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Alanine scanning analysis and structure–function relationships of the frog-skin antimicrobial peptide temporin-1Ta‡

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The increasing resistance of bacteria and fungi to the available antibiotic/antimycotic drugs urges for a search for new anti-infective compounds with new modes of action. In line of this, natural CAMPs represent promising and attractive candidates. Special attention has been devoted to frog-skin temporins, because of their short size (10–14 residues long) and their unique features. In particular, temporin-1Ta has the following properties: (i) it is mainly active on Gram-positive bacteria; (ii) it can synergize, when combined with temporin-1Tl, in inhibiting both gram-negative bacterial growth and the toxic effect of LPS; (iii) it preserves biological activity in the presence of serum; and (iv) it is practically not hemolytic. Rational design of CAMPs represents a straightforward approach to obtain a peptide with a better therapeutic index. Here, we used alanine scanning analogs to elucidate the contribution of the side chains of each amino acid residue to the peptide's antimicrobial and hemolytic activity. Beside providing insight into the biophysical attributes and the critical positions within the peptide sequence, which govern the antimicrobial/hemolytic activity of this temporin isoform, our studies assist in optimizing the design of temporin-based lead structures for the production of new anti-infective agents. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: temporin; alanine scanning; antimicrobial peptides; frog skin

Introduction

Because of the increasing frequency of bacterial and fungal strains resistant to existing drugs, the identification of alternative antibiotics with a new mode of action to slow down the alarming trend of resistance is vital. Primitive innate defence mechanisms in the form of gene-encoded CAMPs [1–3], originally discovered in insects [4,5], are now considered well-suitable candidates for the design and development of new therapeutics [6–9]. These relatively low-molecular weight peptides were later found to be present in all living organisms, sometimes in large quantities freely circulating or sequestered in compartments throughout the organism [10,11]. However, beside being crucial components of the host innate immunity, CAMPs also mediate the adaptive immune response, by causing mast-cell degranulation, by acting as chemo-attractants [1,12] or by blocking the LPS-induced cytokine production and subsequent septic shock syndrome [13–15]. In contrast to conventional antibiotics, most of which work by interfering with a specific biochemical reaction within the cell, numerous CAMPs are believed to perturb the microbial membrane in a non-receptor-mediated fashion. Despite substantial variation in their chain length and structural conformation, most CAMPs do possess: (i) a size ranging from 11 to 50 amino acids; (ii) a net positive charge at neutral pH; and (iii) an amphiphatic structure upon contact with the membrane of the target cell [16–18]. The search for novel CAMPs as lead structures of natural origin has led to the discovery of interesting peptides from the skin glands of amphibian anura. Among them, a marked attention has been devoted to the short and mildly cationic temporins. Initially isolated from the skin of the European red frog *Rana temporaria*

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Abbreviations used: *CAMP, cationic antimicrobial peptide; CFU, colony-forming units; CH_3CN, acetonitrile; DIEA, N,N-diisopropylethyl-amine; DPC, dodecylphosphocholine; Et_3SiH, triethylsilane; HBTU, 2-(1 H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate; LC-MS, liquid-mass spectroscopy; LPS, lipopolysaccharide; MHB, Mueller-Hinton broth; RP-HPLC, reversed-phase high performance liquid chromatography.*

