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In Vivo Efficacy of Anuran Trypsin Inhibitory Peptides against Staphylococcal Skin Infection and the Impact of Peptide Cyclization

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***Staphylococcus aureus* is a virulent pathogen that is responsible for a wide range of superficial and invasive infections. Its resistance to existing antimicrobial drugs is a global problem, and the development of novel antimicrobial agents is crucial. Antimicrobial peptides from natural resources offer potential as new treatments against staphylococcal infections. In the current study, we have examined the antimicrobial properties of peptides isolated from anuran skin secretions and cyclized synthetic analogues of these peptides. The structures of the peptides were elucidated by nuclear magnetic resonance (NMR) spectroscopy, revealing high structural and sequence similarity with each other and with sunflower trypsin inhibitor 1 (SFTI-1). SFTI-1 is an ultrastable cyclic peptide isolated from sunflower seeds that has subnanomolar trypsin inhibitory activity, and this scaffold offers pharmaceutically relevant characteristics. The five anuran peptides were nonhemolytic and noncytotoxic and had trypsin inhibitory activities similar to that of SFTI-1. They demonstrated weak *in vitro* inhibitory activities against *S. aureus*, but several had strong antibacterial activities against *S. aureus* in an *in vivo* murine wound infection model. pYR, an immunomodulatory peptide from *Rana sevosa*, was the most potent, with complete bacterial clearance at 3 mg · kg^{-1}. Cyclization of the peptides improved their stability but was associated with a concomitant decrease in antimicrobial activity. In summary, these anuran peptides are promising as novel therapeutic agents for treating infections from a clinically resistant pathogen.**

Staphylococcus aureus is one of the most virulent and opportunistic pathogens, causing increasing numbers of nosocomial and community-acquired infections, and is a leading cause of skin and soft tissue infections (1–3). These Gram-positive cocci produce a myriad of virulence factors that allow the bacteria to attach to host cells, to invade tissues, to evade the host immune system, and to release an array of exoproteins and toxins (4, 5). Contagious *S. aureus* skin infections can lead to severe muscle or bone infections that ultimately can spread to the lungs or heart (6). The primary treatment involves prescription of β-lactam antibiotics such as penicillins and cephalosporins, along with clinical wound-cleaning procedures (6, 7). Strains resistant to antibiotics have been emerging since the 1960s, however, especially methicillin-resistant *S. aureus* (MRSA), which is most common in nosocomial skin infections (8). Alarmingly, there have been reports of *S. aureus* strains that are resistant to the drug of last resort, vancomycin (9).

Antimicrobial peptides are now recognized as novel alternative therapeutic agents for infection control (10). Several hypotheses have been examined regarding the mode of action of antimicrobial peptides, including autolysin activation, lipopolysaccharide (LPS) permeabilization, fatal depolarization of the energized bacterial membrane, formation of barrel-stave pores that cause leakage of cellular contents, activation of processes that degrade the cell wall, membrane thinning/thickening, impairment of essential intracellular targets after internalization, and disturbance of the distribution of cellular membrane lipids (11–15). Attributes of antimicrobial peptides that make them viable candidates for development as anti-infective therapeutic agents include broad-spectrum antimicrobial activity, novel mechanisms of action and ease of manipulation, and synthesis and tailoring of peptide sequences (16, 17). Conversely, major obstacles to the successful development of antimicrobial peptides as therapies include their potential toxicity, high manufacturing costs, and degradation due to proteases, heat, or extremes of pH (10). The design of peptides that are stable against these processes while maintaining their antimicrobial properties would be a breakthrough in this field.

In amphibians, antimicrobial peptides play an important role in defense against pathogenic microbes. The skin secretions of anuran frogs contain a cocktail of compounds, with various biological activities, that have potential for drug development (18). Among these secreted compounds are the anuran antimicrobial peptides, which have a broad range of antibacterial and antifungal activities (19). These ribosomally synthesized peptides of 8 to 63 amino acids have high affinity for microbial cell membranes (20). They also have serine protease inhibitory activity (21–23).

The amphibian antimicrobial peptides shown in Table 1 contain a conserved Bowman-Birk inhibitor (BBI)-like (24) trypsin inhibitory loop (CWTKSIPPKPC) and share high sequence homology and structural similarities with sunflower trypsin inhibitor 1 (SFTI-1), a 14-amino-acid cyclic peptide discovered in sun-

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TABLE 1 Characteristics of SLF peptides from anuran skin secretions that were highly similar to SFTI-1

Peptide	Origin	Sequence[a]	Peptide property						
						Composition (%)			
			NC	aHI	pI	HY	BA	NE	AC
ORB	*Odorrana grahami*	AALKG-CWTKSIPPKPCFGKR	5.9	−0.42	11.0	40	25	35	0
ORB2K	*Odorrana grahami*	LKG-CWTKSIPPKPCFGK	4.9	−0.44	10.6	35	24	41	0
pYR	*Rana sevosa*	YLKG-CWTKSIPPKPCFS-R	4.9	−0.87	10.9	39	22	39	0
Rana-E[b]	*Rana esculenta*	SAPRG-CWTKSIPPKPC--K	4.9	−1.22	10.5	29	24	47	0
Rana-T[b]	*Rana temporaria*	GALRG-CWTKSIPPKPC--K	4.9	−0.88	10.5	35	25	41	0
SFTI-1	*Helianthus annuus*	GRC-TKSIPPI-CFP-D	0.9	−0.13	8.3	36	14	43	7

