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ARTICLE

# Effect of antimicrobial peptides from *Galleria mellonella* on molecular models of *Leishmania* membrane. Thermotropic and fluorescence anisotropy study

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## Abstract

Antimicrobial peptides are molecules of natural origin, produced by organisms such as insects, which have focused attention as potential antiparasitic agents. They can cause the death of parasites such *Leishmania* by interacting with their membrane. In this study, additional information was obtained on how the anionic peptide 2 and cecropin D-like peptide derived from *Galleria mellonella* interact with liposomes that mimic the composition of the *Leishmania* membrane. In order to do this, lipid bilayers consisting of dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine, dimyristoylphosphatidylserine, and dimyristoylphosphatidylglycerol were constructed. The effect of the peptides on these membranes was evaluated using calorimetry analysis and fluorescence spectroscopy. The results obtained using differential scanning calorimetry indicated a concentration-dependent effect on membranes composed of phosphatidylserine and phosphatidylglycerol, showing a preference of both peptides for anionic lipids. The binding of the peptides drastically reduced the transition enthalpy in the phosphatidylserine and phosphatidylglycerol liposomes. The results suggest that the mode of action of anionic peptide 2 and cecropin D-like peptide is different, with a higher effect of cecropin D-like on the anionic lipids, which led to changes in the main transition temperature and a complete solubilization of the vesicles. Interactions between peptides and phosphatidylcholine, which is the most abundant lipid on the surface of *Leishmania* cells, were evaluated using isothermal titration calorimetry and the anisotropy of fluorescence of DPH. The peptides had a slight effect on the gel phase of the phosphatidylcholine, with changes in the anisotropy correlated with that observed by DSC. The results showed a selectivity of these peptides toward some lipids, which will direct the study of the development of new drugs.

## Introduction

Antimicrobial peptides (AMPs) have been shown to have not only antibacterial and anti-fungal activity. Some studies have demonstrated the potential of these peptides as antiparasitic agents [1]. In accordance with this, studies on the potential of these peptides in the development of new alternatives for the treatment of diseases caused by parasites, such as leishmaniasis, are necessary. This disease is endemic in 98 countries and an estimated 12 million people are infected around the world [2]. Control of this disease has been complicated because a vaccine against this parasite is not available and current drugs are highly toxic, causing multiple adverse effects and the development of resistance to these drugs has been reported in endemic areas [3, 4].

AMPs are molecules naturally produced by a wide diversity of organisms (animals, plants, bacteria, protists, fungi, and archaea) as part of the first line of defense in response to the attack of pathogens [5]. In insects, the AMPs present in hemolymph directly attack the membrane of the microorganisms in response to pathogen invasion, and this causes membrane destabilization and the death of the cell [6]. Several types of peptide belonging to families

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such as, e.g., cecropins, defencins, and lebocins have been found in insects [7–9]. Peptide synthesis in insects can be inducible and depends on the type of pathogen, which varies between Gram-positive bacteria, Gram-negative bacteria, and fungi [10].

AMPs have specific features that are fundamental to their biological activity. These molecules are oligopeptides with a variable number of amino acids; they may have a molecular weight up to 10 kDa. They have usually been classified according to their secondary structure, adopting alpha-helical conformations, beta-sheets stabilized by disulfide bridges, or some have a linear structure [9]. According to current knowledge, most AMPs have a cationic nature, although some biological active peptides may have an anionic or a neutral charge at physiological pH [11]. Other important properties of these molecules are a high degree of hydrophobicity and amphipathic character. Generally, 50% of the amino acids in the primary sequence of the AMPs have hydrophobic residues [7, 12], which creates two faces, a polar and a hydrophobic one. Such an amphipathic character is important for the peptide action mode [13].