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[19], new members were then identified in skin secretions of other ranid frogs of both American and Eurasian origin, enlarging the temporin family to more than 100 different isoforms [20]. Temporins are among the smallest CAMPs (10–14 residues long, except for temporin-SHf and temporin-LTa bearing 8 and 17 amino acids, respectively [21,22]), and with a net charge ranging from +2 to +3. Indeed, with the exclusion of temporin-1Ja, carrying an aspartic acid (net charge 0), and a few isoforms devoid of basic or acidic amino acids (net charge +1, due to the free N-terminal amino group), all the remaining members have only a single or a double positively charged residue in their sequence [20]. This low cationic character is unique and is an exception compared to known CAMPs from other sources [23,24]. In addition, unlike the majority of Ranidae CAMPs, such as brevinins, ranalexins, ranatuerins, and esculentins [25,26], temporins lack the C-terminal heptapeptide ring stabilized by a disulfide bridge and are amidated at their carboxyl end as a result of a post-translational enzymatic reaction [27]. Generally, temporins are active particularly against Gram-positive bacteria, Candida species, and fungi, including antibiotic-resistant strains [28,29]. A large number of studies has revealed that their bacterial killing process is rapid and concomitant with the perturbation of the microbial membrane [20,30]. Furthermore, recent papers have shown the existence of a synergistic effect in two pairs of temporins (temporin-1Ta + temporin-1Tl and temporin-1Tb + temporin-1Tl) in inhibiting both the Gram-negative bacterial growth and the toxic effect of the outer membrane component LPS [31,32]. Interestingly, LPS was found to be the key molecule underlying the molecular mechanism of both types of synergism [33], and this finding represents the first case reported so far in the literature. While temporin-1Tl is highly active on a wide range of microorganisms, but with a lytic effect on red blood cells, temporin-1Ta is mainly active on Gram-positive bacteria and is not hemolytic. This might be linked to the lower α-helical content of temporin-1Ta than Tl, and, as a result, to its inability to deeply insert into the hydrophobic core of zwitterionic membranes, such as those of eukaryotic cells [34]. Importantly, rational design of CAMPs represents an attractive approach to the improvement of their antimicrobial properties.

Here, to address the influence of each amino acid residue of temporin-1Ta on the selective activity of this peptide, an Ala-scanning analysis was carried out. Besides getting insight into the structure–function relationships of this temporin isoform, the results of our studies provide important information for the optimization of temporin-based anti-infective agents and the design of temporin analogs to be employed in biophysical studies for a better understanding of their membrane-active properties.

Materials and Methods

Peptide Synthesis

N^{α}-Fmoc-protected amino acids, HBTU, HOBt, and Rink amide resin were purchased from GL Biochem Ltd (Shanghai, China). Peptide synthesis solvents, reagents, as well as CH_3CN for HPLC were reagent grade and were acquired from commercial sources and used without further purification unless otherwise noted. The syntheses of temporin-1Ta analogs were performed in a stepwise manner via the solid-phase method [35]. For example, for the synthesis of Ta, N^{α}-Fmoc-Leu-OH was coupled to Rink amide resin (0.5 g, 0.7 mmol NH_2/g). The following protected amino acids were then added stepwise: N^{α}-Fmoc-Ile-OH, N^{α}-Fmoc-Gly-OH, N^{α}-Fmoc-Ser-OH, N^{α}-Fmoc-Leu-OH, N^{α}-Fmoc-Val-OH, N^{α}-Fmoc-Arg(Pbf)-OH, N^{α}-Fmoc-Gly-OH, N^{α}-Fmoc-Ile-OH, N^{α}-Fmoc-Leu-OH, N^{α}-Fmoc-Pro-OH, N^{α}-Fmoc-Leu-OH, and N^{α}-Fmoc-Phe-OH. Each coupling reaction was accomplished using a threefold excess of amino acid with HBTU and HOBt in the presence of DIEA. The N^{α}-Fmoc protecting groups were removed by treating the protected peptide resin with a 25% solution of piperidine in DMF (1 × 5 min and 1 × 20 min). The peptide resin was washed three times with DMF and the next coupling step was initiated in a stepwise manner. All reactions were performed under an Ar atmosphere. The peptide resin was washed with DCM (3×), DMF (3×) and DCM (4×), and the deprotection protocol was repeated after each coupling step. The N-terminal Fmoc group was removed as described above and the peptide was released from the resin with TFA/Et_3SiH/H_2O (90 : 5:5) for 3 h. The resin was removed by filtration and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether to give a white powder which was purified by RP-HPLC on a semi-preparative C18-bonded silica column (Vydac 218TP1010, 1.0 × 25 cm) using a gradient of CH_3CN in 0.1% aqueous TFA (from 10 to 90% in 30 min) at a flow rate of 1.0 ml/min. The product was obtained by lyophilization of the appropriate fractions after removal of the CH_3CN by rotary evaporation. Analytical RP-HPLC indicated a purity >98% and molecular weights were confirmed by LC-MS (6110 Quadrupole, Agilent Technologies) (Table 1).

