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ORIGINAL ARTICLE

Antimicrobial activity and interactions of cationic peptides derived from *Galleria mellonella* cecropin D-like peptide with model membranes

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Antimicrobial peptides are effector molecules of the innate immune system against invading pathogens. The cationic charge in their structures has a strong correlation with antimicrobial activity, being responsible for the initial electrostatic interaction between peptides and the anionic microbial surface. This paper contains evidence that charge modification in the neutral peptide Gm cecropin D-like (WT) improved the antimicrobial activity of the modified peptides. Two cationic peptides derived from WT sequence named as ΔM1 and ΔM2, with net charge of +5 and +9, respectively, showed at least an eightfold increase in their antimicrobial activity in comparison to WT. The mechanism of action of these peptides was investigated using small unilamellar vesicles (SUVs) as model membranes. To study permeabilization effects of the peptides on cell membranes, entrapped calcein liposomes were used and the results showed that all peptides induced calcein release from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) SUVs, whereas in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), POPC/POPG and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE)/POPG SUVs, only ΔM1 and ΔM2 induced a notable permeabilization. In addition, interactions of these peptides with phospholipids at the level of the glycerol backbone and hydrophobic domain were studied through observed changes in generalized polarization and fluorescence anisotropy using probes such as Laurdan and DPH, respectively. The results suggest that peptides slightly ordered the bilayer structure at the level of glycerol backbone and on the hydrophobic core in 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) SUVs, whereas in 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)/DMPG SUVs, only ΔM1 and ΔM2 peptides increased the order of bilayers. Thus, peptides would be inducing clustering of phospholipids creating phospholipid domains with a higher phase transition temperature.

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INTRODUCTION

Antimicrobial Peptides (AMPs) perform many important functions in innate immunity of insects, they are found in the hemolymph where their role is to kill invading microorganism. A special feature of AMPs is that it is less likely for bacteria to develop resistance against them[1] since AMPs execute their biological activity via a non-specific mechanism, targeting mainly bacterial cell membranes, unlike conventional antibiotics. The majority of these peptides are formed by <50 residues and have a net positive charge which is related with their lytic activity.[2] Furthermore, the charge is a property that is important due to the interaction of AMPs with the anionic membrane being largely dependent on electrostatic attraction.[2–5] Efforts to isolate or discover new naturally occurring AMP molecules against multi-resistant bacteria are expensive and time-consuming. Therefore, synthesis by solid phase is a useful method for peptide modification that could tackle this bottleneck and thus, evaluate modifications of the charge and other structural parameters to improve their antimicrobial activity.

AMPs have been classified in different types of molecules such as (1) linear α-helical peptides without cysteine residues, for example, cecropins; (2) β-sheet stabilized by disulfide bridges, for example, defensins; (3) peptides with predominance of proline and/or glycine residues.[6] The interactions of AMPs induces membrane damage via a detergent-like disaggregation or pore formation due to electrostatic and hydrophobic interactions with the head groups or the acyl chains of the phospholipids respectively.[2,7] AMPs have a wide spectrum of antimicrobial activity since they possess heightened activity against Gram-positive, Gram-negative bacteria, fungi, parasites, enveloped virus and even cancer cells due in part to electrostatic interactions.[8]

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Our research focusses on evaluating two cationic synthetic peptides called ΔM1 and ΔM2, which were derived from cecropin D-like from *Galleria mellonella*.[16] Cecropin D-like is composed of 39 residues, is neutral at pH 7.4 and shows antibacterial activity against Gram-positive and Gram-negative bacteria. The charge in the peptide analogs ΔM1 and ΔM2 was increased through substitution of negative glutamic and aspartic acid residues with positive arginine and lysine residues. The neutral amino acid glutamine was also substituted with lysine, and all substitutions were performed in the first 18 amino acids of the NH_2-terminal. Nevertheless, the minimal IC for ΔM1 and ΔM2 analog peptides was lower than that of cecropin D-like wild type. Using the self-quenching of the fluorescence dye calcein and the membrane probes Laurdan (6-lauroyl-2-dimethylaminonaphthalene) and DPH (1,6-diphenyl-1,3,5-hexatriene), we found that the peptide analogs induce membrane permeabilization and alterations in the thermotropic behavior of small unilamellar vesicles (SUVs).

MATERIALS AND METHODS

Biologicals

Escherichia coli (ATCC 25922), *Pseudomona aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA).

Construction of charge substitution peptides and hydrophobic index

Amino acid substitutions, based on Bordo and Argos studies,[9] were introduced into the wild-type sequence to generate ΔM1 and ΔM2 peptides. The net charge of each peptide after exchanging residues was calculated as the addition of the basic residues. Peptide hydrophobicity, defined as the mean of hydrophobicity values from all residues within a peptide according to a standard scale,[10] was calculated using Heliquest software (http://heliquest.ipmc.cnrs.fr/).

Peptide synthesis, purification and sequence characteristics

Peptides were synthesized by solid-phase method[11] using Fmoc (9-fluoroenyl-methoxycarbonyl) as a protector group on the NH_2-terminal region in an automatic synthesizer 433 A Applicated Biosystems (University of Lausanne, Switzerland). After that, peptides were obtained with 95% purity by reverse phase-high performance liquid chromatography using a Vydac C-18 preparative column and applying a mixture of: (A) H_2O with TFA 0.1% (v/v) and (B) acetonitrile containing TFA 0.1% (v/v) as mobile phase. For the elution of peptides, the following gradient program was used: 30 min with 5–70% of B at 1 ml min^{-1} and detection at 220 nm. Finally, the molar mass of purified peptides was determined by matrix assisted laser desorption ionization-time of flight MS.[12]

Wheel projections of three peptides were conducted according to the first eighteen residues of the NH_2-terminal region sequence as this is the region where the residue substitutions were performed in ΔM1 and ΔM2 peptides. These projections were calculated using bioinformatic software (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi).

