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Antibacterial activities of temporin A analogs

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Abstract Temporin A (TA) is a small, basic, highly hydrophobic, antimicrobial peptide amide (FLPLIGRVLSGIL-NH_2) found in the skin of the European red frog, *Rana temporaria*. It has variable antibiotic activities against a broad spectrum of microorganisms, including clinically important methicillin-sensitive and -resistant *Staphylococcus aureus* as well as vancomycin-resistant *Enterococcus faecium* strains. In this investigation the antimicrobial activity and structural characteristics of TA synthetic analogs were studied. For antibacterial activity against *S. aureus* and enterococcal strains, the hydrophobicity of the N-terminal amino acid of TA was found to be important as well as a positive charge at amino acid position 7, and bulky hydrophobic side chains at positions 5 and 12. Replacing isoleucine with leucine at amino acid positions 5 and 12 resulted in the greatest enhancement of antibacterial activity. In addition, there was little difference between the activities of TA and its all-D enantiomer, indicating that the peptide probably exerts its effect on bacteria via non-chiral interactions with membrane lipids. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Temporin A; Antibiotic peptide; D amino acid; *Staphylococcus aureus*; *Enterococcus faecalis*; *Enterococcus faecium*

1. Introduction

Temporin A (TA) is a small, basic, highly hydrophobic, antimicrobial peptide found in the skin of the European red frog, *Rana temporaria* [1]. It is one of a group of 21 peptides, collectively known as the temporins, that are among the smallest antibacterial peptides to have been isolated from animal sources [1–5]. They are active against both Gram-positive and Gram-negative bacteria and most are non-toxic to human red blood cells at the concentrations required to kill bacteria.

TA has been synthesized previously by solid phase methods [1,6]. We showed that the purified synthetic peptide had antibacterial activity against clinically important Gram-positive bacteria, with moderately good activity against both methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus* (MSSA and MRSA, respectively), but a weaker activity against vancomycin-resistant strains of *Enterococcus faecium* (VRE).

Previous work has shown that it is possible to improve the activities, or reduce the toxicities, of naturally occurring peptide antibiotics by producing synthetic analogs with modified primary and/or secondary structures [7]. Such research usually also leads to a better understanding of the structural features of a peptide that are important for its antibiotic and/or toxic properties. This work was primarily aimed to improve the activity of TA against clinically important bacterial strains by synthetic modifications of its primary structure and, secondarily, to obtain some information about important structural features of the peptide. The types of modifications included: (1) synthesis of enantiomeric TA (i.e. TA containing all-D amino acids) to produce an antibiotic that would resist enzymatic proteolysis, (2) substitution of Ile with Ala or Leu in order to retain the hydrophobic character of Ile positions within the TA while modifying the amino acid side chain size and chirality (the side chain of Ile contains a chiral carbon, so that L-Ile in TA has two isomeric forms), (3) substitution of Arg with Lys at the amino acid position 7 in TA (i.e. conversion of TA to temporin F (TF) [1]) to examine the effect of minor modifications in the side chain structure and positive character of this position, (4) substitution of N-terminal Phe with Lys, in both the all-L and all-D enantiomers of TA, to examine the effect of reducing the amphipathicity of the peptide and increasing its basicity (from +2 to +3) and (5) to study the structural features of TA with circular dichroism (CD) spectroscopy and nuclear magnetic resonance (NMR).

2. Materials and methods

2.1. Peptide synthesis, purification and characterization

Analogs were designed based on the known amphipathic α-helical structure of TA (Fig. 1) [5]. Peptides were synthesized as C-terminal amides by solid phase methods using Fmoc protected amino acids, purified by preparative reverse phase-high performance liquid chromatography (RP-HPLC), and analyzed for purity by analytical RP-HPLC, for composition by amino acid analysis (AAA) and for mass by electrospray ionization mass spectrometry (ESI-MS), as previously described [6,8]. Molecular weights were calculated using the 'Compute pI/MwTool' program [9] and molecular models were generated with the Swiss PDB Viewer program [10].