Table 1. Analytical data for temporin-1Ta and its analogs

Peptide	HPLC[a] k'	MS (M+H) Found[b]	MS (M+H) Calculated
1Ta	6.86	1397.13	1396.79
Ta A1	7.87	1321.90	1320.69
Ta A2	8.40	1355.70	1354.71
Ta A3	8.09	1370.51	1370.75
Ta A4	7.98	1355.54	1354.71
Ta A5	8.00	1355.81	1354.71
Ta A6	7.55	1411.10	1410.82
Ta A7	10.37	1312.62	1311.68
Ta A8	9.23	1368.69	1368.74
Ta A9	8.13	1355.18	1354.71
Ta A10	8.85	1381.80	1380.79
Ta A11	7.41	1411.30	1410.82
Ta A12	8.21	1354.62	1354.71
Ta A13	7.66	1354.36	1354.71

[a] k' [(peptide retention time – solvent retention time)/solvent retention time].
[b] Molecular masses were determined by LC-MS.

Microorganisms

The strains used for the antimicrobial assays were the following: the Gram-negative bacteria *Escherichia coli* ATCC 25922, *Yersinia pseudotuberculosis* YPIII; the Gram-positive bacteria *Bacillus megaterium* Bm11, *Staphylococcus aureus* Cowan I, *Staphylococcus capitis* 1, and the yeast *Candida albicans* ATCC 10231.

Antimicrobial Assay

Susceptibility testing was performed by adapting the microbroth dilution method outlined by the Clinical and Laboratory Standards Institute using sterile 96-well plates (Falcon, NJ, USA). Aliquots

(50 µl) of bacteria in mid-log phase at a concentration of 2×10^6 CFU/ml in culture medium (MHB) were added to 50 µl of MHB broth containing the peptide in serial twofold dilutions in 20% ethanol. The ranges of peptide dilutions used were 1.25–40 µM. The same procedure was followed with yeast strains, but using Winge medium [36]. Inhibition of microbial growth was determined by measuring the absorbance at 600 nm, after an incubation of 18–20 h at 37 °C (30 °C for yeasts), with a 450-Bio-Rad Microplate Reader. Antimicrobial activities were expressed as the MIC, the concentration of peptide at which 100% inhibition of microbial growth is observed after 18–20 h of incubation.

Hemolytic Assay

The hemolytic activity of the peptides was determined using fresh human erythrocytes from healthy donors. Blood was centrifuged and the erythrocytes were washed three times with 0.9% NaCl. Peptides dissolved in 20% ethanol were added to the erythrocyte suspension (5%, v : v), at a final concentration ranging from 1.25 to 40 µM in a final volume of 100 µl. Samples were incubated with agitation at 37 °C for 40 min. The release of hemoglobin was monitored by measuring the absorbance (Abs) of the supernatant at 540 nm. Control for zero hemolysis (blank) consisted of erythrocytes suspended in the presence of peptide solvent (20% ethanol at a final concentration of 0.6%). Hypotonically lysed erythrocytes were used as a standard for 100% hemolysis. The percentage of hemolysis was calculated using the following equation: % hemolysis $= [(Abs_{sample} - Abs_{blank})/(Abs_{total\ lysis} - Abs_{blank})] \times 100$.

Circolar Dicroism

All CD spectra were recorded using a JASCO J710 spectropolarimeter at 25 °C with a cell of 1 mm path length. The CD spectra were acquired by the range from 260 nm to 190 nm 1 nm bandwidth, four accumulations, and 100 nm/min scanning speed. The CD spectra of the peptides at a concentration of 100 µM were performed in water (pH = 7.4), in SDS (20 mM) and in DPC (20 mM) micellar solutions.