[a] Peptides contain a Bowman-Birk inhibitor (BBI) reactive loop (enclosed in dashed box) and a disulfide bond (black lines). SFTI-1 has a naturally cyclic backbone (gray line). NC, net charge; aHI, grand average of hydropathicity index; pI, isoelectric point; HY, hydrophobic amino acids; BA, basic amino acids; NE, neutral amino acids; AC, acidic amino acids.
[b] Ranacyclin peptides are abbreviated as Rana.

flower seeds (*Helianthus annuus*) (25). SFTI-1 is the smallest and one of the most potent BBIs. The structure of SFTI-1 has an extensive hydrogen bonding network and a disulfide bond connecting two β-strands, which form a tightly folded scaffold with two distinct loops, namely, the trypsin binding loop (loop 1) and the secondary loop (loop 2) (26). Loop 1 contains the active site residue for trypsin inhibitory activity, and loop 2 contains the site for backbone cyclization in the natural biosynthetic process. The anuran peptides studied have additional conserved residues in the binding loop (W and P) and additional residues in the secondary loop, where only the glycine preceding the first cysteine residue is conserved. Based on these sequence similarities, we have termed these anuran peptides with similarity to SFTI-1 the SFTI-1-like frog (SLF) peptides.

The cyclic nature of SFTI-1 contributes to its stability and its resistance to degradation by proteases (26), which is a limitation of linear antimicrobial peptides. Cyclization has been used as one of the strategies in drug design to improve stability; for example, cyclization of the antimicrobial peptide pyrrhocoricin improved its *in vivo* pharmaceutical properties (27). The *in vitro* stability and antimalarial activity of the antimicrobial peptide gomesin, from the Brazilian spider *Acanthoscurria gomesiana*, were also improved after backbone cyclization (28).

In this study, a series of SLF peptides were evaluated for their *in vivo* efficacy in a murine *Staphylococcus aureus* wound infection model. Three of the active peptides were cyclized in an attempt to improve their stability. The structures of the most active peptide and its cyclic analogue were determined to elucidate the effects of cyclization on their antimicrobial and trypsin inhibitory activities.

MATERIALS AND METHODS

Peptide synthesis and purification. 9-Fluorenylmethoxycarbonyl (Fmoc)-based chemistry was used to synthesize linear peptides on an automated synthesizer (Symphony; Protein Technologies, Inc.). 2-Chlorotrityl resin (Novabiochem) was used to assemble the peptide chain, and amino acids were Fmoc deprotected using 30% piperidine (Auspep Pty. Ltd.) in dimethylformamide (DMF). Fmoc-protected amino acids (CS Bio Co.) were coupled with HCTU [*O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate] (Peptide International) and DIPEA (*N*,*N*-diisopropylethylamine) (Auspep Pty. Ltd.) in DMF (RCI Labscan Ltd.) (28). The cyclic peptides were synthesized by *t*-butyloxycarbonyl (Boc)-based solid-phase peptide synthesis using standard protocols (29, 30). A thioester-based linker (β-mercaptopropionic acid) was used to facilitate cyclization by native chemical ligation (NCL) (31). Previously described standard protocols for hydrogen fluoride cleavage were used to cleave the peptides from the resin (32). The cleaved linear reduced peptides were purified by C_{18} reverse-phase (RP)-high-performance liquid chromatography (HPLC) using a gradient of buffer B (90% acetonitrile in 0.05% aqueous trifluoroacetic acid [TFA]) and buffer A (0.05% aqueous TFA) of 1% per minute. Peptide cyclization was achieved by native chemical ligation with a procedure involving two steps, i.e., cyclization in the presence of a reducing agent (TCEP [tris(2-carboxyethyl)phosphine]) followed by oxidation, with both steps involving overnight stirring at room temperature in 0.1 M ammonium bicarbonate buffer (pH 8.5). Peptides were purified by RP-HPLC at each step. The molecular weight and purity of the peptides were confirmed by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS).

Structure determination by NMR spectroscopy. ^{1}H nuclear magnetic resonance (NMR) measurements were carried out with a Bruker Avance-600 spectrometer for all peptides. Each peptide (~3 mg) was dissolved in 90% H_2O-10% D_2O (99.9 and 99.99%, respectively; Cambridge Isotope Laboratories, Woburn, MA) at pH 5, and DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) was added as a chemical shift reference. Two-dimensional NMR data were recorded in phase-sensitive mode using time-proportional phase incrementation for quadrature detection in the t_1 dimension (33). Total correlation spectroscopy (TOCSY) experiments used an MLEV-17 spin-lock sequence (34) with a mixing time of 80 ms, and nuclear Overhauser effect spectroscopy (NOESY) experiments used mixing times of 100 to 200 ms (35). Amide proton temperature sensitivity and deuterium exchange experiments were conducted to determine hydrogen-bonding constraints. Talos+ was used for prediction of the Φ and Ψ backbone angles (36). Structural calculations were carried out with CYANA 3.0, using torsion angle dynamics. The 20 lowest-energy structures with the best MolProbity scores were selected for the final ensemble (37).

Trypsin inhibition. Trypsin inhibition constants (K_i) of the peptides were measured by a rapid microplate assay using a modification of the method described by Erlanger et al. (38). Reactions were conducted in a 96-well plate containing 15 μl of 0.45 mg/ml bovine pancreatic trypsin (Sigma-Aldrich), 5 μl of buffer (50 mM Tris, 20 mM $CaCl_2$ [pH 8.2]), 125 μl of 0.435 mg/ml L-BAPNA (*N*α-benzoyl-L-arginine-4-nitroanilide hydrochloride) (Sigma-Aldrich), and peptides at final concentrations ranging from 6.67 mM to 0.065 mM. The reaction was quenched with 25 μl of

30% acetic acid after 10 min of incubation at 25°C. A PowerWave XS plate reader (Bio-Tek) was used to measure the absorbance of *p*-nitroanilide (pNA) at 410 nm.