It has been recently documented that AMPs show a selectivity toward the microbial surface, which differs from the host cells in terms of composition. Membranes from prokaryotic organisms and in particular from eukaryotic organisms such as *Leishmania* have an anionic character due to the presence of phospholipids (phosphatidylserine and phosphatidylglycerol) and negatively charged polysaccharides (such as lipophosphoglycan) [14], unlike the phospholipids present in mammalian cells, which are zwitterionics. AMPs can induce the destabilization of the membrane through pore formation or they may cause the membrane to destabilize by a mechanism similar to that of detergents [6]. This leads to the loss of membrane integrity and the possible death of the cell.

The greater wax moth *Galleria mellonella* (*G. mellonella*) has been successfully used as a biological model for studying the immune response in insects [15, 16]. A large arsenal of AMPs has been identified in its hemolymph, which, like in other insects, has been shown to vary depending on the type of microorganism inoculated to *G. mellonella* larvae [17]. At least 18 defense peptides with different biochemical and antimicrobial properties have been identified in *G. mellonella* so far [11, 18, 19]. Two of these peptides, anionic peptide 2 and cecropin D-like peptide, were identified in our research group when larvae were challenged with *Leishmania* parasites [20].

Because the biological membranes are highly complex, to simplify the investigations, extensive use is made of membrane molecular models that consist of lipid bilayers that allow to vary the composition and concentration [21]. The use of membrane models that mimic the surface of microorganisms allows us to evaluate the biological activity of molecules such as AMPs that interact with membranes. The aim of this research was to evaluate the interactions between peptides anionic peptide 2 and cecropin D-like peptide isolated from *G. mellonella* and lipids representative of the membrane of the *Leishmania* parasite. A high-sensitivity differential scanning calorimetry (DSC) study was carried out to determine the thermotropic behavior of representative membrane models consisting of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE), dimyristoylphosphatidylserine (DMPS), and dimyristoylphosphatidylglycerol (DMPG) lipids under the influence of both peptides. These analyzes showed a high level of interaction between both peptides and the negatively charged phospholipids phosphatidylserine and phosphatidylglycerol, and a very low effect with the zwitterionic lipids phosphatidylcholine and phosphatidylethanolamine. Using fluorescence spectroscopy of DPH and isothermal titration calorimetry (ITC), a low level of interaction between peptides and PC could also be confirmed.

## Materials and methods

### Peptides synthesis

Peptides were synthesized according to the sequence reported previously; the peptide anionic 2 as access number P85216 (UniProt Database), "TKNFNTQVQNAFDSDKIKSEVNNF IESLGKILNTEKKEAPK", this corresponds to 41 amino acids from the original peptide sequence; the net charge at physiological pH is +1; cecropin D-like peptide as access number P85210 (UniProt Database), "ENFFKEIERAGQRIRDAIISAAPAVETLAQAQ KIIKGGD", this corresponds to an original sequence of 39 amino acid as net charge of zero at physiological pH. The peptides were synthesized according to the solid-phase method, created by GenScript® (GenScript Corporation, Piscataway Township, USA).

### Preparation of multilamellar vesicles (MLVs)

Dehydrated DPPC, DPPE, DMPC zwitterionic lipids and DMPS, DMPG anionic lipids (Avanti Polar Lipids, Alabaster, AL, USA) were dissolved in chloroform and ethanol: chloroform (70:30% v/v). An appropriate amount of the lipid stock solution was dried under a gentle stream of nitrogen until a thin film formed on the wall of the test tube. Lipid films were hydrated with water for DPPC; 10 mM HEPES in pH 7.4 buffer for DPPE; 10 mM HEPES, 50 mM NaCl, 1 mM EDTA in pH 7.4 buffer for DMPS; 10 mM HEPES, 500 mM NaCl, 1 mM EDTA in pH 7.4 buffer for DMPG. The mixtures were heated at the temperature above

that of the main phase transition temperature ($T_m$) of the pure lipid (about 50 °C for DPPC; 65 °C for DSPC; 70 °C for DPPE; 45 °C for DMPS, and 40 °C for DPPG) and vigorously vortexed for 5 min.