Results

Antibacterial Activity

In order to assess the role of each individual amino acid in the antimicrobial activity of temporin-1Ta, we performed an Ala scan study by systematically replacing single residues with the neutral amino acid Ala. The antimicrobial activity of these analogs was analyzed by the microbroth dilution method against two Gram-negative bacteria (*E. coli* ATCC 25922 and *Y. pseudotuberculosis* YPIII), three Gram-positive bacteria (*S. aureus* Cowan I, *B. megaterium* Bm11 and *S. capitis* 1), and the yeast *C. albicans* ATCC 10231. The results are shown in Table 2. The data pointed out that the following substitutions had marked impact on the antimicrobial activity of the natural peptide, exhibiting multiple MIC values (2-4-8-fold higher) than those of temporin-1Ta, against all microorganisms. These were: (i) the replacement of the N-terminal Phe (Ta A1); (ii) the substitution of the hydrophobic Leu in positions 2, 4, 9, and 13 (Ta A2, Ta A4, Ta A9, and Ta A13); (iii) the change of Ile in positions 5 and 12 (Ta A5 and Ta A12); (iv) the change of the positively charged Arg^7 (Ta A7), as well as (v) the replacement of the hydrophobic Val^8 (Ta A8). In contrast, substitution of Pro^3, Gly^6, Ser^{10} or Gly^{11} with Ala (Ta A3, Ta A6, Ta A10, Ta A11) preserved the antibacterial activity of the parent peptide on *Y. pseudotuberculosis, B. megaterium*, and *S. capitis*, and increased its activity against both the Gram-positive bacterium *S. aureus* and the yeast *C. albicans,* giving two to fourfold lower MICs (Table 2).

Hemolysis

Hemolytic activity was determined against human erythrocytes, as described in the Experimental section. The data highlighted a correlation between the antibacterial and the hemolytic activities. Indeed, the most active antimicrobial analogs (Ta A3, Ta A6, Ta A10, and Ta A11) also displayed the highest hemolytic activity at a peptide concentration range from 20 to 40 µM, while a comparable hemolysis caused by the parent peptide 1Ta was observed at the lower concentrations (Table 3). An exception was given by Ta A3 and Ta A11, whose lytic effect was found to be two to threefold more potent than that of 1Ta, also at 10 µM. Interestingly, a considerable enhancement of hemolytic activity was induced by the presence of Ala at the N-terminus of the natural temporin (Ta A1), as well as by the replacement of the single basic residue Arg^7 (Ta A7) (Table 3). Conversely, all the other analogs with Ala substitution in positions 2, 4, 5, 8, 9, 12, and 13 appeared to be almost devoid of toxic effect on human erythrocytes (Table 3).

Conformational Studies

The secondary structure of the analogs Ta A1-A13 was investigated using CD spectroscopy in water, SDS/water, and DPC/water solutions. CD spectra in water (pH 7.4) demonstrated the presence of disordered conformers for all compounds, with a minimum close to 198 nm (data not shown). In contrast, in SDS and DPC micelles solution, which were used to mimic the negatively charged (microbial) and the zwitterionic (eukaryotic) cell membranes, respectively, the shape of the CD spectra indicated that peptides adopted a defined secondary structure. In particular, the CD spectra displayed two minima around 209 and 222 nm, characteristic of α-helix structures (Figure 1). Helical contents were predicted from the CD spectra using the SOMCD method [38] (Table 2). Generally, helical contents observed in SDS and DPC micelle solutions were similar, with a certain reduction in SDS (about 5–10%) compared to DPC. The highest helical content was noted in Ta A3, Ta A6, and in Ta A11 (about 70% and 60% in DPC and SDS, respectively). In these peptides, the replacement of a proline (Ta A3) or a glycine residue (Ta A6 and Ta A11) clearly increased the helical content compared to that of the native peptide 1Ta (55% and 50% in DPC and SDS, respectively). An increase of the α-helical content with respect to 1Ta was also observed in Ta A7. In contrast, replacement of the aliphatic residues $Leu^{2,4,9}$, Ile^5, and Val^8 reduced the helical percentage. In particular, Ta A5 and Ta A9 showed the lowest helicity (about 50% and 46% in DPC and SDS, respectively) probably due to the central position of the replaced amino acids along the peptide sequence. Note that earlier studies had evidenced that in both SDS and DPC micelles, temporin-1Ta adopted an amphiphatic α-helix when considering the central residues 6–9 [34]. Finally, no variation in the α-helical content was detected when Ile^{12} and the C-terminal Leu^{13} were replaced, whereas substitution of Phe^1 or Ser^{10} (Ta A1 or Ta A10) increased the helical content of the native peptide in DPC (60 or 56%, respectively), but not in SDS solution.