***In vitro* antimicrobial assays.** The MICs of peptides against *S. aureus* were determined using the standardized serial dilution method, according to North Carolina Science Leadership Association (NCSLA) guidelines (39). Overnight colonies of *Staphylococcus aureus* (strain ATCC 29213) were suspended and standardized at 0.5 units by a turbidity method, followed by dilution in Mueller-Hinton (MH) broth. For MIC determinations, peptides were added at 1 to 200 μM concentrations from a stock solution, and gentamicin was used as a positive control. Each well of the 96-well plate contained 90 μl of bacteria (1×10^5 cells) in MH agar and 10 μl of peptide solution. Ampicillin (40 mg · ml^{-1}) and phosphate-buffered saline (PBS) were used as positive and negative controls, respectively. Each data point was measured in triplicate. The polypropylene plates were incubated for 24 h at 37°C. Peptide MICs were determined as the lowest tested concentration that resulted in 100% development inhibition, in comparison to the negative control. The MIC is defined as the lowest concentration of an antimicrobial compound that completely inhibits the growth of the organism, as detected by the unaided eye (40).

Hemolytic assays. All peptides were tested for their abilities to hemolyze red blood cells (RBCs). Using a previously described method (41), serially diluted peptides (final concentrations of 0.4 to 50 μM) were incubated with human RBCs in 96-well plates for 1 h at 37°C and then were centrifuged at 150 relative centrifugal force (RCF) for 5 min. The absorbance of the supernatant, containing plasma and lysed RBCs, was measured at 415 nm with a PowerWave XS plate reader (Bio-Tek). Triton X-100 (0.1%) and PBS were used as positive and negative controls, respectively. Melittin, a cationic hemolytic peptide, was used as a positive control and was tested at initial concentrations ranging from 0.4 to 50 μM. Percent hemolysis was calculated using the absorbance of maximum lysis in the positive-control samples (Triton X-100).

Cell cytotoxicity. The cytotoxic effects of anuran peptides on human foreskin fibroblast (HFF-1) cells and melanoma (MM96L) cells were evaluated using an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay. Cells were seeded in 96-well plates at 3×10^3 cells/well (100 μl) with 15% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) for HFF-1 cells (Gibco) or 10% FBS in RPMI 1640 medium for MM96L cells, supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate (Gibco). All cells were incubated at 37°C in 5% CO_2 for 24 h. The medium was removed and replaced with fresh serum-free medium (90 μl/well), and then peptides were added. All peptides were tested in triplicate, with final peptide concentrations ranging from 1 to 200 μM, and were incubated with cells for 2 h. A negative vehicle control (water) and a positive control (1% Triton X-100) were included in the assay. After 2 h of incubation, 10 μl of MTT (5 mg/ml in PBS) was added to each well, and the cells were further incubated for 3 h before the removal of supernatant. The formazan crystals formed in each well were dissolved in 100 μl dimethylsulfoxide (DMSO), and absorbance was measured at 600 nm using a BioTek PowerWave XS spectrophotometer. Data were analyzed using the GraphPad Prism program, and 50% inhibitory concentration (IC_{50}) values were obtained from the sigmoidal dose-response curves.

Acute toxicity. Acute toxicity assays were based on the method described by Navon-Venezia and coworkers (42), with intraperitoneal (i.p.) administration of the tested peptides to groups of 10 C57BL/6 mice. Each mouse was injected with 0.5 ml of a solution of freshly prepared pyR or cpYR in PBS. The doses of peptides administered per mouse were 0, 5, 10, 25, 50, and 100 mg · kg of body $weight^{-1}$. Animals were inspected for adverse effects for 30 min, and survival was monitored for 6 h thereafter.

***In vivo* antimicrobial activity.** Female C57BL6 mice (6 to 8 weeks of age) were obtained from CEMIB-Unicamp (Brazil). The study was approved by the Animal Use Committee at the Institute of Biological Sciences, University of Brasilia. Briefly, groups of mice ($n = 4$/group in each experiment) were anesthetized with a combination of ketamine and xylazine, their backs were shaved, and the surgical area was disinfected with 70% ethanol. A 1-cm incision was made, and 25 μl with 2×10^9 CFU *S. aureus* was pipetted into the incision. The mice were euthanized at 7 days after surgery, and the wound tissue was excised, weighed, and homogenized in 1 ml of PBS. Serial dilutions of the homogenates were plated in triplicate on mannitol salt agar plates, and results were expressed as CFU/gram of tissue. Peptides were administered to animals daily. Peptides were solubilized in water for injection at two different concentrations, 1.5 mg · kg^{-1} and 3.0 mg · kg^{-1}, and were injected into the wound in a 25-μl volume; PBS was used as a negative control and 10 mg · kg^{-1} neomycin sulfate as a positive control.