2.2. Bacterial strains and media

MSSA strain K52 and MRSA strain K35, as well as the VRE strains 18578 and 18172, which carried the *van*A and *van*B genes, respectively, were clinical isolates originating from the Laboratory of Bacteriology, Section of Clinical Microbiology, HUCH Laboratory Diagnostics, Helsinki University Central Hospital [6]. Additionally, *S. aureus* strain ATCC 25923 and *Enterococcus faecalis* strain ATCC

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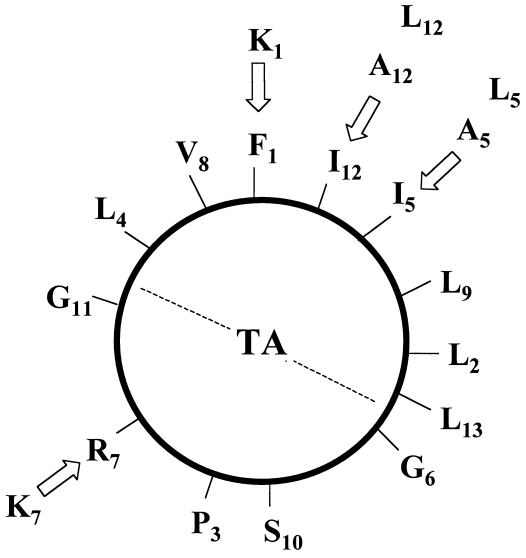

Fig. 1. Helical wheel diagram for TA, showing the relative positions of constituent amino acids when the peptide is in a helical conformation, and the positions of amino acid replacements described in the text. The numbers of the amino acid positions, 1 through 13, increase around the circumference of the circle in a clockwise direction. For DTA, the numbers would increase in a counterclockwise direction. The dotted line indicates that the helix surface can be divided into hydrophobic and hydrophilic halves, demonstrating the amphipathic nature of the peptide.

29212 were used for testing. For the experiments, bacterial strains were maintained on chocolate agar plates by subculturing every 3–4 weeks.

2.3. Determination of minimal inhibitory concentration (MIC) and bacterial killing

MIC values and bacterial killing were determined as described [6]. Briefly, bacterial suspensions corresponding to a McFarland value of 1.0 were diluted 1:1000 in 2-fold concentrated Mueller–Hinton broth containing Ca^{2+}, and an aliquot (25 μl) was mixed with an aliquot (25 μl) of a 2-fold dilution of TA analogs in deionized H_2O. The MIC was defined as the lowest concentration of the analog inhibiting visible growth after incubation at 37°C for 20 h.

Bacterial killing was determined by cultivating 10 μl of a suitable dilution, made from microdilution wells, on Mueller–Hinton agar plates and by counting colony forming units after an overnight incubation at 37°C. For colony counting only plates having 20–500 bacterial colonies per plate were used.

2.4. CD and NMR spectroscopy

The CD measurements were carried out with a Jasco 720 spectropolarimeter using a 1 mm quartz cell at 25°C. The peptide samples (0.1 mM) were prepared in H_2O–trifluoroethanol (TFE) solutions (0–80 vol% TFE). For each sample five spectra were recorded at the scan rate of 20 nm/min and averaged.

NMR measurements were carried out both in H_2O and D_3-TFE/H_2O (3:7, v/v). Samples of freeze-dried peptides were dissolved to obtain 1 mM solutions. The pH was adjusted to 6.0 with a few μl of dilute HCl (pH not corrected for the deuteron or solvent effects). The samples were prepared to 300 μl in a Shigemi microcell. The NMR spectra were acquired with a 600 MHz Varian Unity NMR spectrometer. Homonuclear two-dimensional spectra, correlation spectroscopy, relayed correlation spectroscopy, total correlation spectroscopy (with mixing times of 30, 55 and 180 ms) and nuclear Overhauser enhancement (with mixing times of 50, 100, 200 and 400 ms), were collected at 20°C. The data were processed with the Felix 98 software.

3. Results

3.1. Peptide synthesis, purification and characterization

The peptides were pure, as shown by analytical RP-HPLC, and had the expected amino acid compositions and molecular weights, as shown by AAA and ESI-MS (Table 1).

