluted to 5 µM in Hanks' balanced salt solution (HBSS) without Ca²⁺. *E. coli* and *S. aureus* were washed three times in HBSS buffer, incubated with Fura 2-AM at 37°C for 45 min, washed three times as described above to remove residual Fura 2-AM, and mixed with P3. Fluorescence intensity data were collected via multiple Varioskan Flash measurements and converted into [Ca²⁺]_i values using the equation $[Ca^{2+}]_i = K_d (F_o/F_s) (R - R_{min}) / (R_{max} - R)$, where K_d is the dissociation constant (224 nmol/liter), F_o and F_s are the fluorescence intensities at 380 nm after addition of 1 mM EGTA and 2.2 mM Ca²⁺, respectively, R is the fluorescence ratio at 340/380 nm, and R_{max} and R_{min} are the fluorescence ratios F_s at 340 nm/ F_s at 380 nm and F_o at 340 nm/ F_o at 380 nm, respectively.

CD analysis. Circular dichroism (CD) spectra were captured using a 1-mm quartz cell and a Jasco-810 spectrometer. The wavelength range was 250 nm to 190 nm, with a bandwidth 0.5 nm, a scan speed of 100 nm/min, a data pitch of 2 nm, and a response time of 1 s. The final P3 concentration was 1× the *E. coli* MIC. CD spectra were captured in 0.01 M phosphate buffer (pH 7.4). Lipid membrane configurations were assessed by recording the CD spectra of peptides in lipid membranes.

In vivo assay. ICR mice (weight, 15 to 20 g) were obtained from Xinxiang Medical University and housed individually in cages with free access to food and water. All of the animals were euthanized via anesthesia at the end of the experiments. All of the procedures and animal handling were approved by the Ethics Committee of the Henan Institute of Science and Technology. The *in vivo* toxicities of peptides in ICR mice were evaluated by intraperitoneal (i.p.) injections of dose gradients in PBS; the control received PBS alone. A 7-day mortality was recorded. The 50% lethal dose (LD₅₀) was calculated using the up-and-down method (21).

Murine bacteremia model. A model of bacteremia was established in ICR mice via i.p. injection of a 200-µl suspension (approximately 1×10^9 CFU/ml) of an *E. coli* MDR strain isolated from human blood. Amp served as a positive antimicrobial control. The animals were assigned to seven groups (10 animals per group): (i) P3, (ii) JH-0, (iii) JH-1, (iv) JH-2, (v) JH-3, (vi) Amp, and (vii) untreated. One hour after inoculation, peptides (20 to 60 mg/kg) or Amp (40 mg/kg) was injected i.p., and survival to 24 h and 48 h after treatment was recorded. Two hours after injection, 20-µl blood samples were collected from tail veins, and bacterial counts were determined by plating blood dilutions on LB agar plates.

Statistical analysis. MIC values and 50% lethal concentrations (LC₅₀s) are presented as the geometric means of data from three separate experiments. Fisher's exact test was used to compare mortality rates between groups, and survival rates were compared via Kaplan-Meier analysis. The significance of a difference in mean viable bacterial counts was accepted when the *P* value was <0.05, calculated using the Kruskal-Wallis test.

FIG 1 Comparison of amino acid sequences from positions 97 to 114 (box) of the hemoglobin α -subunits of bovines (GenBank accession no. NP-001070890.2), humans (GenBank accession no. P69905), sheep (GenBank accession no. P68240), and deer (GenBank accession no. P21379). The dots represent the most common amino acids.

RESULTS

Sequence characteristics. P3 is an 18-residue peptide with modest antimicrobial activity and weak hemolytic activity (15). A series of analogs were created by substituting or removing specific amino acids in efforts to enhance P3 antimicrobial activity. To reduce the cost of peptide synthesis, we designed a smaller peptide, JH-0, by removing three amino acids from each of the C- and N-terminal regions of P3. To enhance P3 antimicrobial activity, JH-2 and JH-3 were synthesized by replacing certain amino acids with arginine. The primary sequences, molecular masses, and net charges of all of the peptides are shown in Table 1. Sequence alignment showed that the primary structure of P3 included amino acids 97 to 114 of the α -subunit of bovine hemoglobin, which is very similar to the same hemoglobin region of humans, pigs, deer, and sheep (Fig. 1). Some hemoglobin peptides from bovines and humans are active against bacteria (22, 23). Therefore, the ob-

served high level of sequence similarity may be important for understanding the antimicrobial activity of hemoglobin or its segments (24).