Serum stability. The linear and cyclic forms of pYR and SFTI-1 peptides were evaluated in serum stability assays using an established method (30). Human AB serum (Sigma) was used in this assay. Lipids were removed by centrifugation at 13,000 rpm for 15 min prior to the assay. Peptides (initial concentration, 2 mg/ml) diluted 10-fold in serum (test) or PBS (negative control) were incubated at 37°C. At each time point (0, 3, 6, 10, and 24 h), triplicate aliquots were taken from each peptide sample in serum or PBS and denatured with equal volumes of 6 M urea, followed by 10 min of incubation at 4°C. The serum proteins were then precipitated with an equal volume of 20% trichloroacetic acid, with a 10-min incubation at 4°C followed by centrifugation at 13,000 rpm for 15 min. The supernatant was stored at 4°C until analysis. The relative concentration of the intact peptide, in comparison with the level at the start of the experiment, was measured by ultra-high performance liquid chromatography (uHPLC) for each peptide sample, using the integrated area of the peak corresponding to the intact peptide (with UV absorbance at 214 nm) on the HPLC trace. The mean and standard deviation were calculated for each peptide.

RESULTS

Synthesis of antimicrobial peptides. The SLF peptides (Table 1) were synthesized to study their structures, biological characteristics, and *in vivo* antimicrobial efficacy against *S. aureus* in an animal wound infection model. The cyclic versions of the SLF peptides are prefixed with c, and the open or acyclic form of SFTI-1 is prefixed with o. The native open-chain peptides were synthesized using Fmoc-based chemistry and were oxidized in ammonium bicarbonate buffer (pH 8.5). SFTI and the cyclic versions of the anuran peptides were synthesized using Boc-based chemistry with a C-terminal thioester to facilitate cyclization. Cyclization (in the presence of reducing agent) and oxidation were carried out in two separate steps. These conditions led to efficient cyclization of peptides without the requirement for additional linker residues.

Structure determination by NMR spectroscopy. The peptides were analyzed by NMR spectroscopy. The secondary Hα chemical shifts (Fig. 1) were calculated using the measured chemical shifts and the random coil values reported by Wishart et al. (43). Secondary Hα chemical shifts are highly sensitive to structural modifications, and thus they offer an excellent platform to observe structural differences due to amino acid variations (44). The linear and cyclic constructs had comparable chemical shifts, suggesting that the cyclization of the backbone did not have a significant influence on structure. The first proline in the binding loop (CX TKSIPPK/IP) is in a *cis* conformation, consistent with SFTI-1 (32). The other proline residues are in a *trans* conformation in all peptides.

The three-dimensional structures of open and cyclic pYR (Fig. 2) were elucidated using the program CYANA, and the ensembles chosen to represent the final structures were based on the 20 structures from a set of 100 structures with the lowest MolProbity scores (37, 45). Statistics showing the precision and stereochemi-

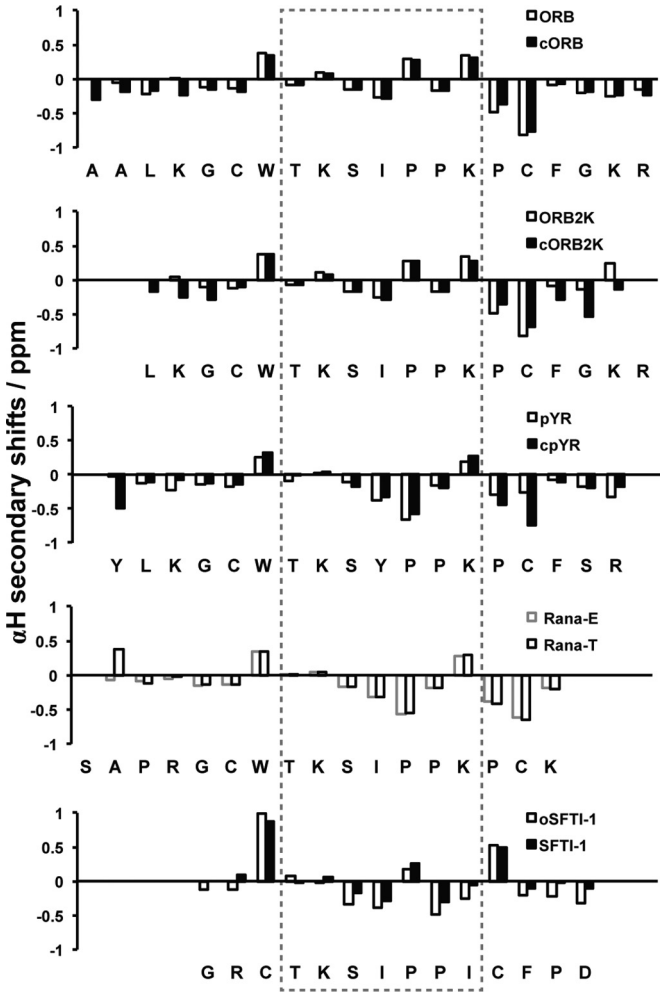

FIG 1 Hα NMR secondary shift analysis of SLF peptides and SFTI-1. Secondary shifts were obtained by subtracting experimental ^{1}H NMR Hα chemical shifts from random coil shifts for the corresponding residue (43). The ORB/ORB2k open and cyclic peptides have similar shifts in loop 1, whereas the Rana-E/T and pYR peptides have similar secondary shifts in loop 1. Loop 1 contains a highly conserved BBI reactive loop (dotted gray box).

