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Biological activities of C-terminal 15-residue synthetic fragment of melittin: design of an analog with improved antibacterial activity

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Abstract Melittin, the 26-residue predominant toxic peptide from bee venom, exhibits potent antibacterial activity in addition to its hemolytic activity. The synthetic peptide of 15 residues corresponding to its C-terminal end (MCF), which encompasses its most amphiphilic segment, is now being shown to possess antibacterial activity about 5–7 times less compared to that of melittin. MCF, however, is 300 times less hemolytic. An analog of MCF, MCFA, in which two cationic residues have been transpositioned to the N-terminal region from the C-terminal region, exhibits antibacterial activity comparable to that of melittin, but is only marginally more hemolytic than MCF. The biophysical properties of the peptides, like folding and aggregation, correlate well with their biological properties.

Key words: Melittin; C-terminal synthetic peptide; Analog with improved activity; Antibacterial activity; Hemolytic activity; Folding; Aggregation

1. Introduction

The emergence of microbes that are resistant to conventionally used antibiotics has triggered considerable interest in the structure-function relationship studies in short antimicrobial peptides in recent years [1]. In addition to naturally occurring short antimicrobial peptides like indolicidin, protegrins and tachyplesins, shorter segments of comparatively larger peptides and proteins like seminalplasmin, cecropins, neuropeptide Y, dermaseptins and lactoferrin have been studied in considerable detail [2–12]. These studies are aimed at not only delineating the structural requirements for selective antimicrobial activity but also to develop peptides that could have potential as therapeutic agents. As cationic antimicrobial peptides are a part of innate immunity in a wide variety of organisms across the evolutionary scale and are known to rapidly kill microorganisms by permeabilizing their membranes, a mechanism different from other antibiotics, resistance is not likely to develop to them easily [1,13–15].

Melittin, a 26-residue peptide, present as the major toxic component of the European bee venom, *Apis mellifera* is characterized by its powerful hemolytic activity [16]. Although it possesses potent antimicrobial activity its overriding cytotoxic effect makes it unsuitable for any therapeutic use. Several reports have appeared describing non-hemolytic analogs of melittin that retain their antimicrobial activity. These analogs include retro, retro-enantio and dia-stereo analogs and all of them are of the same length as melittin [16,17]. In this paper we describe the synthesis and biological activity of the C-terminal 15-residue fragment of melittin as well as an analog of this peptide with antimicrobial activity comparable to that of melittin, but with much reduced hemolytic activity.

2. Materials and methods

2.1. Peptides

Melittin was purchased from Sigma (St. Louis, MO, USA). MCF and MCFA (Fig. 1) were synthesized by manual methods using fluorenylmethoxycarbonyl (Fmoc) chemistry on PAL resin (Advanced Chem Tech, Louisville, KY, USA) [18,19]. All amino acids were added as Fmoc amino acids with coupling mediated by 2(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate/1-oxybenzo-triazole (HBTU/HOBT). The synthesis was performed on 0.05 mmol scale using ~125 mg of resin. Cleavage of the peptide from the resin was effected by acidolysis using trifluoroacetic acid, thioanisole, metacresol and ethanedithiol (10:1:1:0.5). Peptides were checked for purity on HPLC using a Biorad C_4 column. Purified peptides were characterized by liquid secondary ion mass spectrometry, on a VG Autospec-M Mass spectrometer and amino acid analysis on a Pharmacia LKB 4151 Alpha Plus amino acid analyzer.

2.2. Antimicrobial activity

The antimicrobial activity of the peptides was assayed in nutrient broth (6 g of bactonutrient broth and 5 g of NaCl per liter medium) under aerobic conditions. Different concentrations of peptides were added to 1 ml medium containing the inocula of the test organism (~10^6 CFU) in mid-logarithmic phase of growth. Microbial growth was assessed by recording the increase in OD_{600}, after incubating at 37°C or 30°C for 6–9 h. The lowest concentration that resulted in complete inhibition of growth was taken as minimal inhibitory concentration (MIC). The microorganisms used were *E. coli*, *S. aureus*, *P. putida* and *B. subtilis*.