3.2. Bacteriological assays

Changing the chirality of TA, from all-L to all-D (DTA), produced MIC values that were similar to those of native TA, except for *E. faecalis*, where there was a 2-fold improvement in activity (Table 2). MIC values for TA and DTA varied between 2.6 μM and 5.2 μM for staphylococci, and between 10.5 μM and 20.9 μM with enterococci. Interestingly, substitution of Ile in TA with Leu (L512TA) produced a peptide that had the same or improved potency as TA against staphylococci, except for the ATCC strain, and 2-fold improved potency against all enterococcal strains. Conversion of TA to TF (K7TA) reduced potency by 2-fold or less against most test strains, except *S. aureus* K52 where there was a slight improvement in activity, and *E. faecalis* which was equally sensitive to both peptides. MIC values were 4.4–5.2 μM for staphylococci and 17.5–20.9 μM for enterococci. The antibacterial effect of TA was strongly reduced when the amino-terminal Phe of TA or DTA was replaced by L- or D-Lys, respectively. MIC values were elevated an average of 19-fold for staphylococci and at least 10-fold for enterococci. Replacement of Ile with Ala yielded a peptide without any antibacterial activity (Table 2).

Killing curves for each bacterial strain tested were in good

Table 1
Amino acid sequences, net charges and masses of TA and synthetic analogs at pH 7

Peptide[a]	Amino acid sequence[b]	NC	Mass	
			Calculated[c]	Found[d]
TA	FLPLIGRVLSGIL-NH_2	+2	1396	1396.4
DTA	(same as TA, except all-D amino acids)	+2	1396	1396.4
K1TA	**K**LPLIGRVLSGIL-NH_2	+3	1377	1377.4
DK1TA	(same as K1TA, except all-D amino acids)	+3	1377	1377.4
K7TA	FLPLIG**K**VLSGIL-NH_2	+2	1368	1368.2
A512TA	FLPL**A**GRVLSG**A**L-NH_2	+2	1312	1311.8
L512TA	FLPL**L**GRVLSG**L**L-NH_2	+2	1396	1395.6

NC, net charge.
[a]TA (i.e. Ile-5,12 TA); DTA, all-D TA; K1TA, Lys-1 TA; DK1TA, all-D K1TA; K7TA, Lys-7 TA (i.e. TF [1]); A512TA, Ala-5,12 TA; L512TA, Leu-5,12 TA.
[b]All peptides are amidated at their C-termini, and, unless otherwise indicated, all amino acids are of the L configuration. Single and double amino acids replacements are in bold and underlined.
[c]Mass of amidated peptide calculated by subtracting 2 from value calculated for free carboxyl-terminal peptide.
[d]Mass from ESI-MS [$(M+2H^+$ ion$\times 2)-2$].

Table 2
MIC[a] values for TA and analogs

Bacterial strain	Peptide						
	TA	DTA	K1TA	DK1TA	K7TA	A512TA	L512TA
S. aureus							
K52[b]	5.2	3.1	58.2	49.5	4.4	>99.8	2.8
K35[c]	2.6	3.1	58.2	49.5	4.4	>99.8	2.8
ATCC 25923	2.6	2.6	83.7	41.9	5.2	>167.5	5.2
E. faecalis							
ATCC 29212	20.9	10.5	>167.5	167.5	20.9	>167.5	10.5
E. faecium							
18578[d]	10.5	12.5	>116.3	99.0	17.5	>99.8	5.6
18172[d]	10.5	12.5	>116.3	49.5	17.5	>99.8	5.6

[a]Minimal inhibitory concentration (μM).
[b]Methicillin-sensitive.
[c]Methicillin-resistant.
[d]Vancomycin-resistant.

agreement with MIC values and revealed that DTA was practically as effective in killing staphylococci and enterococci as the native TA (data not shown). Substitution of Arg with Lys at position 7 did not change the killing activity of TA against staphylococcal strains whereas the activity against VRE strains seemed to be slightly weakened. In agreement with MIC values, replacement of Ile at positions 5 and 12 by Leu produced a peptide with slightly enhanced bacterial activity against both staphylococci and enterococci. In contrast, substitution of Ala for the same positions abolished totally the killing activity at the concentrations used in the assays. Replacement of the amino-terminal Phe by Lys, in TA or its D-form, reduced activity against staphylococci and enterococci roughly 10-fold. Additionally, the killing curves indicated that the bacterial populations were very homogeneous and that there was no heterogenic resistance against TA analogs among these strains (data not shown).