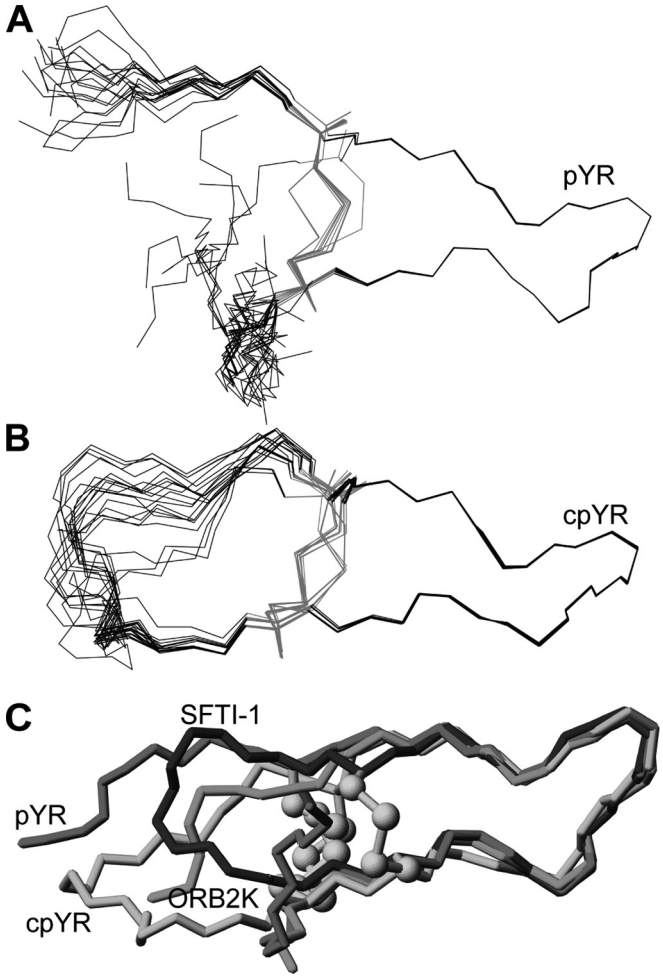

FIG 2 NMR solution structures of open and cyclic pYR. (A and B) Structural ensembles of open (A) and cyclic (B) pYR. Disulfide bonds are highlighted in gray. (C) Three-dimensional structural comparison of pYR peptides with truncated ORB2K (PDB accession number 2O9Q) and SFTI-1 (PDB accession number 1JBL).

cal qualities of the pYR structures are presented in Table 2. In the cyclic form, the loop with the disulfide bond (loop 2) showed less structural variation than the loop with the free termini (loop 1).

***In vitro* biological activity.** The SLF peptides contain a BBI loop similar to SFTI-1. For this reason, the trypsin inhibitory activities of the SLF peptides were assessed and compared with that of SFTI-1 (Fig. 3), which has been reported to have an inhibition constant of 1.7 pM (29). cpYR was found to have trypsin inhibitory activity similar to that of SFTI-1. The other peptides had significantly reduced inhibitory activity, ranging between 13 and 50%, relative to SFTI-1. Cyclization improved the inhibitory activities of all peptides, but opening of the SFTI-1 chain resulted in decreased inhibitory activity, compared to wild-type SFTI-1, consistent with previous studies (26).

The antimicrobial activities of the peptides were measured in an *in vitro* assay with the widely used sensitive *S. aureus* strain ATCC 29213, on MH agar, using a dilution method. Table 3 presents the MIC data for open and cyclic forms of the peptides. pYR was the most potent peptide, with an MIC of 50 μM against *S. aureus*. Rana-E and Rana-T showed MICs of 100 μM, whereas the other open peptides had MICs greater than 100 μM. Backbone cyclization resulted in decreased potency of pYR, while MIC values for cORB and cORB2K were >100 μM. Cyclic and open SFTI-1 forms had no antimicrobial activity at concentrations up to 100 μM.

The hemolytic activities of all peptides were determined in human blood. Melittin, a well-studied and highly hemolytic peptide from honeybee venom (46), and Triton X-100 were used as a positive controls and PBS was used as a negative control in the assay. All peptides were found to be nonhemolytic at up to 50 μM concentrations (data not shown). To further evaluate the toxicity and effects of cyclization of these peptides, the cytotoxicities of open and cyclic pYR and ORB were determined with human foreskin fibroblast (HFF-1) and melanoma (MM96L) cell lines. The peptides were noncytotoxic at up to 200 μM, except for cpYR, which was found to kill 20% of the cells in both cell lines at 200 μM (Fig. 4).

Acute toxicity. Toxicity was assessed *in vivo* for pYR and cpYR.

TABLE 2 Structural statistics for NMR solution structures of pYR and cpYR

Parameter	pYR	cpYR
Experimental restraints[a]		
No. of interproton distance restraints	170	202
No. of intraresidue restraints	39	51
No. of sequential restraints	72	80
No. of medium-range ($i - j$ of <5) restraints	27	27
No. of long-range ($i - j$ of ≥5) restraints	32	44
No. of hydrogen bond restraints[b]	4	4
No. of disulfide bond restraints	2	2
No. of dihedral angle restraints (Ψ and Φ)	20	22
RMS deviation from mean (mean ± SD)[c]		
Backbone atoms (residues 22-45)	1.27 ± 0.53	0.72 ± 0.33
Heavy atoms (residues 22-45)	2.53 ± 0.83	2.13 ± 0.65
Stereochemical quality (mean ± SD)[d]		
Residues in most favored Ramachandran region (%)	96.47 ± 4.83	87.50 ± 2.99
Ramachandran outliers (%)	0.29 ± 1.31	0.31 ± 1.40
Unfavorable side chain rotamers (%)	8.82 ± 7.27	11.00 ± 8.72
Clash score, all atoms (no. of overlaps/1,000 atoms)[e]	13.55 ± 2.30	8.97 ± 3.16
Overall MolProbity score	2.41 ± 0.50	2.74 ± 0.34

[a] Only structurally relevant restraints, as defined by CYANA, are included.
[b] Two restraints were used per hydrogen bond.
[c] RMS, root mean square; SD, standard deviation.
[d] Stereochemical properties were obtained by MolProbity (http://molprobity.biochem.duke.edu).
[e] The clash score is defined as the number of steric overlaps of >0.4 Å per 1,000 atoms.