### Differential scanning calorimetry (DSC)

The peptide stock solution was prepared in water at 1 mM, and the phospholipid vesicles were prepared as described above. The final lipid concentration in each test tube was 1 mM and peptide/lipid molar ratios of 1:100, 1:50, and 1:10 were used. The DSC measurements were made using a NANO DSC Series III System with Platinum Capillary Cell (TA instrument, Lukens, New Castle, USA) with an active volume of 0.3 ml. To prevent the formation of bubbles in heating mode, the samples were degassed prior to being loaded by pulling a vacuum of 30.4–50.7 kPa on the solution for a period of 10–15 min. The sample cell was then filled with 0.3 ml of sample solution, and an equal volume of water (or buffer) was used as a reference. The cells were sealed and equilibrated for ~10 min at the starting temperature. All of the measurements were made using samples under 0.3 MPa pressure. The data collected ranged between 15 °C and 60 °C (DPPC), 40 °C and 90 °C (DPPE), 15 °C and 55 °C (DMPS), and 10 °C and 40 °C (DMPG); at the scan rate 1 °C per minute both for heating and cooling. Thermograms were corrected by subtracting the buffer blank and normalized to the lipid concentration. All samples were prepared and recorded at least three times. Each data set was analyzed and the values of the transition temperatures were calculated using a software package supplied by TA Instruments. The accuracy was ±0.1 °C for the main phase transition temperature and ±1 kJ mol$^{-1}$ for the main phase transition enthalpy.

### Isothermal titration calorimetry (ITC)

Microcalorimetric experiments on peptides binding to DMPC were carried out at 25 °C, using an ITC NANO 2G calorimeter (TA instrument, Lukens, New Castle, USA) characterized by 950 µl of sample cell volume with a 100 µl dosing syringe. During all experiments, the reference cell was filled with deionized water. Before starting the measurement, the apparatus was equilibrated at the desired temperature. The stirring rate provided by the injector paddle rotation was set at 250 rpm. In these experiments, the reaction cell was filled with MLVs, the concentration of which ranged between 5 and 15 mM and the syringe was filled with peptide solution whose concentration ranged from 0.125 to 0.175 mM. All solutions were degassed prior to being loaded by pulling a vacuum of 30.4–50.7 kPa on the solution for a period of 10–15 min. For this an Affinity ITC Degassing Station (TA Instruments) was used. The volume of peptide solution introduced into the reaction cell was 7 µl. The heat of the interaction after each injection measured by the ITC calorimeter was plotted vs time (30 injections by experiment). As a control, the peptide solutions were titrated into the water in the cell. The data were analyzed with the software nano-analyze (TA Instruments, Lukens, New Castle, USA).

### Fluorescence anisotropy of DPH

To estimate the peptide-induced changes in the lipid acyl chain order of the bilayer, a hydrophobic fluorescent probe l,6-diphenyl-l,3,5-hexatriene (DPH), introduced into small unilamellar vesicles (SUVs) formed from DPPC, was used. The SUVs were prepared according to the injection method [22]. Briefly, solutions of DPPC and DPH were mixed and the solvent was evaporated under a gentle stream of nitrogen. Then, the dry residue was dissolved in a small volume of ethanol and slowly injected into the water (or water containing an appropriate amount of peptide) while stirring continuously at a temperature above that of the main phase transition of lipids. The final lipid concentration was 0.5 mM, phospholipid/DPH ratio of 1:1000 and peptide/DPPC ratios were 1:100, 1:50, and 1:10. The experiments were carried out at temperatures ranging between 20 and 55 °C. Fluorescent anisotropy measurements were made on a FluoroMax-P spectrofluorometer (Horiba Scientific, Besheim, Germany) equipped with a thermostated cuvette holder and stirrer. Excitation and emission were set at 360 nm and 450 nm, respectively. The anisotropy value ($r$) was calculated from the equation:

$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}}$$

where $I_{\|}$ and $I_{\perp}$ are the parallel and perpendicular components of DPH emission, with respect to the direction of the polarized excitation. Fluorescence anisotropy measurements were made twice, and the average value with standard deviation error bars is shown.