Antimicrobial activity

The determination of the MIC was performed in compliance with the method described by Wiegand *et al.*,[13] with slight modifications. The bacteria were grown in a preculture overnight at 37 °C gently shaken. The culture was diluted in nutrient broth until an OD_{625} of 0.1 was reached (Approximately 1×10^8 CFU ml^{-1}). Then such culture was diluted by a factor 1:200. 10 μl aliquots of serial dilutions of peptide, being 100 and 0.62 μM, the maximum and minimum concentration, respectively, were mixed with 90 μl of the bacterial suspension reaching a final inoculum of approximately 5×10^5 CFU ml^{-1}. The mixtures were transferred to 96-well plates, incubated at 37 °C for 18–20 h and the absorbance at 625 nm was measured at a Multiskan Go (Thermo). The MIC was reported as the lowest concentration that showed growth inhibition. Phosphate-buffered saline (PBS, 138 mM NaCl, 3 mM KCl, 1.5 mM NaH_2PO_4, 8.1 mM Na_2HPO_4 and pH 7.4) was used as negative control, and oxytetracycline and polymyxin B (1 μM) were used as positive control for Gram-positive and Gram-negative bacteria respectively.

Hemolytic activity

Erythrocytes were isolated from fresh human peripheral blood. They were washed three times with PBS by centrifugation for 5 min at 3000 *g* and resuspended in PBS. Peptides at different concentrations (115, 80, 57.5, 40, 25 and 12.5 μM), were added to 4% human erythrocytes in PBS and incubated at 37 °C for 1 h. After, mixtures were centrifuged at 4000 *g* for 5 min. Aliquots of the supernatant were transferred to 96-well plates. Hemolysis was measured by absorbance at 540 nm with a Multiskan Go (Thermo). For negative and positive controls, erythrocytes in PBS (blank) and erythrocytes in PBS with 5 μM of melittin (positive) were used respectively. The hemolysis percentage was calculated using the following equation: [(Abs in the peptide solution − Abs in PBS)/(Abs in Melittin − Abs in PBS)] × 100.[14] Hemolytic concentration 50 (HC_{50}) is the necessary concentration of peptide to induce 50% of lysis of erythrocytes under physiological conditions.

Preparation of small unilamellar vesicles

Dehydrated anionic and zwitterionic lipids were dissolved in the solvent $CHCl_3$:CH_3OH (70:30, % v/v) and $CHCl_3$, respectively. The samples were dried under nitrogen gas and placed under vacuum overnight to remove the residual solvent. Films were hydrated with Hepes buffer (10 mM Hepes, 110 mM KCl, 0.01 mM EDTA, 0.03 mM $CaCl_2$, pH:7.4) or calcein buffer (10 mM Hepes, 110 mM KCl, 0.01 mM EDTA, 0.03 mM $CaCl_2$, 50 mM Calcein, pH:7.4). The mixtures were homogenized by vortex for 2 min. After that, each mixture was incubated by 10 min at 37 °C, above the temperature of phase transition (T_m). This cycle was repeated three times. Multilamellar vesicles were then extruded above the phase transition temperature of the lipid mixtures through 100 nm diameter pore filter (Whatman, Clifton, NJ, USA) using a hand held extruder (Avanti Polar Lipids, Alabaster, AL, USA) 20 times to obtain SUVs. Each SUV was named with the abbreviations of the phospholipids that compose it: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), POPC/POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE)/POPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), DMPC/DMPG and POPE/DMPG. All phospholipids mixtures were performed at 3:1 ratio.

Calcein release experiments

Calcein-loaded SUVs were separated from bulk dye through size-exclusion chromatography using Sephadex 50 (Sigma-Aldrich, St Louis, MO, USA). The calcein SUVs total phospholipid concentration was adjusted to a final concentration of 25 μM and they were exposed to different concentrations of peptides: 0.5, 0.35, 0.25 and 0.05 μM, at 37 °C. Fluorescence measurements were performed in an ISS-PC1 spectrofluorometer (ISS, Champaign, IL, USA) using λ_{exc} and λ_{em} of 490 and 519 nm, respectively. The fluorescence intensities were recorded as a function of time (0–300 s). The percentage of calcein released was calculated as: $[(F_t - F_{contr})/F_{tot} - F_{contr})] \times 100$, where F_t is the fluorescence signal measured at a time t under the influence of peptides, F_{contr} is the fluorescence signal measured at the same time t in absence of peptides, and F_{tot} is the total fluorescence signal obtained after complete disruption of the liposomes by 1% Triton X-100.

Laurdan generalized polarization

The influence of peptides on gel toliquid-crystalline transition at the level of the glycerol backbone as well as on phase transition temperature (T_m) was determined using Laurdan (6-lauroyl-2-dimethylaminonaphthalene) probe (Molecular Probe, Eugene, OR, USA). The emission spectral shift of Laurdan fluorescence results from changes in dipolar relaxation phenomena due to fluctuating membrane hydration. On excitation, the dipole moment of Laurdan increases notably and water molecules surrounding the probe reorientate around the new dipole.[15] Fluorescence measurements were performed using an ISS-PC1 spectrofluorometer (ISS) with λ_{exc} 340 nm. The lipid concentration of

liposomes was adjusted to 0.25 mM and Laurdan probe was added to a lipid: probe ratio of 1:500. Then, peptides were added to a final concentration of 2.5 μM (1 peptide/100 lipids) under continuous agitation and increasing the temperature from 12 to 37 °C, 1 °C each two minutes. The generalized polarization (GP) value was obtained using the following equation:

$$GP = I_{440} - I_{490} / I_{440} + I_{490}$$

where I_{440} and I_{490} are the fluorescence intensities at emission wavelengths of 440nm (gel phase) and 490 nm (liquid-crystalline phase) respectively. The melting temperature was calculated by maximum of the first derivative of GP values as a function of temperature, corresponding to the inflection point of the curve. The slope of the transition gives information about the cooperativity of the process.