First, acute toxicity was examined after i.p. administration of a single dose of each peptide to groups of C57BL/6 mice ($n = 5$ mice/group). No immediate adverse events were noted for the peptides at doses of up to 25 mg · kg^{-1} (Table 4). Toxicity level I, involving narrowing of the eyes, was noted minutes after injection in mice injected with doses of peptides above 50 mg · kg^{-1}, except for one animal with pYR at 50 mg · kg^{-1}. Toxicity level II, involving crouching and cuddling, was observed in a few mice injected with doses of pYR or neomycin sulfate above 50 mg · kg^{-1}. No deaths were observed for any of the treated mice, and most mice recovered 2 h after treatment.

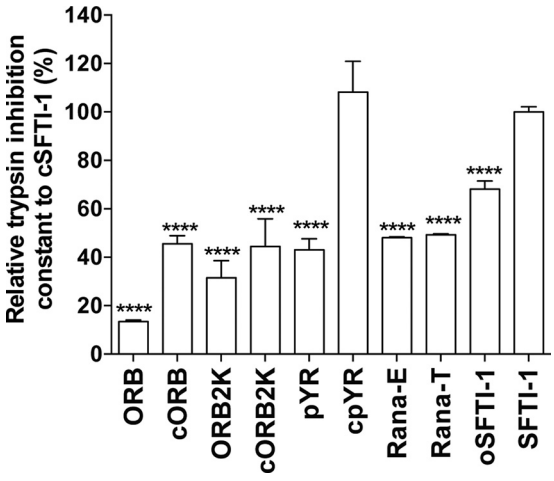

FIG 3 Relative trypsin inhibitory activities of SLF peptides, compared with SFTI-1. SFTI-1 has an equilibrium dissociation constant K_i of 17 pM (29). The statistical significance was calculated with respect to SFTI-1 by one-way analysis of variance (ANOVA) and the *post hoc* Bonferroni test. ****, $P < 0.0001$.

***In vivo* antimicrobial activity.** The therapeutic efficacy of the anuran defense peptides against *S. aureus* was studied in a murine wound infection model (Fig. 5). The wound was infected with 2×10^9 CFU of *S. aureus* ATCC 29213 at the start of therapy, and changes in bacterial loads were evaluated after 7 days of daily antimicrobial therapy with antimicrobial peptides. The negative-control group of mice (untreated group) was given PBS instead of antimicrobial agent, whereas the positive-control group was treated with neomycin sulfate antibiotic at 10 mg · kg^{-1} (antibiotic-treated group). Neomycin sulfate is an antibiotic from the aminogylcoside family that is used in topical ointments, and this has been used to study *S. aureus* infections (47, 48). The bacterial recoveries for the antibiotic-treated group and the untreated group were found to be 4.1 ± 1.0 and 10.9 ± 0.3 $\log_{10}$CFU/g of tissue, respectively.

The antimicrobial peptides were administered daily at the site of infection at doses of 1 or 3 mg · kg^{-1}. The bacterial recovery for

TABLE 3 *In vitro* efficacy of SLF peptides against *Staphylococcus aureus* strain ATCC 29213

Peptide	MIC (μM)
ORB	>100
cORB	>100
ORB2k	>100
cORB2k	>100
pYR	50
cpYR	75
Rana-E	100
Rana-T	100
oSFTI-1	>100
SFTI-1	>100
Gentamicin	48

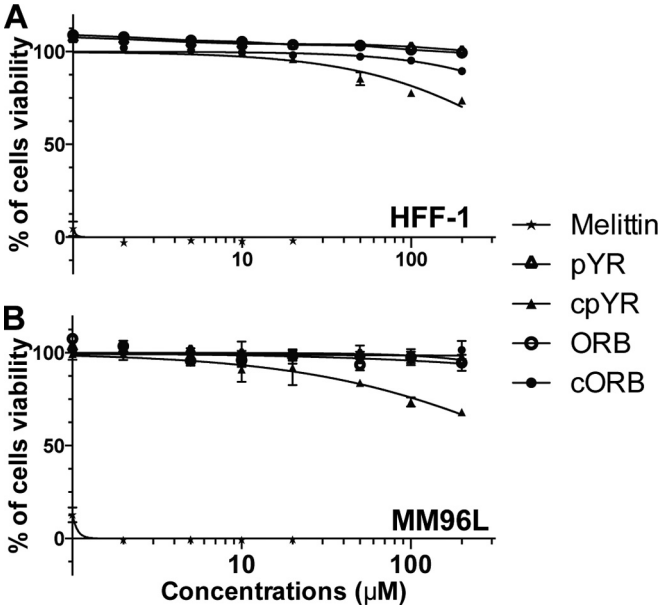

FIG 4 Cytotoxic activity of selected ORB and pYR peptides against noncancerous (HFF-1) (A) and cancerous (MM96L) (B) cells. Both open and cyclic forms of the peptides were tested, and melittin was used as a positive control (0% viability corresponds to 100% cell death).

pYR at 3 mg · kg^{-1} was found to be 4.3 ± 0.7 $\log_{10}$CFU/g of tissue, which was equivalent to the antibiotic control (20 mg · kg^{-1}). Lowering the dose of pYR by one-third increased the bacterial recovery 1.5-fold. The other open-form anuran peptides significantly reduced the numbers of $\log_{10}$CFU/g of tissue, compared to the untreated group of mice; however, they were less efficient than pYR.