## Results

### Differential scanning calorimetry

The calorimetry experiments were carried out in order to determine if there is interaction between lipids and peptides; for this the changes in the heat flux associated with the phase transition were observed. These experiments were conducted with synthetic peptides, anionic peptide 2 (AP-2) and cecropin D-like peptide, using the pure phospholipid liposomes described above. All the experiments were carried out with three different molar ratios 1:100, 1:50,

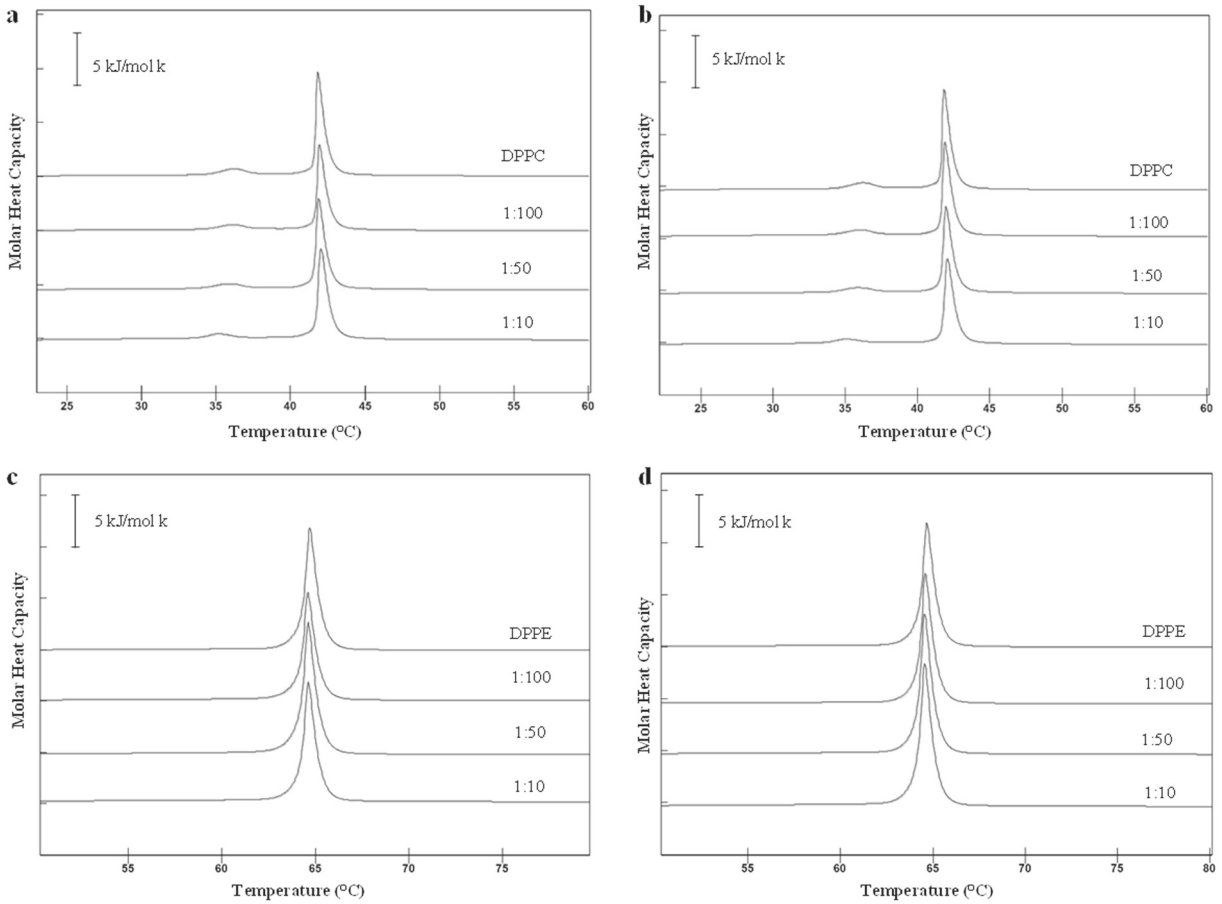


**Fig. 1** Measurement of differential scanning calorimetry obtained for multilamellar DPPC and DPPE liposomes in the presence of anionic peptide 2 (AP-2) (**a**, **c**) and cecropin D-like (**b**, **d**)

**Table 1** Thermodynamic parameters of the pretransition and main phase transition of pure DPPC multilamellar liposomes and peptide/DPPC mixtures determined from heating scans

| **DPPC + peptide** | | **Pretransition heating** | | **Main transition heating** | |
|---|---|---|---|---|---|
| | Molar ratio (peptide:lipid) | $\Delta H$ (kJ mol$^{-1}$) | $T_p$ (°C) | $\Delta H$ (kJ mol$^{-1}$) | $T_m$ (°C) |
| DPPC | | 1.35 ± 0.8 | 35.5 ± 0.1 | 15.4 ± 0.8 | 41.8 ± 0.1 |
| AP-2+ DPPC | 1:100 | 1.13 ± 0.8 | 36.1 ± 0.1 | 14.0 ± 0.8 | 41.9 ± 0.1 |
| AP-2+ DPPC | 1:50 | 0.87 ± 0.8 | 35.4 ± 0.1 | 17.4 ± 0.8 | 41.9 ± 0.1 |
| AP-2+ DPPC | 1:10 | 1.16 ± 0.8 | 35.2 ± 0.1 | 17.2 ± 0.8 | 42.1 ± 0.1 |
| Cecropin+ DPPC | 1:100 | 1.15 ± 0.8 | 36.0 ± 0.1 | 16.1 ± 0.8 | 41.9 ± 0.1 |