Inner membrane permeability of *E. coli* W 160-37 was monitored by measuring the β-galactosidase activity in the cells (in which the enzyme has been previously induced with 5×10^{-4} M IPTG), in the presence of different concentrations of the peptides. *Ortho*-nitrophenyl galactoside (ONPG) was used as the substrate. Cells incubated in the absence of the peptides served as the control. OD measurements were made at 550 and 420 nm and ($A_{420}-1.75\times A_{550}$) was taken as a measure of β-galactosidase activity [6].

2.3. Hemolytic activity

The hemolytic activities of the peptides were evaluated with rat erythrocytes. Erythrocytes were isolated from heparinized blood by centrifugation and washed thrice with phosphate buffered saline (PBS). Aliquots of 1 ml suspension containing 10^7 cells in Eppendorf tubes were incubated at 37°C in duplicates for 30 min with gentle mixing. The tubes were then centrifuged and the absorbance of the supernatants was measured at 540 nm. The lysis obtained with water was considered 100%. Osmotic protection experiments were done by including 30 mM of one of the osmoticants, D-mannitol, sucrose, raffinose or PEGs of molecular weights 600 or 1000 in the hemolytic assay.

2.4. Circular dichroism (CD) studies

CD spectra were recorded using a JASCO-J-715 spectro polarimeter under nitrogen flush in 0.1 cm path length cells at 25°C. Spectra were recorded between 250–200 nm and average of three recordings was taken. Calibration was carried out with d-camphorsulfonic acid. Data are represented as mean residue ellipticities. Fractional helicities were

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Melittin : GIGAVLKVLT$_{10}$TGLPALISWI$_{20}$KRKRQQ-CONH$_2$

MCF : GLPALISWI KRKRQQG-CONH$_2$

MCFA : GLKKLISWI KRAAQQG-CONH$_2$

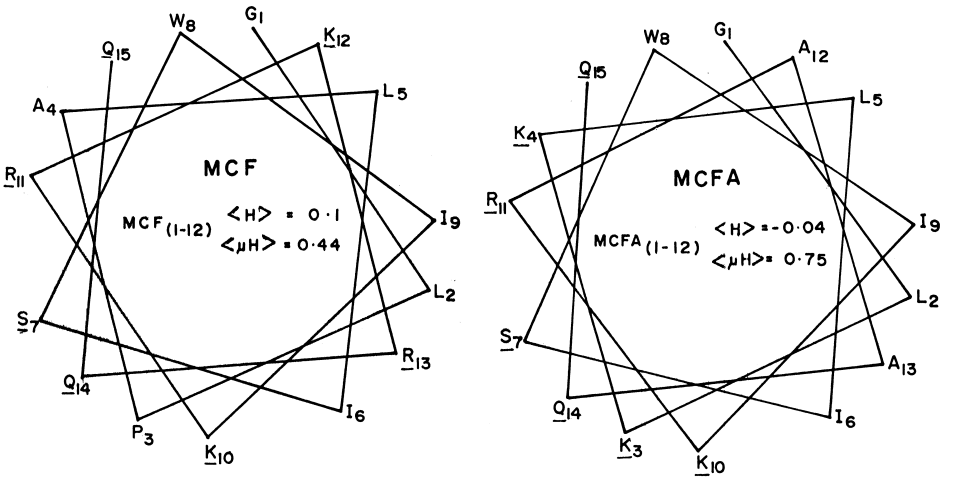

Fig. 1. Sequnces of melittin, MCF and MCFA. Helical wheel projections of MCF and MCFA are also shown. Polar residues are underlined.

calculated by using the formula $f_h = ([\theta]_{222} - [\theta^0_{222}])/[\theta]^{100}_{222}$, where $[\theta]_{222}$ is the experimentally observed ellipticity at 222 nm. Values of −28 400 and −2000 were taken for 100% ($[\theta]^{100}_{222}$] and 0% ($[\theta]^0_{222}$) helix content respectively [20].

2.5. Light scattering

Aggregation of melittin, MCF, and MCFA as a function of concentration in aqueous solutions was monitored by 90° angle scattering on a spectroflourimeter by fixing both excitation and emission monochromators at 600 nm.

2.6. Fluorescence spectroscopy

All steady state fluorescence spectra and scattering were recorded on a Hitachi 650-10S spectrofluorimeter at 25°C. Emission spectra of the peptides were recorded between 320 and 400 nm with the excitation monochromator set at 280 nm.