Backbone cyclization of the peptides resulted in a decrease or loss of antimicrobial activity. The cyclic analogue (cpYR) of the most active open peptide (pYR) completely lost therapeutic efficacy even at 3 mg · kg^{-1}. cORB and cORB2K showed reduced bacterial clearance in wounds by 1 to 1.5 $\log_{10}$ CFU/g of tissue, compared to their respective open forms.

Serum stability. The open and cyclic forms of pYR and SFTI-1 were incubated in human serum (Fig. 6), and the cyclic peptides were found to have improved stability over the open forms over 24 h. SFTI-1 did not degrade at all, whereas 57.2 ± 0.9% of cpYR degraded after 24 h. oSFTI-1 was more stable than pYR.

DISCUSSION

Antimicrobial peptides are found in both animal and plant species and are used in host defense mechanisms. There is growing interest in exploring the potential of antimicrobial peptides as antibiotics to combat the decreased efficacy of conventional small-molecule antibiotics (49, 50). Some antimicrobial peptides kill microbes directly, while others are effective by modulating the innate immune system (51). The ability of antimicrobial peptides to neutralize endotoxemia/sepsis and to stimulate host innate responses while dampening potentially deleterious inflammatory responses offers an added advantage over small-molecule antibiotics (17). In this study, five antimicrobial peptides of anuran origin, containing a reactive site loop of BBIs, were evaluated for their therapeutic efficacy to treat *S. aureus*-infected wounds in mice. Three active peptides were backbone cyclized in an attempt to improve their biological properties.

Several SLF peptides have been reported to have *in vitro* antimicrobial activity against a range of microbes, but only ORB and ORB2K have been tested against *S. aureus* ATCC 25923 (21–23). In light of these observations, we evaluated the *in vitro* antimicrobial activities of five SLF peptides against *S. aureus* strain ATCC 29213, a strain isolated from wounds (Table 3). ORB and ORB2K were found to have much greater inhibitory activities than reported (21). Variations in the antimicrobial susceptibility testing methods have been found to have significant effects on the MICs of compounds, as demonstrated using the cysteine-rich antimicrobial peptide protegrin-1, for example (52). Differences are also dependent on the characteristics of the antimicrobial agents, including hydrophobic moments, exposed charges, amphipathicity, and peptide flexibility (53, 54). pYR was found to be the most active SLF peptide, while others had moderate-to-poor antimicrobial activity. Loop 2 of these SLF peptides has significant sequence diversity, compared to loop 1, and this diversity in the length and number of hydrophobic and cationic residues might be responsible for the observed variations in antimicrobial activity.

The high sequence similarity of the anuran peptides to SFTI-1, a stable and naturally cyclic peptide, guided this study toward the development of cyclic antimicrobial peptides that could potentially have improved stability and biological activities. Therefore,

TABLE 4 Evaluation of acute toxicity in mice treated with pYR, cpYR, or neomycin sulfate (positive control)

Dose (mg · kg^{-1}) and effect[a]	No. of mice (n = 5/group)		
	Neomycin sulfate	pYR	cpYR
0			
No effect	5	5	5
Toxicity level I	0	0	0
Toxicity level II	0	0	0
5			
No effect	5	5	5
Toxicity level I	0	0	0
Toxicity level II	0	0	0
10			
No effect	5	5	5
Toxicity level I	0	0	0
Toxicity level II	0	0	0
25			
No effect	3	4	5
Toxicity level I	2	1	0
Toxicity level II	0	0	0
50			
No effect	2	2	4
Toxicity level I	0	2	1
Toxicity level II	3	1	0
100			
No effect	2	1	3
Toxicity level I	0	2	2
Toxicity level II	3	2	0

[a] Toxicity grading was as follows: level I, narrowing of eyes; level II, crouching and cuddling.