DPH fluorescence polarization

DPH fluorescence anisotropy is dependent on the natural dynamic motions of the bilayer and the shift of its values are indicative of changes of the ordering in domains where the probe is located. The lipid and peptide concentrations were adjusted as mentioned in Laurdan assays and DPH probe (Molecular Probe) was added in a lipid:probe ratio of 1:500. Peptides were added to under continuous agitation and increasing the temperature from 12 to 37 °C. Fluorescence polarization was measured with an ISS-PC1 spectrofluorometer (ISS) using λ_{exc} and λ_{em} of 349 and 426 nm respectively. Fluorescence anisotropy (r) was calculated using the equation: $r = I_0 - I_{90}/I_0 + 2 \times I_{90}$, where I_0 is the fluorescence intensity when angle between polarizers is 0°, I_{90} is the fluorescence intensity when angle between polarizers is 90°. The melting temperature (T_m) was obtained as mentioned above.

Statistical analysis

Biological and biophysical assays were carried out at least in triplicate. The LSD multiple range tests were used to determine whether statistically significant differences occurred among the mean values obtained. HC_{50} was determined using an adjusted-correlated model equation. All statistical analyses were performed using the software Statgraphics Centurion XV.

RESULTS

Peptide design and sequence characteristics

Peptide WT is a cecropin-D of *Galleria mellonella* composed of 39 residues (p85210, UniProt KB)[16] with a neutral charge at pH 7.4. This cecropin was selected as the template peptide in this study to change its neutral charge to a positive charge in the last 18 residues at NH_2-terminal region. The increase of the net charge was achieved by replacing of E6R, E8R and Q12K for ΔM1 and for ΔM2 previous substituitions were also performed, but additionally E1R and D16K (Table 1, bold letters). Peptide ΔM1, with three substitutions in the polar face, resulted in a net charge increased from 0 to +5. On the other hand, peptide ΔM2 with five substitutions resulted in a net charge increased from 0 to +9 (Table 1).

Residues substitutions in the polar face of the helix did not alter the non-polar face in the modified peptides (Figure 1). In these first 18 residues the polar face consisted of 10 residues with 5 residues conserved in all peptides (Asn 2, Lys 5, Arg 9, Arg 13 and Arg 15). The non-polar face in this region consisted of 8 residues: two Phe, three Ile, two Ala and one Gly residue. However, the hydrophobicity index of the peptides decreased after the substitutions from the initial 0.232 seen in WT to 0.193 and 0.178 for ΔM1 and ΔM2, respectively.

Antimicrobial, hemolytic activity and therapeutic index

Antibacterial activity of the peptides were evaluated (Table 2) against Gram-positive and Gram-negative bacteria strains as the MIC. Cationic peptides showed the highest activity especially against Gram-negative bacteria. In *E. coli,* ΔM1 and ΔM2 peptides exhibited MICs of 2.5 and 1.5 μM, respectively. Additionally in *P. aeruginosa*,

Table 1 Amino acids sequences and properties of WT, ΔM1 and ΔM2 peptides

Peptide name	*Sequence*	*Q*[a]	*<H>*
	1 10 20 30 39		
WT	NH-ENFFKEIERAGQRIRDAIISAAPAVETLAQAQKIIKGGD-Ac	0	0.232
ΔM1	NH-ENFFK**R**I**R**RAG**K**RIRDAIISAAPAVETLAQAQKIIKGGD-Ac	+5	0.193
ΔM2	NH-**R**NFFK**R**I**R**RAG**K**RIR**K**AIISAAPAVETLAQAQKIIKGGD-Ac	+9	0.178

Abbreviation: <H>, hydrophobicity.
[a]Q = charge.

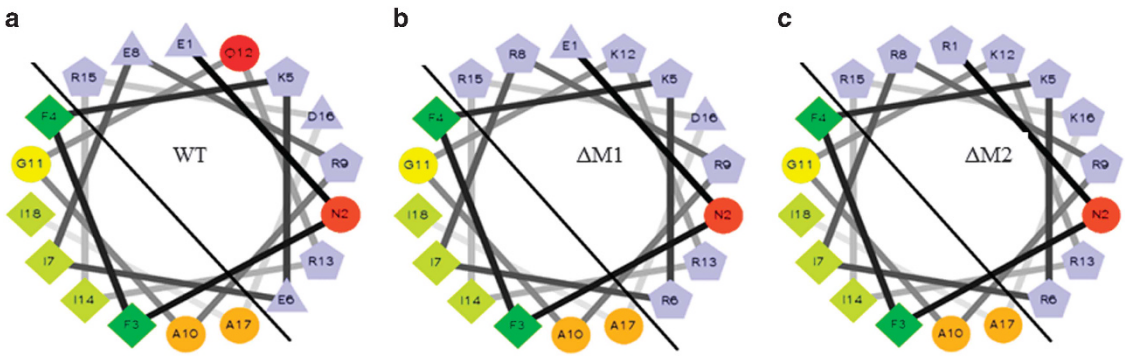

Figure 1 Helical wheel projections of the first 18 residues of the NH_2-terminal region sequence of each peptide. (**a**) Cecropin D-like *G. mellonella* (WT) peptide; (**b**) ΔM1 peptide; (**c**) ΔM2 peptide. By default, the output presents the hydrophilic residues as circles, hydrophobic residues as diamonds and potentially positively charged as pentagons. Hydrophobicity is color coded as well: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue. Helical wheels are adapted from http://rzlab.ucr.edu/scripts/wheel/wheel.cgi.