Table 2. Temporin-1Ta Ala-scan comparison with respect to charge, H, μ_H, % helicity, and MIC on different microbial strains

					% Helix		MIC (μM)					
							Gram-negatives		Gram-positives			Yeasts
Peptides	Amino acid sequences	Charge	H	μ_H	DPC	SDS	*E. coli*	*Y. pseudo-tuberculosis*	*S. aureus*	*B. megaterium*	*S. capitis*	*C. albicans*
1Ta	FLPLIGRVLSGIL-NH_2	+2	0.22	0.35	55	50	>40	20	5	2.5	5	5
Ta A1	ALPLIGRVLSGIL-NH_2	+2	0.2	0.33	60	50	>40	>40	>40	20	>40	>40
Ta A2	FAPLIGRVLSGIL-NH_2	+2	0.2	0.34	54	49	>40	40	20	5	20	10
Ta A3	FLALIGRVLSGIL-NH_2	+2	0.25	0.33	69	57	>40	20	2.5	2.5	2.5	1.25
Ta A4	FLPAIGRVLSGIL-NH_2	+2	0.2	0.36	53	49	>40	40	40	5	20	10
Ta A5	FLPLAGRVLSGIL-NH_2	+2	0.19	0.31	50	46	>40	>40	40	20	40	20
Ta A6	FLPLIARVLSGIL-NH_2	+2	0.23	0.35	71	65	>40	20	2.5	2.5	5	2.5
Ta A7	FLPLIGAVLSGIL-NH_2	+1	0.38	0.21	63	52	>40	>40	>40	>40	>40	>40
Ta A8	FLPLIGRALSGIL-NH_2	+2	0.2	0.34	54	51	>40	40	20	5	20	10
Ta A9	FLPLIGRVASGIL-NH_2	+2	0.2	0.34	51	46	>40	>40	>40	>40	>40	>40
Ta A10	FLPLIGRVLAGIL-NH_2	+2	0.26	0.32	56	50	>40	20	2.5	2.5	5	2.5
Ta A11	FLPLIGRVLSAIL-NH_2	+2	0.23	0.35	71	61	>40	20	2.5	2.5	5	2.5
Ta A12	FLPLIGRVLSGAL-NH_2	+2	0.19	0.32	55	49	>40	>40	40	20	>40	20
Ta A13	FLPLIGRVLSGIA-NH_2	+2	0.2	0.35	55	50	>40	>40	>40	20	>40	40

Mean residue hydrophobicity and hydrophobic moment were calculated using the Eisenberg scale of hydrophobicity [37].