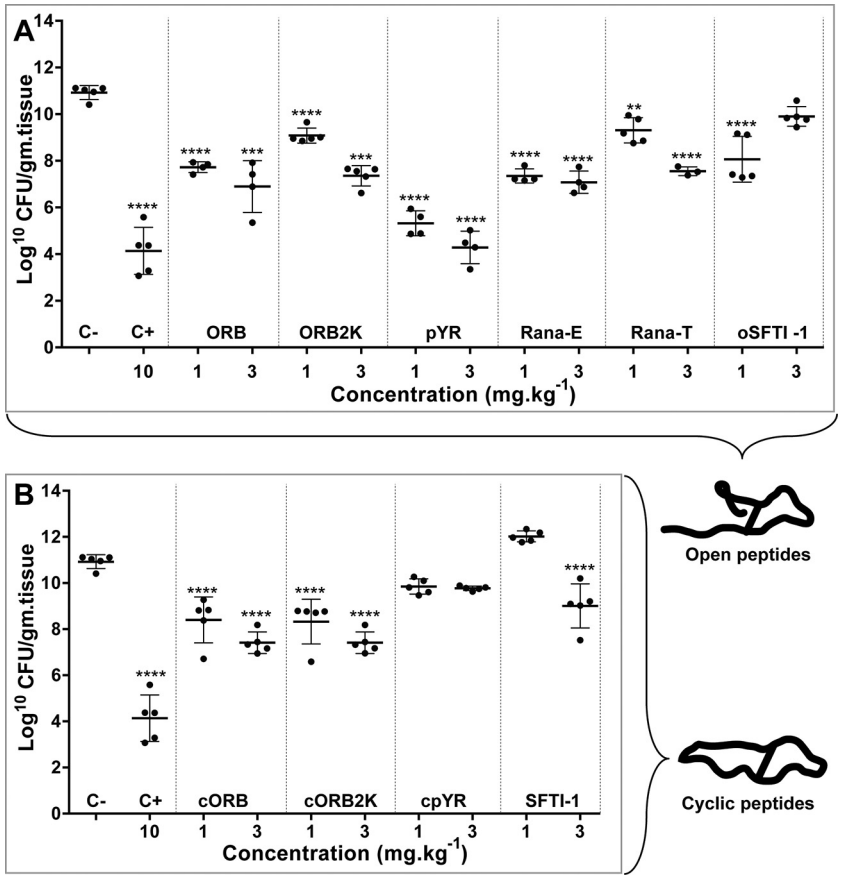

FIG 5 *In vivo* efficacy of SLF peptides against *S. aureus* in the murine thigh infection model. Both open (A) and cyclic (B) forms were tested at 1 and 3 mg · kg^{-1} (5 animals tested per compound). PBS and neomycin sulfate (3 mg · kg^{-1}) were used as negative (C−) and positive (C+) controls, respectively. The statistical significance was calculated with respect to the untreated group (C−) by one-way ANOVA and the *post hoc* Bonferroni test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

pYR, ORB, and ORB2K were cyclized using a method previously used for SFTI-1 (29, 32). Cyclization of a peptide backbone has been used previously to effectively improve *in vitro* stability, but the proximity between the N and C termini has a significant influence on the ease of cyclization (55, 56). Apart from contributing to structural stability, head-to-tail cyclization provides useful insights into the significance of the C and N termini of peptides in biological activity and folding (57). The structures of pYR, ORB, and ORB2K were found to be well suited for cyclization, as the overall folding was maintained following cyclization. Cyclization of pYR decreased the *in vitro* antimicrobial activity but improved the stability of the peptide in serum. The decrease in antimicrobial activity might be related to the loss of flexibility in residues near the termini and the positive charge at the N terminus. Since the antimicrobial activities of ORB, ORB2K, and SFTI-1 were above the upper limit of the *in vitro* assay, the impact of cyclization on the stability of these peptides was not examined.

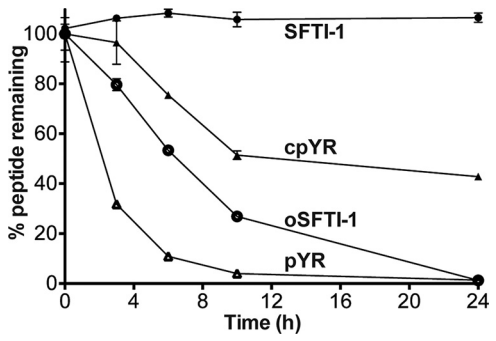

FIG 6 Serum stability of open and cyclic forms of pYR and SFTI-1. Backbone-cyclized pYR and SFTI-1 had better stability in human serum than did their open forms.

The specificity of the peptides for bacterial cells was examined by analysis of their hemolytic activities and their cytotoxicities for mammalian cells. None of the peptides was found to exhibit hemolytic activity. Open and cyclic forms of pYR and ORB were not cytotoxic to human foreskin fibroblast or melanoma cells. The lack of hemolytic and cytotoxicity activity with human cells indicated that the SLF peptides are selective for bacterial cells. pYR, cpYR, and neomycin sulfate did not cause acute toxicity at clinically used doses (10 mg · kg^{-1}), while mild toxicity at doses of 25, 50, or 100 mg · kg^{-1} was seen in a few animals from each group. Toxic effects were resolved after 2 h in most cases.

We evaluated the *in vivo* efficacy of the SLF peptides for the treatment of *S. aureus* skin infections in the widely used C57BL mouse model. The relationship between the *in vitro* efficacy and the *in vivo* efficacy of antimicrobial agents remains unclear, and

prediction of *in vivo* activity is very challenging, due to variations in potency that can occur due to many uncontrolled parameters in the host systems (58, 59). Despite the challenges of using *in vitro* potency to predict i*n vivo* potency, we found that the most potent peptide in the *in vitro* study, pYR, was also the most potent peptide in treating *S. aureus* skin infections in mice. pYR (at 3 mg · kg^{-1}) reduced bacterial loads in the wounds similarly to the positive-control compound neomycin sulfate (at 10 mg · kg^{-1}), a broad-spectrum antibiotic that is used in topical ointments and is most effective against *S. aureus* (48).

In conclusion, this study has investigated a class of β-sheet multifunctional antimicrobial peptides that are effective in treating *S. aureus* skin infections in a murine model. Although cyclization was shown previously to enhance stability and bioactivity (28, 60), in this case an improvement in stability was observed but bioactivity was reduced. Structural or charge distribution changes upon cyclization might be responsible for the decreased antimicrobial activity. The choice of peptide for further development (open versus cyclic) reflects a balance between stability and bioactivity. Further studies are needed to investigate the mechanisms of action of these peptides, to fully explore their potential for clinical use.

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