3. Results

The sequences of melittin, C-terminal 15-residue synthetic fragment of melittin, MCF, and its analog MCFA are shown in Fig. 1. Additional glycine amide was introduced at the C-terminal end of both MCF and MCFA for synthetic convenience. Helical wheel diagrams of MCF and MCFA along with their mean hydrophobicity ($<H>$) and hydrophobic moments ($<\mu H>$) are presented in Fig. 1.

Melittin has earlier been shown to possess antibacterial activity and hence the antimicrobial activity of the three peptides melittin, MCF and MCFA were assessed. The results are presented in Table 1. Melittin exhibited antibacterial activity on all the strains tested with MICs ranging between 5 and 35 μg/ml. MCF also exhibits activity against three of the organisms tested, but the MICs are higher and it is ∼5–7 times less active as compared to melittin. MCFA, on the other hand, has MICs comparable to that of melittin. Thus a minor rearrangement in the sequence of MCF which results in increased $<\mu H>$, leads to considerable increase in its antimicrobial activity. A majority of antimicrobial peptides are known to exert their activity by permeabilizing their membranes, hence the ability of the three peptides to permeabilize the cytoplasmic membrane of *E. coli* was examined by assessing the influx of ONPG into *E. coli* in the presence and absence of the peptides. The data shown in Fig. 2 indicate that all the three peptides indeed open up additional pathways for ONPG uptake besides the normal lac permease route. The relative abilities of these peptides to permeabilize the cytoplasmic membrane parallel their MICs against *E. coli*.

The hemolytic activities of the three peptides are compared in Fig. 3a. Melittin exhibits 100% lytic activity at 1 μg/ml concentration. MCF shows ∼50% lysis at 100 μg/ml reaching a value of 100% beyond 300 μg/ml. Thus MCF is 300 times less active than melittin. MCFA is comparatively more hemo-

Table 1
Antimicrobial activity of melittin and its analogs

Peptide	MIC (μg/ml) against			
	E. coli W 160-37	*S. aureus* 8530	*B. subtilis*	*P. putida* NCIM 2102
Melittin	5–10	10–15	2–4	25–35
MCF	35–45	25–35	15–25	>200
MCFA	10–15	5–10	2–4	50–60

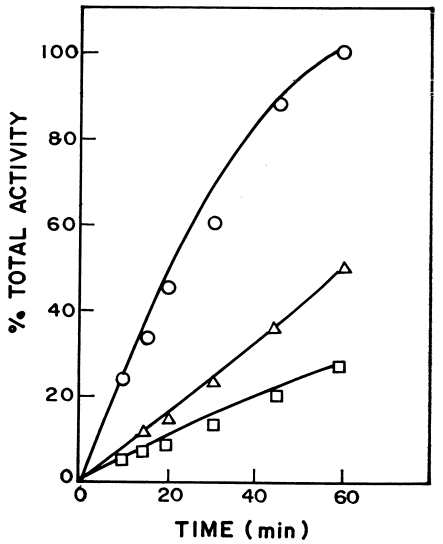

Fig. 2. Effect of melittin (○), MCF (□) and MCFA (△) on the influx of ONPG into *E. coli* W 160.37. Peptide concentrations of 20 μg/ml were used in the assay.

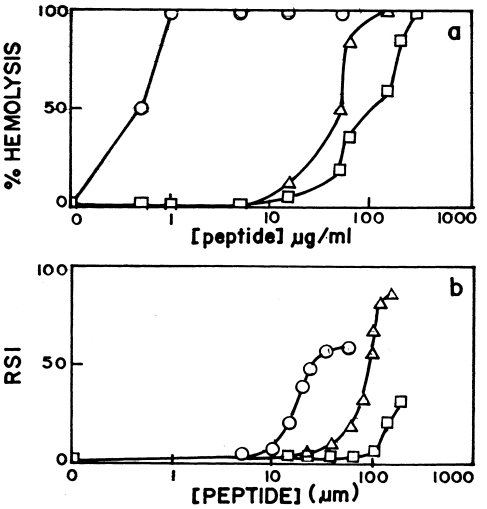

Fig. 3. (a) Hemolysis of rat erythrocytes as a function of peptide concentrations (symbols used are same as in Fig. 2). (b) Light scattering (90° angle) of aqueous solution of peptides. Peptides were in 10 mM HEPES buffer (pH 7.4).