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Mean Hydrophobicity *H* and Mean Hydrophobic Moment μ_H

Eisenberg consensus scale was used to calculate both the mean hydrophobicity *H* and the mean hydrophobic moment μ_H [37]. The mean hydrophobicity is a measure of the amino acid residue relative affinities for hydrophobic phases. The hydrophobic moment is a measure of the amphiphilicity or asymmetry of hydrophobicity of a polypeptide chain's segment. The helical wheel presentation of temporin-1Ta is given in Figure 2. We noted that temporin-1Ta analogs with *H* ranging from 0.23 to 0.26 (Table 2) and with a mean hydrophobic moment μ_H calculated to range between 0.32 and 0.36, had the same or a two to fourfold lower MIC than that of the natural temporin ($H = 0.22$ and $\mu_H = 0.35$), against all the tested microorganims, but with an increased hemolytic activity (Tables 2 and 3). A different behavior was manifested by the analog lacking the single basic residue, Ta A7. Indeed, despite its high hydrophobicity ($H = 0.38$) and hemolytic activity, ranging from 15 to 52% at a peptide concentration between 2.5 and 20 μM (Table 3), Ta A7 was the least active peptide against both bacteria and fungi, together with Ta A9 ($H = 0.2$) (Table 2). Otherwise, peptide analogs whose hydrophobicity was lower than that of temporin-1Ta were almost devoid of antimicrobial and hemolytic activities, except for Ta A1, where the loss of the N-terminal Phe increased the lytic effect on red blood cells (Table 3). Importantly, the hydrophobic moment was not found to influence the biological activity of the peptide.

Discussion

All living organisms are constantly exposed to multiple harmful microbes and the capacity to overcome infections is essential for their survival. Therefore, several host defence mechanisms have been engendered during evolution, including the generation of fast acting weapons, such as the CAMPs [39–41]. Among the several sources for natural CAMPs, amphibian skin is the major one [42], especially that of frogs of the genus *Rana,* which has a worldwide distribution with approximately 250 species [26]. Their synthesis is transcriptionally regulated by the NF-κB/IκBα machinery [43] and modulated by exposure to microorganisms [44]. In these animals, CAMPs are stored in granules of holocrine-type dermal glands and released into the skin secretion, as a reaction to stress or injury [25]. Remarkably, temporins represent the largest family and are among the shortest α-helical CAMPs found in nature to date. In addition, they have unique properties: (i) they contain only one or two basic amino acids; (ii) most of them are not toxic toward mammalian cells; (iii) they have a fast killing activity; (iv) some of them synergize in both the antimicrobial and anti-endotoxin activities; (v) they partially preserve antimicrobial activity in serum [45]; and (vi) they are endowed with chemotactic activity towards human phagocytes [46], which represents an important link between the innate and adaptive immune system, recruiting immune cells to the sites of infection. Note that the first stage of a CAMP in selecting the target microorganism includes its electrostatic attraction to the cell surface, which contains anionic molecules such as LPS in Gram-negative bacteria [47], and teichoic acids in Gram-positive bacteria. Following electrostatic binding, the CAMP reaches the cell membrane, composed predominantly of phosphatidylethanolamine and the negatively charged phosphatidylglycerol, and alters its permeability by making transmembrane pores or destroying the membrane's structure in a detergent-like manner [17]. Importantly, several studies have pointed out that temporins are membrane-active CAMPs. However, the range of the crucial peptide parameters required for their molecular mechanism is still not yet completely known.

We have presented a structure-activity study of the frog-skin peptide temporin-1Ta by replacing each individual amino acid with Ala. This standard Ala-positional scanning has identified four analogs (Ta A3, Ta A6, Ta A10, and Ta A11) with a higher hydrophobicity and a higher percentage of α-helix compared to the natural peptide, in both DPC and SDS. All of them display not only the same or a better antimicrobial activity than temporin-1Ta but also a higher lytic effect on human erythrocytes. It is worthwhile noting that all these substitutions reside at the hydrophilic face of the peptide (Figure 2). However, substitution of Arg with Ala (Ta A7), also located at the polar face of the peptide, suppresses its antimicrobial effect against all microorganisms (Table 2). This is in agreement with reports of other authors [48] and is probably due to the loss of the single positively charged residue (Arg) in the sequence, causing a weaker electrostatic interaction with the microbial membrane. In comparison, when amino acid substitutions with the neutral and inert Ala take place at the hydrophobic side of the molecule (Ta A1, Ta A2, Ta A4, Ta A5, Ta A8, Ta A9, Ta A12, and Ta A13), (Figure 2) a clear decrease or almost abolishment of the antimicrobial activity occurs, along with a significant decrease in the hemolytic activity. Ta A1 is an exception, because it exhibits two to threefold higher hemolytic activity compared to the parent peptide (Table 3).