Table 2 MIC, HC_{50} and TI of the peptides against bacteria and hRBC

		E. coli ATCC 25922		*P. aeruginosa ATCC 27853*		*S. aureus ATCC 25923*	
Peptide	*HC_{50} in hRBC (μM)*	*MIC (μM)*	*TI*	*MIC (μM)*	*TI*	*MIC (μM)*	*TI*
WT	>115	40	ND	42	ND	>100	ND
ΔM1	1752.3	2.5	701	5	350	50	35
ΔM2	735.4	1.25	588	2.5	294	5	147

Abbreviations: HC_{50}, hemolytic concentration 50; hRBC, human red blood cells; MIC, minimal inhibition concentration; ND, not determined; TI, therapeutic index.

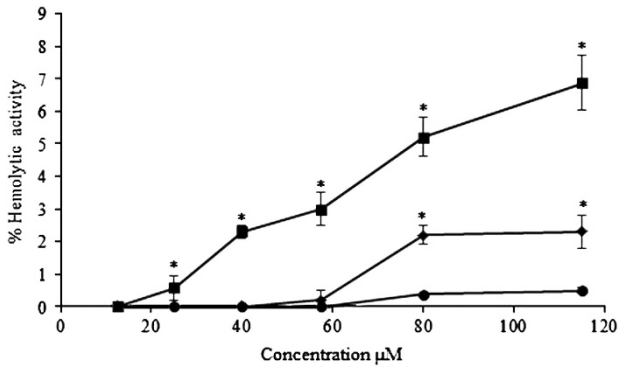

Figure 2 Hemolytic activity of WT (●), ΔM1 (♦) and ΔM2 (■) was measured using human red blood cells. Data is the average of at least three independent experiments by duplicated ± s.d. Error bars represents the s.d. *Significant difference to the WT peptide, $P<0.01$.

cationic peptides exhibited MICs of 5 and 2.5 μM for ΔM1 and ΔM2, respectively. On the other hand, Gram-positive bacteria was less sensitive to WT and ΔM1 peptides at the evaluated concentrations (Table 2), ΔM2 peptide showed the highest antimicrobial activity against *S. aureus*, reaching a MIC of 5 μM, whereas ΔM1 reached a MIC 10-fold larger than that exhibited by ΔM2. WT peptide had antimicrobial properties against Gram-negative bacteria with MICs observed at around 40 μM and no effect was detected on Gram-positive bacteria.

ΔM2 peptide exhibited high activity against both Gram-positive and Gram-negative bacteria, having the maximum antimicrobial effect with at least a 19-fold increase in activity in comparison to effect observed from WT peptide. ΔM1 peptide had at least an 8-fold increase in bactericidal effect in regard to WT peptide on Gram-negative bacteria. Therefore, the increase of net charge in the polar face showed a substantial increase in antimicrobial activity of the peptides.

The cytotoxic effects of peptides on red blood cells was followed by monitoring the hemoglobin release from erythrocyte cell suspensions with six different concentrations ranging from 12.5 to 115 μM. A concentration-response effect with the modified peptides was observed with a significant hemolytic effect ($P<0.01$) in comparison with WT peptide (Figure 2). The ΔM2 peptide had the highest hemolytic activity with a HC_{50} of 735.4 μM and 6.88% hemolysis at 115 μM, whereas ΔM1 had a lower hemolytic effect with a HC_{50} of 1752.3 μM and 2.3% hemolysis at 115 μM. On the other hand, WT peptide showed a very slight hemolytic activity at the maximum evaluated concentration.

The therapeutic index (TI) is a widely accepted parameter to represent the specificity of antimicrobial reagents for prokaryotic versus eukaryotic cells[17,18] and was calculated by: HC_{50}/MIC (Table 2). ΔM1 peptide had a TI slightly higher than that exhibited by ΔM2 in Gram-negative bacteria, whereas in *S. aureus*, ΔM2 had a TI of 147, 4.2-fold higher than that obtained for ΔM1. TI could not be evaluated for the WT peptide because the HC_{50} was not found in the maximum evaluated concentration.

Membrane permeability experiments

After evaluating the antimicrobial activity of the peptides, membrane permeability experiments were performed by measuring the release of calcein entrapped within SUVs prepared from representative lipids of Gram-negative bacteria. Both the neutral and cationic peptides show activity on POPG SUVs from the lowest concentration, with ~85% of the calcein released at a peptide to lipid ratio of 1:100 (0.25 μM of peptide) (Figure 3). However, WT peptide has a slight activity on the other liposome mixtures (Figure 3a). When cationic peptides are added to membrane models, the permeabilization activity decreases according to the following liposomes order: POPG > POPC/POPG > POPC > POPE/POPG (Figures 3b and c). On POPC/POPG liposomes, both peptides exhibited a maximal release activity around 70% at the highest concentration, whereas on POPC SUVs they reached an activity of around 40%. The minimal activity was shown in POPE/POPG SUVs, with an activity around 10% at the highest concentration.

The results of the calcein release titration curve showed that it saturates at 0.25 μM (1:100 peptide:lipid ratio), independently of the SUV composition the curves showed a steady activity after this ratio (Figure 3). Therefore, a final comparison of the calcein release activity of the peptides at 0.25 μM was performed (Figure 4). On POPG liposomes, all peptides showed activity, but ΔM1 peptide effect is significantly higher than that exhibited by ΔM2 and WT peptides ($P<0.01$), whereas on POPC liposomes both cationic peptides, ΔM1 and ΔM2, have an activity significantly ($P<0.01$) higher than WT peptide. In addition, on liposomes composed by lipid mixtures ΔM2 peptide have the highest activity ($P<0.01$) unlike WT peptide, which shows almost no activity (Figure 4).