Antimicrobial activities of P3 and its analogs. The bacterial MICs of P3 and its analogs are shown in Table 2. P3 exhibited modest antimicrobial activities against Gram-negative and Gram-positive bacteria and fungi, grown in LB and YPD broth, respectively. The arginine-substituted analogs exhibited much stronger antimicrobial activities. Compared to P3, JH-3 exhibited higher antimicrobial activities against all of the seven tested microbes, with MIC values of 3.125 to 12.5 $\mu\text{g/ml}$. The MICs of JH-0 were similar to those of JH-2, except those for the *C. albicans* standard and clinical strains. The antimicrobial activities of P3 and its ana-

TABLE 2 Antimicrobial activities of P3 and its analogs

Strain	MIC ($\mu\text{g/ml}$) of ^a :					
	P3	JH-0	JH-1	JH-2	JH-3	Amp
<i>E. coli</i>						
ATCC 25922	12.5	6.25	3.125	6.25	3.125	0.5
Clinical strain ^b	12.5	12.5	6.25	12.5	3.125	>200
<i>S. aureus</i>						
ATCC 29213	25	6.25	6.25	12.5	6.25	0.5
Clinical strain ^b	12.5	6.25	6.25	6.25	3.125	>200
<i>P. aeruginosa</i> clinical strain	25	6.25	12.5	6.25	6.25	>200
<i>C. albicans</i>						
ATCC 90029	50	>200	>100	25	12.5	—
Clinical strain	25	>200	>100	25	6.25	—

^a A minimal level of 99.9% inhibition is indicated. >, no activity detected at the concentration indicated. —, not assayed.

^b Resistant to three or more of the following antibiotics: gentamicin, amikacin, piperacillin, levofloxacin, imipenem, and colistin.

FIG 2 Toxicity of P3 and its analogs *in vitro* and *in vivo*. (A) Hemolytic activities of the peptides at various concentrations upon incubation with human red blood cells for 1 h. (B) Survival of HaCaT cells after exposure to peptides for 24 h. (C and D) Acute toxicities of the peptides. Shown is survival of mice after injection of peptides i.p. (C) or s.c. (D).

FIG 3 Bacterial survival after treatment with peptides. Killing kinetics of P3 and JH-3. *E. coli* ATCC 25922 (A), an *E. coli* clinical strain (B), *C. albicans* ATCC 90029 (C), and *C. albicans* (D) (6×10^5 CFU/ml) were exposed to P3 and JH-3 at $2 \times \text{MIC}$ for 0, 5, 10, 20, 30, 40, 50, and 60 min.

logs did not significantly differ among *E. coli* and *S. aureus* clinical strains and standard strains. The data also indicated that bacterial resistance to Amp did not affect the antimicrobial activities of P3 or its analogs.

Toxicity of P3 and its analogs. Because the peptides are promising novel antimicrobial agents, their hemolytic capacities and cytotoxicities were assessed. All of the peptides tested exhibited low hemolytic activities against rabbit red blood cells. Even at 400 $\mu\text{g}/\text{ml}$ for 1 h, no peptide caused more than 20% hemolysis (Fig. 2A). Peptide cytotoxicities were tested using the HaCaT cell line. After incubation with P3 and JH-3 at 400 $\mu\text{g}/\text{ml}$ for 1 h, the HaCaT viabilities were approximately 85% and 94%, respectively (Fig. 2B). JH-3 was less cytotoxic than P3 and the other analogs.

Drug cytotoxicity is of major concern. In the present study, the acute toxicities of P3 and its analogs *in vivo* were evaluated in ICR mice via single i.p. or subcutaneous (s.c.) peptide injections over a wide range of doses (Fig. 2C). PMB, a clinically acceptable antimicrobial polypeptide, served as a control. Upon i.p. injection, the LD_{50} values of P3 and JH-3 were approximately 160 mg/kg and 180 mg/kg, respectively, which were 3-fold higher than that of PMB (50 mg/kg). After single s.c. injections of P3 or JH-3 at 240

mg/kg, no mortality was noted within 7 days; the LD_{50} of PMB was 80 mg/kg.