Overall, our data have indicated that hydrophobicity and cationicity, more than helicity, are the main critical biophysical properties that determine the antimicrobial potency of temporin-1Ta, whereas the mean peptide hydrophobicity and helicity represent the main parameters that govern the peptide's toxicity towards eukaryotic cells. In fact, a direct correlation between the helical content in DPC and the hemolytic activity could be detected (Tables 2 and 3). The most hemolytic peptides of the series are those (Ta A1, Ta A3, Ta A6, Ta A7, and Ta A11) adopting the highest helical content in DPC, while the less hemolytic ones have the lowest helical percentage (Ta A5 and Ta A9). However, an exception is given by Ta A12 and Ta A13: indeed, despite their negligible hemolytic activity, their helical content is similar to that of 1Ta. Probably, highly hydrophobic residues at the C-terminus, such as Ile^{12} and Leu^{13}, are needed for the peptide interactions with zwitterionic membranes. Noteworthy, because the cationicity is a fundamental requisite for the 'carpet-like' mechanism of action of CAMPs and both helicity and hydrophobicity play a crucial role in the membrane interaction of CAMPs that act via the 'barrel-stave' mechanism [17], our findings seem to fit well with the 'carpet-like' and the 'barrel-stave' models that explain the antimicrobial and hemolytic activities of temporins, respectively [34,49]. Nevertheless, in regard to the latter model, which includes the formation of a transmembrane pore, it should be recalled that temporins are not long enough to span a phospholipid bilayer as α-helices, a property requiring a length of 20 amino acids for a 30 Å thick phospholipid bilayer [50]. Therefore, these peptides tend to form holes in a more elaborated way, perhaps due to their end-to-end dimerization [51].

The finding that enhancement of hydrophobicity increases the hemolytic and antibacterial activities of temporin-1Ta is consistent with what has already been found in magainin-2 [52]; on the contrary, no correlation between hydrophobicity and hemolytic/antimicrobial activity could be detected in other members of the temporin family, such as temporin-1Tl analogs, the percentage of helicity being the principal parameter controlling

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Table 3. Hemolytic activity of temporin-1Ta Ala-scan on human erythrocytes, at different peptide concentrations

Peptide concentration (μM)	% Hemolysis													
	Peptide													
	1Ta	Ta A1	Ta A2	Ta A3	Ta A4	Ta A5	Ta A6	Ta A7	Ta A8	Ta A9	Ta A10	Ta A11	Ta A12	Ta A13
40	27.5	62.5	15	100	11.5	3.5	97	39	10.5	3.5	70	100	1	3.5
20	12.5	39	5.5	89.5	10	2.5	27.5	52.5	10	4	14	92.5	0.5	3.5
10	9.5	11.5	2.5	23.5	8	1.5	10.5	39	12	1.5	5.5	32.5	0	3
5	3.5	8	1.5	4.5	6.5	0.5	3.5	27.5	8.5	1	5.5	13	0	0
2.5	0.5	5.5	0.5	2	2	0.5	3.5	15	6.5	1	1	5.5	0	0
1.25	0.5	2	0	0	2	0	1	6	1.5	0	0	3.5	0	0