Hydration changes of phospholipids (generalized polarization of Laurdan)

After determining through the calcein release experiments that 1:100 peptide:lipid ratio disrupts the membrane integrity, we studied the changes in the physicochemical properties of the bilayers by monitoring the temperature dependence on Laurdan probe. Laurdan fluorescent spectrum is sensitive to the polarity of membrane, therefore it provides information of the molecular dynamics of the glycerol backbone level estimated from the GP parameter. An increase in the GP value is associated with highly ordered bilayers, whereas a decrease in the GP value is associated with higher fluidity of membranes. As elucidated, clear gel and liquid phases with a defined T_m were observed in the sigmoidal curve (Figure 5), such as it has been reported in previous studies.[19–21] The results of the cationic peptides on DMPG liposomes showed that the addition of peptides induce a slight but significant GP increase ($P<0.01$) at and above the phase transition temperature (Figure 5a). In addition, peptides provoke a small shift in the T_m from 24 to 24.5 °C (Table 3). The results of the incorporation of ΔM1 and ΔM2 peptides on DMPC/DMPG SUVs showed a mild increase the GP values above the phase transition (Figure 5c) but did not induce a significant increase of T_m.

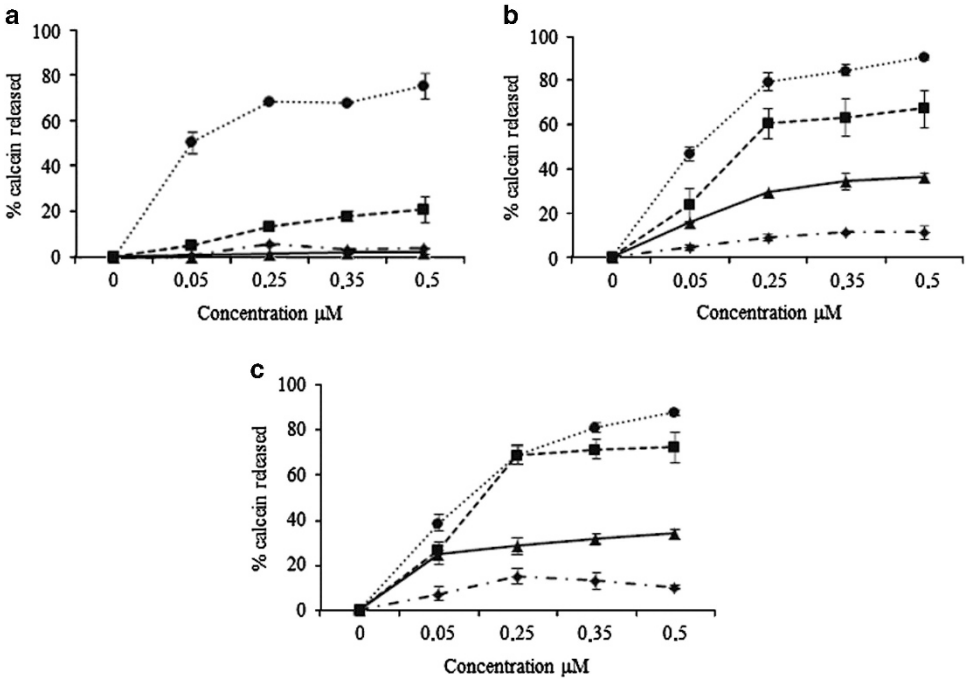

Figure 3 Release of calcein from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG; ···●···), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; —▲—), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine POPE/POPG (3:1;--◆--) and POPC/POPG (3:1; ---■---) liposomes, on increase of the concentration at 25 °C. Tested peptides were (**a**) WT, (**b**) ΔM1 and (**c**) ΔM2. The ordinate shows the percentage of calcein release after addition of peptides as a fraction of the total amount released by 1% triton X-100. Data are the average of at least three independent experiments ± s.d. Error bars represent the s.d.'s.

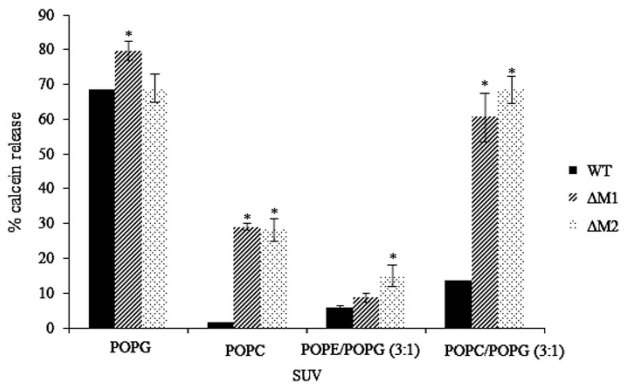

Figure 4 Measurement of calcein release from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE)/POPG (3:1) and POPC/POPG (3:1) small unilamellar vesicles (SUVs), on the effect of a concentration of 0.25 μM at 25 °C. Results are given in percent and peptides are represented as: WT (black bars), ΔM1 (striated bars) and ΔM2 (dotted bars). Data is the average of at least three independent experiments ± s.d. Error bars represent the s.d.'s. *Significant difference to the WT peptide, $P<0.01$.

In contrast, the incubation of the peptides on DMPC and POPE/DMPG SUVs did not affect the GP parameter (Figures 5b and d), indicating that the hydration of the polar groups of the phospholipids are not altered by the peptides. The cooperativity of the transition deduced as the steepest point of the curve (Figure 5 and Table 3) changed slightly under peptides effect at all liposomes.

Changes in membrane fluidity of bilayers (DPH fluorescence anisotropy)

To follow the disturbing effect of the peptides on the hydrophobic domain of bilayers, the changes in fluorescence anisotropy of DPH on temperature were measured at a 1:100 peptide:lipid ratio (Figure 6). The analysis of the results reflect that the incorporation of the peptides on DMPG SUVs induced an shift in the T_m from 24 to 26.12 °C (Table 4). The same mild shift on the T_m was observed in the previous experiments with Laurdan. There was no significant change in the anisotropy below and above the phase transition temperature (Figure 6a).