P3 and JH-3 rapidly kill *E. coli* via disruption of the cytoplasmic membrane. To determine the antimicrobial mechanism of action of P3 and JH-3, the kinetics of killing of *E. coli* 25922, the *E. coli* clinical strain, *C. albicans* ATCC 90029, and the *C. albicans* clinical strain were investigated. JH-3 killed *E. coli* and *C. albicans* more rapidly than P3 (Fig. 3). At a concentration of $2 \times \text{MIC}$, JH-3 reduced the inoculum numbers by $>90\%$. P3 required 40 min and 50 min to induce 99.99% cell death of *E. coli* 25922 and *C. albicans* ATCC 90029, respectively, indicating that the antibacterial activities of P3 and JH-3 were time dependent.

Membrane disruption assays were also used to explore the killing kinetics of P3. Permeabilization of bacterial cytoplasmic membranes by peptides was investigated by measuring bacterial PI uptake. PI only penetrates damaged membranes, staining DNA red. As shown in Fig. 4, after incubation with *E. coli* at 37°C for 30 min, PI penetrated the cytoplasmic membrane, creating red fluores-

FIG 4 Effect of P3 on *E. coli* bacterial membrane permeability. *E. coli* was incubated with P3 ($1 \times \text{MIC}$) (B) or PBS (control [A]) for 30 min. The bacteria were stained with PI and viewed under a fluorescence microscope to assess bacterial membrane integrity. No fluorescence is evident in panel A. However, in panel B, some bacteria exhibit red contours, reflecting PI fluorescence. Bar, 10 μm .

FIG 5 Scanning electron micrographs of *E. coli* cells not exposed (A) or exposed (B) to P3 ($1 \times \text{MIC}$) for 30 min. Bar, 1 μm . The *E. coli* cells are generally normal in appearance, but bacterial membrane holes are evident in panel B, caused by the P3 treatment.

FIG 6 CD spectra of P3 in sodium phosphate buffer, pH 7.2 (dotted line), and a phospholipid membrane (solid line). A positive band at around 197 nm and two negative bands near 208 nm and 225 nm are apparent (solid line). One negative band may be noted near 203 nm.

cence absent from the control. Next, SEM was used to study bacterial morphology and structure in the presence or absence of P3. *E. coli* cell surfaces developed many obvious indentations and pores after incubation with the peptides (Fig. 5), showing that the rapid *E. coli* antimicrobial action of P3 may be attributable to permeabilization and disruption of the cytoplasmic membrane.

The mode of action of P3. To explore how P3 disrupted the bacterial membrane, the relationship between P3 secondary structure and bioactivity was investigated via CD spectroscopy; we sought to determine P3's conformation within a simulated membrane. P3 yielded a positive band at about 197 nm and two negative bands near 208 nm and 225 nm, suggesting that an ordered α -helical conformation was assumed in the lipid environment. In contrast, P3 was disordered in PBS (Fig. 6), suggesting that the α -helical conformation was induced by the lipid membrane and was crucial in terms of membrane disruption.

To explore how the peptides compromised bacterial metabolism, Fura 2 fluorescence assays were used to monitor real-time fluctuations in $[Ca^{2+}]_i$ levels. Fura 2-AM is converted into Fura 2 by an esterase of the bacterial cytoplasm; the maximum excitation wavelength of Fura 2 shifts from 380 nm to 340 nm upon binding of calcium. As shown in Fig. 7, after peptide addition, the concentration of free calcium ions in the *E. coli* cytoplasm increased more rapidly than in *S. aureus*, consistent with the stronger antibacterial activity of peptides against *E. coli* than *S. aureus*. The $[Ca^{2+}]_i$ concentration rose substantially after incubation with peptides for longer than 30 min, in accordance with the bacterial killing kinet-

ics and the PI assay data, suggesting that P3 triggers the release of intracellular calcium stores.

Efficacy of P3 and its analogs *in vivo*. To explore the protective effects exerted by P3 and its analogs *in vivo*, bactericidal efficacies against an *E. coli* clinical strain were assessed in a mouse model of bacteremia. The *E. coli* LD₅₀ was 1×10^8 CFU/ICR mouse after i.p. injection, and all of the mice died within 24 h. In the peptide-treated groups, mortality significantly decreased (Fig. 8) ($P < 0.005$). Moreover, survival upon JH-3 treatment (90%) was significantly higher than that of the Amp-treated or untreated group ($P < 0.05$), in which all of the mice died within 24 h. This indicated that Amp was not active against the clinical *E. coli* strain. At 40 mg/kg, JH-3 reduced the bacterial count from 10^8 CFU/ml to approximately 10^5 CFU/ml (Fig. 9).