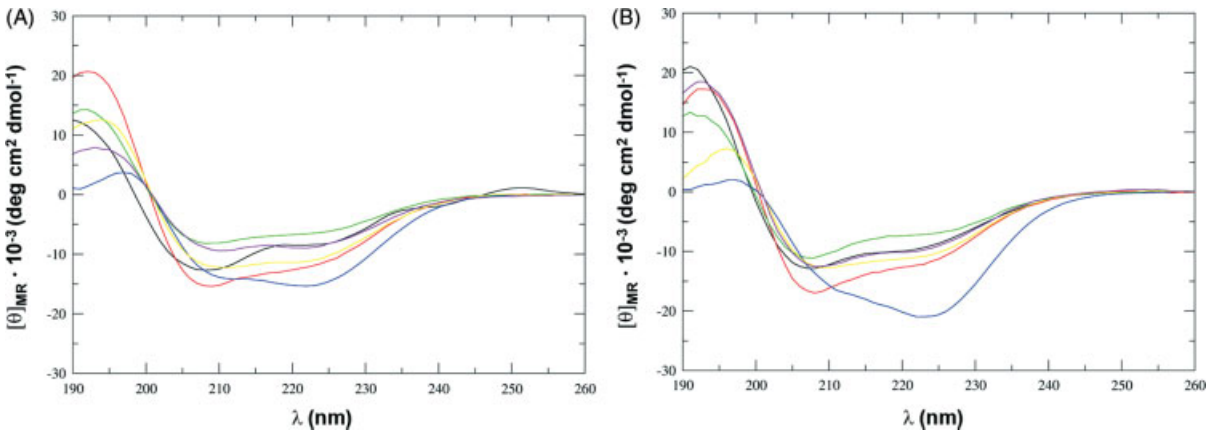

Figure 1. CD spectra of 1Ta analogs in (A) DPC, (B) SDS (1Ta, purple line; Ta A1, black line; Ta A3, red line; Ta A5, green line; Ta A6, blue line; Ta A7, yellow line). For the sake of clarity, Ta A2, Ta A4, Ta A8, Ta A10, Ta A12, and Ta A13 spectra were not reported since they were almost superposable with those of 1Ta. Similarly, Ta A9 and Ta A11 spectra were not shown because very similar to those of Ta A5 and Ta A3, respectively. $[Q]_{MR}$: mean residue molar ellipticity.

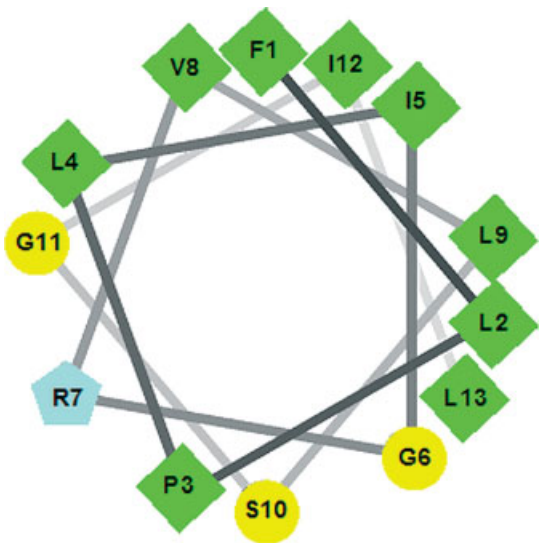

Figure 2. Helical wheel projection of temporin-1Ta. Green squares, yellow circles, and blue pentagons refer to non polar, polar uncharged, and basic residues, respectively.

the peptide's hemolytic effect (Mangoni ML *et al*., unpublished results). Obviously, a large number of factors (peptide's charge distribution, hydrophobicity, helicity, oligomeric state, amphiphilicity, and composition of the target cell membrane) cooperate in modulating the ability of a CAMP to disturb the organization of microbial membranes, and the target cell selectivity and potency of a CAMP cannot be easily predicted.

In conclusion, we can state that despite other structure–function relationship studies that have been performed with some analogs of temporin-1Ta [48,50,53], this work is the first report demonstrating the contribution of each amino acid residue to the antimicrobial/hemolytic activity of temporin-1Ta, by the standard alanine-positional scanning. The correlation found between the net charge, helicity, hydrophobicity, and hydrophobic moment due to a single substitution has increased our knowledge on the physicochemical attributes and structural properties underlying the biological activity of temporin-1Ta, and should assist in optimizing the design and manufacturing of temporin-based lead structures for the development of new anti-infective agents with expanding properties.

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