Regarding the experiments on DMPC/DMPG SUVs, only cationic peptides (ΔM1 and ΔM2) induced an minor increase of the anisotropy above the T_m (Figure 6c). The results on DMPC SUVs showed that the addition of the peptides had no effect on the ordered hydrophobic core of bilayers (Figure 6b).

DISCUSSION

In this research, it is presented evidence of the evaluation of the antimicrobial activity of a wild-type peptide known as cecropin D-like and two cationic modified peptides derived from the natural sequence. We evaluated the peptides on Gram-negative and Gram-positive bacteria, and cytotoxic effects on human red blood cells. In addition, the biophysical interaction of the peptides with synthetic lipids was investigated to understand their biological activity. We have employed a combination of fluorescence experiments to evaluate the disrupting effect of the peptides and the interaction at different membrane depths. The analysis of the results showed that the cationic peptide variants that were synthesized exhibited an antimicrobial activity significantly higher than the WT peptide. It is accepted that, the charge increase is accompanied by an increase in the antimicrobial

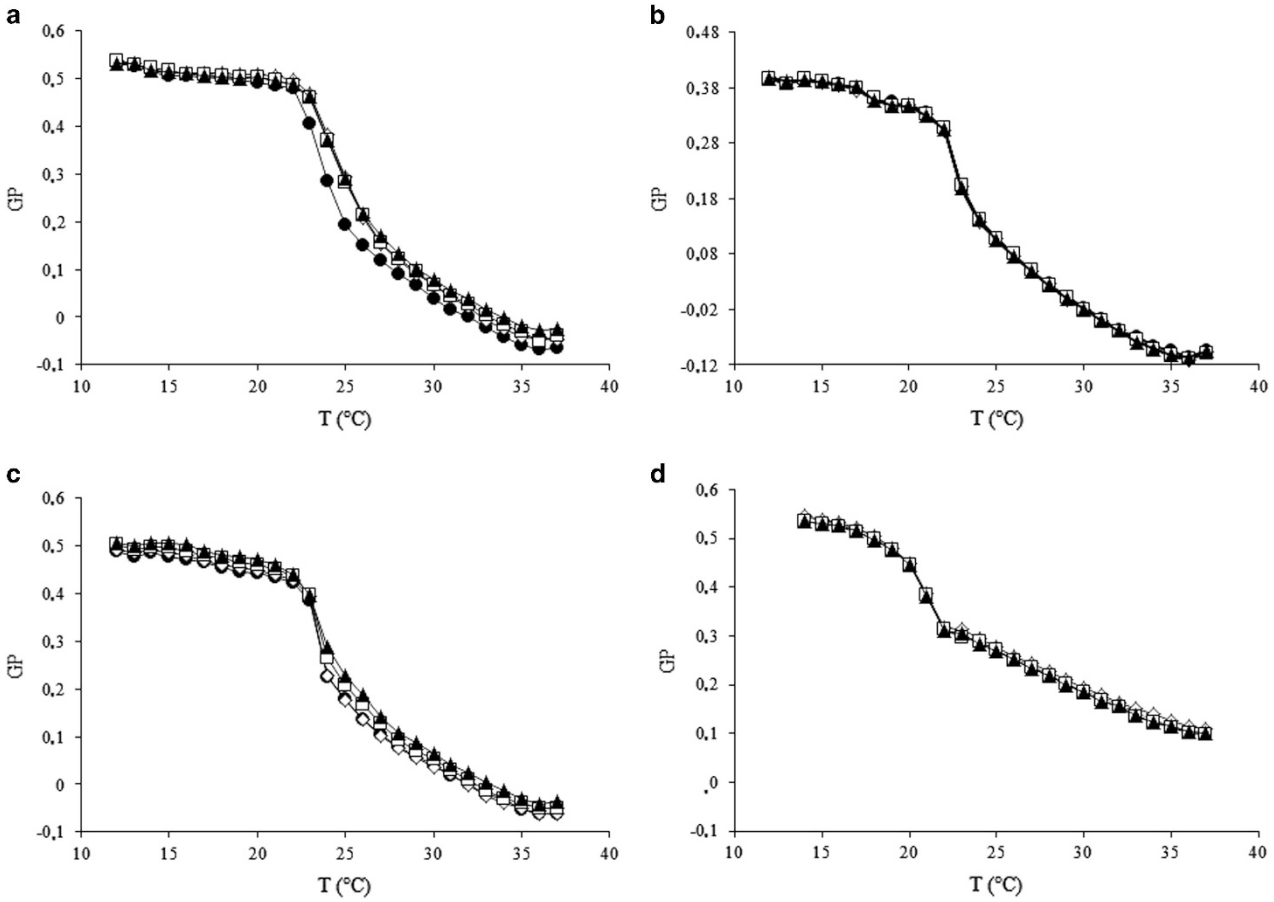

Figure 5 Generalized polarization (GP) values as a function of temperature under the effect of treatments: hepes (●), WT (♦), ΔM1 (■) and ΔM2 (▲) at peptide:lipid molar ratio of 1:100 (2.5 μM). Data were obtained from (**a**) DMPG, (**b**) DMPC, (**c**) DMPC/DMPG (3:1) and (**d**) POPE/DMPG (3:1) liposomes prepared in 10 mM Hepes. The excitation wavelength was 340 nm and the emission wavelengths were 440 nm (gel phase) and 490 nm (liquid-crystalline phase).