3.3. Determination of peptide conformation by circular dichroism and NMR

CD spectroscopy was used to determine the conformation of TA in solution conditions. In H_2O, TA gave spectra with a minimum close to 200 nm and an overall shape suggesting a random coil conformation, as shown for the wild type in Fig. 2. A gradual change from a random coil to an α-helical conformation was observed when increasing amounts of TFE were added to the TA peptide in H_2O. The maximal effect was obtained already with 30% TFE.

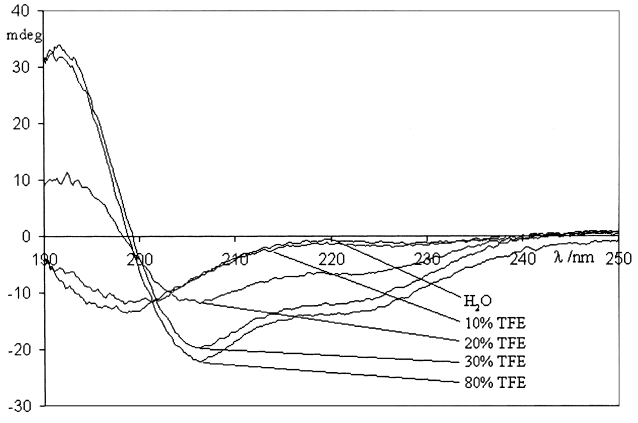

Fig. 2. CD spectra of TA in H_2O and TFE. In water, TA has a random coil structure, but adopts a mainly α-helical structure in TFE, the maximal effect is obtained already in 30% TFE solution.

NMR spectroscopy was used to further assess the structure of the TA peptide. In water, TA was largely in a random coil conformation as indicated by the typical crowding of the NH resonances due to fast exchange with water [11]. The chemical shifts of the alpha protons (chemical shift index, CSI) are sensitive for the secondary elements in proteins [12]. According to CSI, the TA peptide adopts α-helical structure in

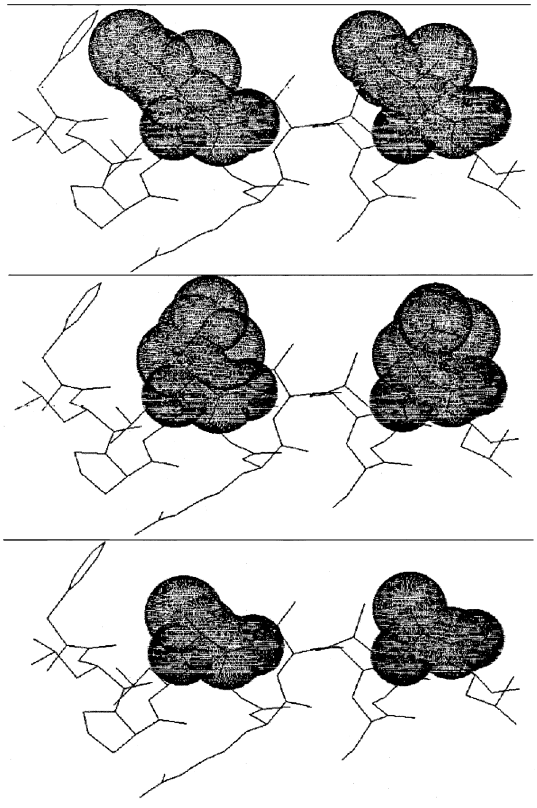

Fig. 3. Molecular models of TA (top), L512TA (middle), and A512TA (bottom), showing the carbon skeletons of each peptide in the same orientation, and the van der Waals surfaces of Ile, Leu and Ala. The N- and C-termini of the peptide are located on the left and right sides of the figure, respectively. Each peptide was modeled as a complete α-helix using the Swiss PDB Viewer program [10].

TFE/water (3:7, v/v) solutions (data not shown). These findings agree well with the CD data (see above).