lytic than MCF showing 100% lysis at 100 μg/ml, but as compared to melittin it is still 100 times less active. Melittin exerts its hemolytic effect by colloid-osmotic mechanism [21]. In order to determine whether MCF and MCFA also follow a similar mechanism, lysis of red blood cells by the three peptides was assessed in the presence of osmoprotectants sucrose, maltose, raffinose and PEGs 600 and 1000. Virtually the first three molecules provided no protection to lysis (results not shown). But, in the presence of PEG 600 and PEG 1000 no hemolytic activity was observed. Thus, osmoprotectants of >20 Å gave protection to hemolysis caused by all the peptides, suggesting that all the three peptides indeed followed colloid-osmotic mechanism and the size of the lesions made by them is ~ 20 Å.

Amphiphilic peptides like melittin show a tendency to aggregate in aqueous solution as a function of concentration [22]. Hence the aggregation properties of MCF and MCFA were compared with that of melittin. The data shown in Fig. 3b indicate that while melittin aggregates between 10 μM and 50 μM concentrations, MCF has the ability to aggregate beyond 100 μM. MCFA exhibits concentration dependent aggregation beyond 50 μM. The scatter values also indicate that the aggregates of MCFA may indeed be bigger than melittin aggregates. The aggregation properties of the peptides correlate well with their ability to cause hemolysis.

Melittin adopts a tetrameric helical structure in aqueous medium at high ionic strength [23]. Hence the folding of melittin and its shorter analogs, MCF and MCFA, were investigated by both circular dichroism, as well as by fluorescence spectroscopy, as all the three peptides contain a single Trp residue each. The results are summarized in Table 2. A steady decrease in θ_{222} value, in the case of melittin, as a function of ionic strength is discernible. This indicates an increase in its α-helical content. Such increases in α-helical contents are not observed in the cases of MCF and MCFA, indicating the inability of these peptides to fold at high salt concentrations. Melittin also exhibits a pronounced salt induced blue shift, from 351 to 339 nm, in its emission maximum (λ_{max}). Such a blue shift has been attributed to the formation of tetrameric aggregates. MCF and MCFA do not exhibit any such blue shift, indicating again the absence of any salt induced folding or aggregation in these short peptides, unlike melittin.

Alcohols are known to induce monomeric α-helical structure in melittin as opposed to increased ionic strength, which favors tetramerization [24,25]. In order to investigate the behavior of MCF and MCFA in alcohols, CD spectra and fluorescence emission spectra (Fig. 4a and b) of the three peptides were recorded in the presence of various concentrations of MeOH. While all the three peptides occur in unordered conformation in the absence of MeOH, addition of MeOH stabilizes the α-helix in all the three peptides (Fig. 4a). The results shown in Fig. 4b reveal blue shifts in the emission maximum in the case of all the three peptides in methanol rich phases. Thus while melittin can fold into α-helix conformation both in the tetrameric form (at high ionic strength) and in monomeric form (in methanol), the shorter analogs can

Table 2
Salt induced folding of melittin and its analogs

[NaCl] (M)	Melittin				MCF				MCFA			
	$-\theta_{222}$	$[f_h]$	λ_{max}	$[\Delta\lambda]$	$-\theta_{222}$	$[f_h]$	λ_{max}	$[\Delta\lambda]$	$-\theta_{222}$	$[f_h]$	λ_{max}	$[\Delta\lambda]$
0	4213	[0.08]	350	[0]	1389	[0]	350.4	[0]	2913	[0.03]	353.6	[0]
0.3	4716	[0.10]	349	[−1]	3015	[0.04]	351.4	[+1.4]	3497	[0.05]	355	[+1.4]
0.6	5108	[0.11]	346	[−4]	3584	[0.05]	352	[+2.0]	4404	[0.09]	356	[+2.4]
0.9	7184	[0.19]	341	[−9]	3750	[0.06]	351	[+0.6]	4500	[0.09]	356	[+2.4]
1.2	8825	[0.24]	339	[−11]	3879	[0.07]	351	[+0.6]	4589	[0.10]	356	[+2.4]
1.5	13245	[0.41]	338	[−12]	4651	[0.10]	351	[+0.6]	4895	[0.11]	356	[+2.4]