Table 3 Effect of the peptides at a concentration of 2.5 μM on the transition temperature and minimal slope of transition obtained by monitoring of GP values from DMPG and DMPC/DMPG liposomes

	DMPG		*DMPC/DMPG (3:1)*	
Treatment	$T_m \pm$ s.d. (°C)	*Min slope* (dGp/dT_m) ± s.d.	$T_m \pm$ s.d. (°C)	*Min slope* (dGp/dT_m) ± s.d.
Hepes	24 ± 0	−0.1319 ± 0.019	23.5 ± 0	−0.1594 ± 0.01
WT	24.1 ± 0.003	−0.0904 ± 0.003	23.5 ± 0	−0.1672 ± 0.01
ΔM1	24.5* ± 0	−0.0888 ± 0.003	23.5 ± 0	−0.1332 ± 0.02
ΔM2	24.5* ± 0	−0.0885 ± 0.007	23.5 ± 0	−0.1077 ± 0.02

Abbreviations: DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol; GP, generalized polarization; T_m, temperature of phase transition.
*$P<0.01$.

activity.[3–5] An increase in the positive charge induce a higher attraction to the surface charge density of the membrane. The magnitude of this attraction also increases as the peptide approaches the membrane.[22]

Our results demonstrated that Gram-negative bacteria were more sensitive to the peptides under evaluation. Gram-negative bacteria is constituted by a lower fraction of anionic lipids in their membranes in comparison with Gram-positive bacteria. Furthermore, *S. aureus* which was less sensitive to the AMPs tested, includes cationic lipid lysyl-PG in the membrane. Studies has postulate that these lipids repel and avoid the sequestration of cationic peptides in the bacterial membrane.[23] On the other hand, the peptidoglycan barrier of the Gram-positive bacteria constitute a protection against the electrostatic attraction and insertion of peptides reducing the antimicrobial.[3]

The antimicrobial experiments of WT peptide showed no activity against Gram-positive bacteria; however, Cytryńska *et al.*,[16] reported that cecropin D-like peptide (WT) has antimicrobial activity reaching a MIC ranging from 6.9 to 34.4 μM in *E. coli* D31 (*rfa* mutation), *M. luteus, L. monocytogenes* and *S. lueta*, but it was not determined in *E. coli* ATCC 25922 the strain we used (MIC of 40 μM) was found. This is because the maximum concentration of WT used in that study was 34.4 μM, this may be accounted for by the lacking additional 5.6 μM to reach the MIC.

The calcein release assay elucidates the ability of peptides to induce a membrane-disrupting effect. The mechanism responsible for permeabilization is the insertion of amphiphilic molecules into the bacterial membrane, followed by partitioning of the monomer into the lipid bilayer and oligomerization leading to an imbalance between the inner and the outer leaflet and driving shape changes.[20] The results of the calcein experiments provide evidence that peptides have the capability to affect the POPG vesicles through a membrane-disrupting mechanism, which could lead to the loss of the cytoplasmatic content on bacteria cultures.

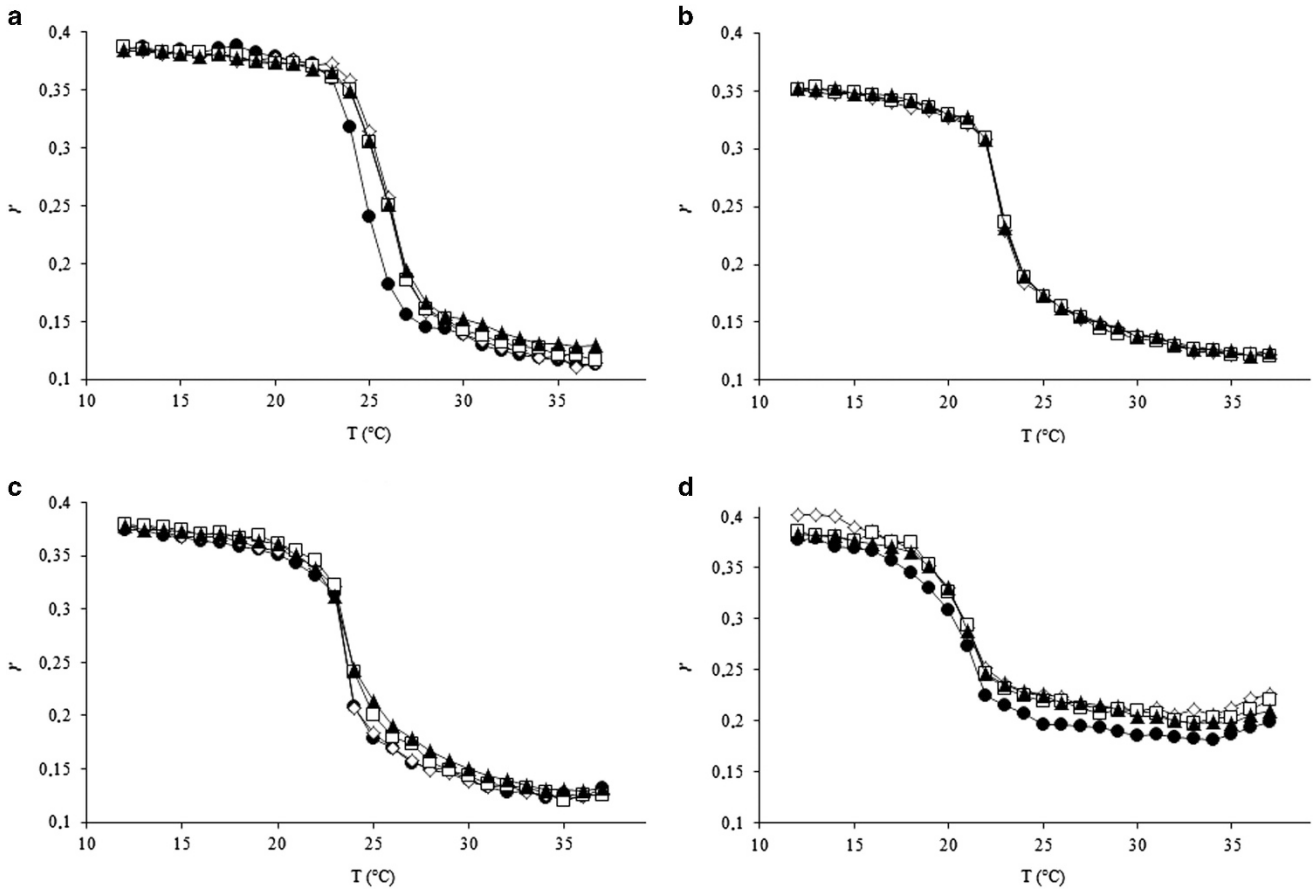

Figure 6 DPH fluorescence anisotropy (*r*) values on increasing temperature under the effect of treatments: hepes (●), WT (♦), ΔM1 (■) and ΔM2 (▲) at peptide:lipid molar ratio of 1:100 (2.5 μM). Data were obtained from (**a**) DMPG, (**b**) DMPC, (**c**) DMPC/DMPG (3:1) and (**d**) POPE/DMPG (3:1) liposomes prepared in 10 mM Hepes. The excitation wavelength was 349 nm and emission wavelength was 426 nm.