DISCUSSION

AMPs are broad-spectrum antimicrobials that are candidate alternatives to conventional antibiotics (25). However, many natural AMPs are large and associated with high synthetic costs, rendering clinical application difficult. Therefore, the potent antimicrobial activity of JH-3 and its low cytotoxicity are encouraging. In contrast to most AMPs, P3 from bovine hemoglobin exhibits potent antimicrobial activity *in vivo* and low cytotoxic and hemolytic activities, indicating that is an excellent candidate for development as a new antimicrobial agent.

The antimicrobial roles played by conserved sequences within AMPs have been studied. In the present work, we minimized the size of P3 with maintenance of antimicrobial activity. We reduced the size of P3 by removing three amino acids from each of the C and N termini. The antimicrobial activities against both Gram-positive and Gram-negative bacteria were thereby increased, but the antifungal ability was lost, indicating that the residual region retained antimicrobial activity. Arginine optimally increases the cationic property of a peptide chain, and several natural amino acids of P3 and JH-0 were replaced with this residue, enhancing antimicrobial activities.

P3 and its analogs exhibited strong antimicrobial activities against both clinical drug-resistant and control strains *in vitro*. It is known that AMPs, including SMAP29 and LL-37, can permeabilize the bacterial cytoplasmic membrane (26, 27), as did P3, which created many membrane pores in the *E. coli* membrane, in line with the barrel stave model (28). It is generally considered that AMPs exhibiting potent antimicrobial activities also have strong hemolytic and cytotoxic activities (29). However, hemolysis did

FIG 7 Release of intracellular calcium in *E. coli* (A) and *S. aureus* (B) treated with P3 (1×MIC).

FIG 8 Survival of mice in the bacteremia model after injection of the MDR *E. coli* clinical strain. Ampicillin (40 mg/kg) (A) and P3 (20, 40, or 60 mg/kg) were given via i.p. injection (B).

not exceed 20% after treatment of red blood cells with P3 or its analogs at 400 μ g/ml, a concentration approximately 60-fold higher than the MIC. JH-3 at 3.125 to 12.5 μ g/ml exhibited potent antibacterial activity but only slight toxicity toward mammalian cells, indicating that the prokaryotic cell membrane was specifically targeted.

An increase in intracellular calcium levels can trigger a rapid chain reaction, including activation of phosphatidase, protease, ATPase, and nuclease in the cytoplasm, causing degradation of phospholipids, proteins, ATP, and nucleic acids. This is the first response of the cell to AMP stimulation (30). Massive calcium release can deplete ATP and is a significant cause of bacterial cell death (31). Some antifungal drugs induce continuous calcium overload. In the present study, intracellular calcium levels rose after peptide addition. However, no extracellular calcium was noted, suggesting that JH-3 acted in the cytoplasm to trigger the release of intracellular calcium stores, in turn causing bacterial death.

In the bacteremia model, 1 h is sufficient to allow bacteria to disseminate throughout the mouse, and this murine model is typically used in the pharmaceutical industry to evaluate the systemic efficacy of antibiotics (32). Unlike Amp, a widely used antibiotic, P3 and JH-3 protected mice against bacteremia induced by clinical drug-resistant *E. coli* strains and rescued mice from bacteremia after i.p. injection. The efficacies of JH-0, JH-1, and JH-2 were also

tested: these peptides exhibited potent antimicrobial activities both *in vitro* and *in vivo*.

In summary, we found that P3 and an arginine-substituted analog, JH-3, exhibited potent antimicrobial activities against drug-resistant strains by disrupting and penetrating the bacterial cytoplasmic membrane and disturbing normal physiological processes. Moreover, P3 and its analogs exerted potent therapeutic effects in a model of bacteremia. Together, the results indicate that P3 and JH-3 are excellent templates for the development of novel antimicrobial agents against infections caused by drug-resistant bacteria.

ACKNOWLEDGMENT

This work was supported by grants from the National Natural Science Foundation of China (no. 31372469).

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FIG 9 Blood bacterial counts in the bacteremia model. Mice received P3 or analogs thereof (40 mg/kg) or ampicillin (40 mg/kg) via i.p. injection ($n = 10$). Each point represents data from a single animal, and the line shows the mean value. *, $P < 0.05$ versus the untreated and ampicillin-treated groups.

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