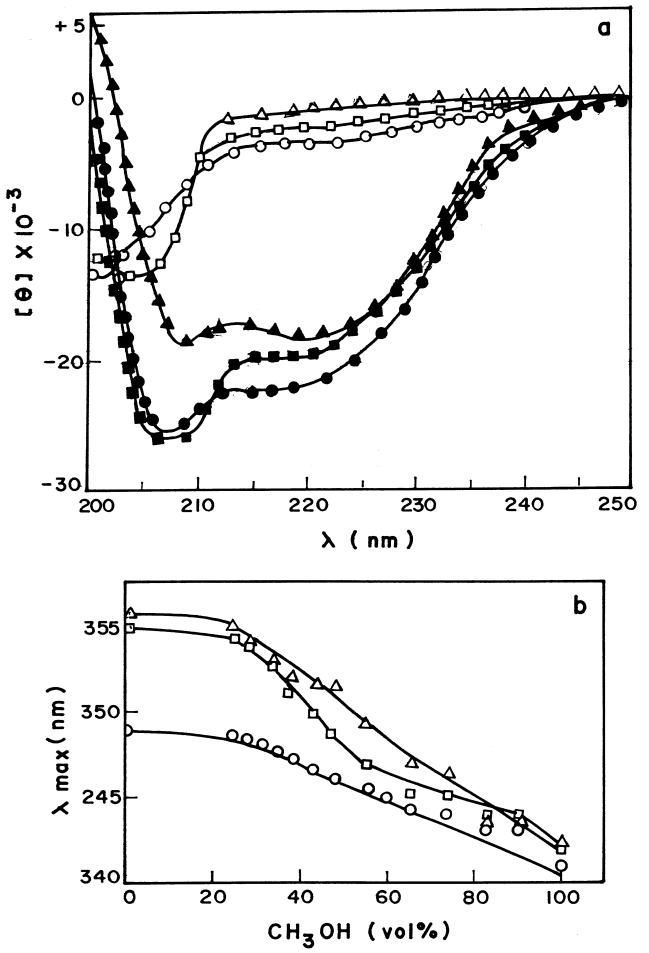

Fig. 4. (a) Circular dichroism (CD) spectra of peptides (melittin (○), MCF (△) and MCFA (□)) in aqueous buffer (open symbols) and methanol (darkened symbols). (b) Fluorescence emission maximum (λ_{max}) as function of methanol content in water. All measurements were made at 25°C using 20 μM peptide.

only exist in the monomeric α-helix conformation, but are incapable of self associating to form higher aggregates at high ionic strengths.

4. Discussion

Melittin is a 26-residue peptide with a predominantly hydrophobic N-terminal segment from 1–20 residues and a cationic tail of six residues between 21–26 [26]. The segment between 1–20, although membrane active, does not possess any lytic activity. Even the cationic segment 21–26 is inactive [27,28]. However, melittin has an 11-residue segment between 12–22, which falls in 'surface seeking' region of the 'hydrophobic moment' plot [29]. Such segments play an important role in the biological activities of several antimicrobial/lytic peptides. Short synthetic peptide, encompassing 'surface seeking' segments have been shown to exhibit antimicrobial and hemolytic activities [6–8,11,30–32]. Thus, it was interesting to see whether a synthetic peptide corresponding to C-terminal 12–26 segment of melittin, encompassing its most amphiphilic region, also possesses biological activity. Hence MCF was synthesized and its properties studied. MCF indeed possessed both antimicrobial and hemolytic activities, albeit to a very small extent compared to melittin. It is very well established that cationicity, mean hydrophobicity ($<H>$) and hydrophobic moments ($<\mu H>$) of a peptide are the determining factors of biological activity of cytolytic peptides [33–37]. Small change in μH values has been demonstrated to modulate these activities of the peptide [37,38]. An examination of the helical wheel diagram of MCF (Fig. 1) indicates that although it happens to be most amphiphilic region of melittin cationic residues 12 and 13 are in the hydrophobic face of the helix. Hence it is possible to increase the amphiphilicity of the peptide by transposing these residues to the hydrophilic face of the helix. These residues were therefore replaced for proline 3 and alanine 4. As Pro residue is known to be a structure breaker this was replaced at position 12 by Ala which is an α-helix stabilizer. These changes increase the μH of 1–12 segment of the peptide considerably (0.42 to 0.75) without much change in mean hydrophobicity. The antimicrobial activity of MCFA is comparable to that of melittin, but its hemolytic activity is less pronounced. Thus it is possible to design short peptides with relatively selective antimicrobial activity, by judiciously positioning the charged amino acids.