Table 4 Effect of the peptides at a concentration of 2.5 μM on the thermal transition temperature and minimal slope of transition obtained by monitoring the DPH fluorescence anisotropy from DMPG and DMPC/DMPG liposomes

	DMPG		*DMPC/DMPG (3:1)*	
Treatment	$T_m \pm$ s.d. (°C)	*Min slope* $(dr/dT_m) \pm$ s.d.	$T_m \pm$ s.d. (°C)	*Min slope* $(dr/dT_m) \pm$ s.d.
Hepes	24 ± 0	−0.092 ± 0.02	23.5 ± 0	−0.1063 ± 0.015
WT	26.1* ± 0.57	−0.0681 ± 0.001	23.5 ± 0	−0.113 ± 0.003
ΔM1	26.1* ± 0.57	−0.0671 ± 0.009	23.5 ± 0	−0.0805 ± 0.006
ΔM2	26.1* ± 0.57	−0.0639 ± 0.006	23.5 ± 0	−0.0678 ±0.006

Abbreviations: DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; T_m, temperature of phase transition.
*$P<0.01$.

Regarding the results with the POPC/POPG (3:1), POPE/POPG (3:1) SUVs, WT induces less permeabilization than ΔM1 and ΔM2 peptides, such as that observed in the antimicrobial activity against Gram-negative bacteria whose membranes maintain the 3:1 PE/PG phospholipid composition.[24] If the global charge of the SUVs is the same and it is determined by the PG content, the main difference probably lies in the terminal amino groups, being these $^{+}N(CH_3)_3$ in POPC and $^{+}NH_3$ in POPE. It is well reported that the head groups of the PC bilayers are more hydrated than PE. This phenomenon allows the incorporation of peptide molecules into POPC intercalated water layers and then a possible insertion into the bilayers. In the case of PE, the polar groups have a higher effective charge, strong electrostatic interactions and direct intermolecular hydrogen bonds are formed between the $^{+}NH_3$ and the PO_4^{-} which reduce the hydration. Besides, cationic peptides interact preferentially with liquid-crystalline POPC rather than POPE bilayers probably due to higher hydration and decreased packing density of the phospholipids in PC as compared with PE.[25,26]

To further characterize the interactions of peptides with phospholipid bilayers, the fluorescent probes, Laurdan and DPH, were employed. Laurdan is sensitive to polar environment,[27] it is considered a sensor at the glycerol backbone level of the lipids. The GP values at transition phase slightly increased with the addition of any of the three peptides to DMPG SUVs. There was a similar effect on DMPC/DMPG SUVs under the effect of the cationic peptides, indicating that these peptides affect the hydration of the polar head region. On the other hand, the anisotropy of DPH had a small increase in DMPG and DMPC/DMPG membranes. The results suggested that the peptide interaction with DMPG and DMPC/DMPG liposomes, changes the fluidity of the bilayer to a more rigid system. The increase in order of the bilayer could be due to the shielding of the polar group charges after peptide binding, avoiding the lateral repulsion between the lipids and establishing peptide-phospholipid domains with higher phase transition temperature.[28] The presence of domains would destabilize

the bilayer inducing efficient calcein leakage from the vesicles.[29] Besides, these results could indicate that cationic peptides also acted through binding and destabilization of the microbial membrane leading to the release of cytoplasmic content from bacteria.

ΔM1 and ΔM2 charged peptides had a concentration-dependent effect in the hemolytic activity. ΔM2 (+9) induced the highest hemoglobin release because it have a stronger interaction with erythrocyte surface than ΔM1. WT had a low hemolytic effect, these results are correlated with the permeabilization results in zwitterionic PC SUVs, where the membrane surface has an electronic nature similar to erythrocytes membrane since they are generally composed by zwitterionic phospholipids.[24] Cationic peptides showed higher hemolytic activity perhaps due to an initial electrostatic interaction through the negatively charged groups of the membrane including the phosphate and sialic acid-containing carbohydrate moieties.[30]

The concentration necessary to release hemoglobin was dramatically higher compared with that necessary to kill bacteria. This is because the erythrocyte membrane consists mainly of zwitterionic phospholipids, which would hinder the emergence of electrostatic interactions with the cationic peptides; additionally, such membranes possess cholesterol contributing to the rigidity and strength against the permeator effect of the peptides.[24]

In conclusion, the increase of basic residues on the polar face in a template AMP was associated with an enhance the antimicrobial activity mainly against Gram-negative bacteria. Furthermore, the increase of charge is also associated with a permeabilization effect in SUVs and the possible formation of domains in the membrane, suggesting that peptides would be killing bacteria by permeabilizing membrane and releasing cytoplasmic content. On the other hand, increase of the net charge enhances also increase the hemolytic activity, although this activity would not represent a considerable risk for a clinical approach. However, further studies would be necessary to determine the stability of the peptide in '*in vivo*' models. Therefore, this study gives insight into the introduction of cationic residues into peptide sequences with regard to biological activity to be useful in the design and development of molecules with a low hemolytic activity and improved antimicrobial activity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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