4. Discussion

In the present study we demonstrated that it is possible to improve the antibiotic activity of TA. By modifying a single amino acid residue (Ile → Leu) at two positions (5 and 12) in the peptide a 1.9-fold enhancement in antibacterial activity was obtained with *E. faecium* strains, and with one of *S. aureus* strains. Additional studies will be necessary to determine if the improvement in activity requires replacements at both positions, or if replacement at only one position is sufficient. Replacement of Leu with Ile or Ala showed that a bulky, hydrophobic side chain is required at positions 5 and(/or) 12 (see Fig. 3). There are small, but apparently significant, differences between the physical properties of the side chains of Ile and Leu, and larger ones between Ile and Ala. The solvent accessible surface areas of Ile, Leu, and Ala side chains are 175, 170, and 115 $Å^2$, respectively [13], and the relative hydrophobicities are 2.5, 1.8 and 0.5 kcal/mol, respectively, relative to a value of 0 kcal/mol for Gly [14]. The results obtained for the Ile to Ala replacement can be compared to those obtained in experiments with a two-stranded, 70-residue (2×35), α-helical, coiled-coil peptide in which it was shown that the replacement of eight Leu residues, by Ala, destabilized the coiled-coil conformation [15]. The dimer was stabilized by hydrophobic interactions between nine Leu residues in each of its two chains, and their partial replacement reduced the ability of the two chains to pack into an optimal coiled-coil conformation. By analogy, the Ile to Ala replacements in TA may reduce the optimal packing of peptides in an aggregate structure, such as an ion channel. It is also interesting to consider that the side chain of Ile has a chiral center, so that there would be four different isomers of TA, and these isomers may not have equivalent activities.

The TFE solutions mimic quite well hydrophobic conditions. It is known, however, that TFE may introduce α-helical structures in proteins. According to the results of CD and NMR spectroscopy, TA is mainly random coil in water solutions, showing no aggregation behavior. However, even in relatively small TFE concentrations, TA adopts a mainly α-helical structure, which is in agreement with the postulated membrane interaction.

Conversion of the N-terminal Phe of TA to Lys resulted in drastic reduction of antibacterial activity both against *S. aureus* and enterococcal test strains as measured by MIC and killing activity determinations. Most studies of antibiotic peptides from animal sources indicate that amphipathicity of the peptide is important for antibacterial activities [16]. Fig. 1 illustrates the orientation of amino acids in TA when it is in a helical conformation, and it is obvious that the substitution of Lys for Phe at position 1 of TA, when it is converted to K1TA will disrupt the amphipathic character of the peptide, as this substitution will place a charged, hydrophilic amino acid side chain on a side of the helix in which the surface is completely hydrophobic.

In regard to the ion channel hypothesis for the mechanism of action of antibiotic peptides, temporins have all of the features required to satisfy this model except that they are not long enough to span a bilayer membrane as α-helices, a property requiring a length of 20 amino acids for a 30 Å thick membrane [17]. However, it would be possible for the temporins to span a bilayer if two molecules were oriented end-to-end, as has been suggested for ion channels composed of the 15-residue peptide antibiotic, gramicidin [18].

This is the first report of the synthesis of TF (or K7TA), which had a slightly reduced antibacterial activity relative to TA against test organisms. TF and TA are equally hydrophobic by RP-HPLC [1], so the slight reduction in activity accompanying the Arg to Lys replacement may be due to the difference in p*K* values of the side chains of Arg (∼12.5) and Lys (∼10.8) resulting in about a 50-fold reduction in the amount of cationic peptide in solution.

The fact that enantiomeric TA (DTA) was as active as native TA indicates that the mechanism of action of TA on bacteria is not chiral, such as a receptor–ligand interaction. It may be a simple hydrophobic interaction with membrane lipids [19]. Also, since DTA would be less susceptible to proteolysis than TA, it could be a useful replacement for TA in situations where proteolysis is a problem. Based on the results obtained for L512TA and both all-D enantiomers (DTA and DK1TA), it would be reasonable to anticipate that an all-D enantiomer of L512TA would also be at least as active as the all-L isomer, and, in addition, it would be less expensive to produce due to the difference in the cost of D-Ile versus D-Leu.

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