Antibacterial peptides, whose activities stem from their ability to interact with the membranes, are known to compromise the permeability barriers of the bacterial membranes [15]. Melittin and its two short analogs also effectively permeabilized the cytoplasmic membrane of *E. coli* to ONPG, by opening additional pathways for its influx in addition to the protein transporter lac permease, indicating that the underlying mechanism of antimicrobial activity for all the three peptides is likely to be the same.

Most cytolytic peptides are known to lyse the cells by formation of discrete transmembrane pores, which allows small molecules to move across the membrane [39]. However, large molecules like proteins cannot move through these small pores and because of this the cell interior becomes hyperosmotic. This is counter balanced by a net influx of water, which results in swelling of the cell and subsequent lysis. By including osmoprotectants of appropriate size, which cannot penetrate the induced pores, it is possible to counter balance the osmotic drag. By determining the size of the osmoticants that is able to give protection to lysis it is possible to make a rough estimate of the size of the pores induced by the peptide. In the case of these three peptides hemolysis was virtually blocked by PEGs 600 and 1000 and thus the probable size of the induced pores was $\sim$20 Å.

Despite the absence of qualitative differences in the biological activities of the three peptides studied here, quantitative differences are quite evident. The segment 1–7 in melittin is very important in modulating the biophysical as well as biological properties of melittin, as 8–26-peptide is very weakly lytic on model lipid membranes [28,40]. However, there are no reports describing the antimicrobial and hemolytic activities of short C-terminal fragments of melittin. The low antimicrobial and hemolytic activities of MCF could be attributed to the absence of the N-terminal segment of melittin. MCFA, on the other hand, has antimicrobial activity comparable to that of melittin and hemolytic activity considerably more than that of MCF. An examination of the helical wheel diagram of MCF and MCFA reveals the presence of a larger hydrophobic face in MCFA. A larger hydrophobic face, in an amphiphilic peptide, favors the formation of relatively stable transmembrane pores and consequently better biological activity. Such peptides with large hydrophobic face have indeed been

shown to possess strong hemolytic activity [41,42]. The presence of cationic residues on the hydrophobic face in MCF is likely to destabilize transmembrane pores leading to poor biological activity.

In an attempt to correlate the biological activities with the biophysical properties of the peptides, their structural preferences were investigated in aqueous solutions as well as methanol. The association and folding properties of melittin are known to be dependent on its N-terminal 1–11-segment and particularly the polar residue at position 7 seems to play a predominant role [21,43]. As both MCF and MCFA lack this segment it was also interesting to study their folding and aggregation behavior. All the three peptides are unordered in dilute aqueous solution. The CD spectra of the three peptides in methanol are characteristic of α-helical structure, with double minima at 222 λ and 208 λ and cross over at ∼200 λ, indicating their intrinsic preference to acquire α-helical structure [44]. MCF exhibits very poor folding and aggregating properties as a function of peptide concentration as well as at high ionic strength, emphasizing again the importance of the N-terminal segment of melittin for these attributes. However, MCFA's large hydrophobic face would explain its aggregation as a function of peptide concentration. The formation of these aggregates in MCF is likely to be due to non-specific aggregation, as opposed to specific tetramer formation in melittin. One would expect MCFA to form similar aggregates at high salt concentrations also and the failure to detect them remains unclear at this time.

Structure function studies on magainin and cecropins have indicated that deletion of up to three amino acids from the N-terminal end resulted in analogs with considerably reduced antimicrobial activity [45]. Further reduction in length appears to result in complete loss of activity. In this study we have shown that deletion of 11 residues from the N-terminal end of melittin results in a 15-residue peptide (MCF), encompassing its most amphiphilic segment, that exhibits both antimicrobial and hemolytic activities. An analog of this peptide, in which two cationic residues are trans-positioned to the N-terminal region from the C-terminal region that results in increased μH, shows antimicrobial activity comparable to that of melittin, but with marginal hemolytic activity. Thus it is possible to rationally design short peptides with enhanced antimicrobial activity from highly hemolytic peptides on the basis of biophysical principles.

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