| Cecropin+ DPPC | 1:50 | 1.03 ± 0.8 | 36.8 ± 0.1 | 17.0 ± 0.8 | 41.9 ± 0.1 |
| Cecropin+ DPPC | 1:10 | 0.60 ± 0.8 | 34.9 ± 0.1 | 16.7 ± 0.8 | 42.0 ± 0.1 |

1:10 (peptide:lipid); as a reference pure lipid was used. The results obtained from the DSC experiments with the DPPC are shown in Fig. 1a, b. Between 15 and 60 °C, DPPC showed an endothermic peak with a main transition temperature ($T_m$) of 41.8 ± 0.1 °C, with an enthalpy change ($\Delta H$) of 15.4 kJ mol$^{-1}$ (Table 1), corresponding to the conversion of the gel phase ($L_{\beta'}$) to liquid crystalline phase ($L_{\alpha'}$). Additionally, a small peak with a transition temperature of 35.5 °C and $\Delta H$ of 1.35 kJ mol$^{-1}$ (Table 1) was recorded. This corresponds to transition from the gel phase ($L_{\beta'}$) to the ripple-gel phase ($P_{\beta'}$), called pretransition. These results are consistent with previous reports in the literature [23]. The presence of AP-2 induced a slight increase in the main transition temperature (0.3 °C), only at the higher molar ratio peptide/lipid (1:10) (Table 1). For the pretransition peak the results showed a change in the

**Table 2** Thermodynamic parameters of the pretransition and main phase transition of pure DPPE multilamellar liposomes and peptide/DPPE mixtures determined from heating scans

| DPPE + peptide | | Main transition heating | |
|---|---|---|---|
| | Molar ratio (peptide/lipid) | $\Delta H$ (kJ mol$^{-1}$) | $T_m$ (°C) |
| DPPE | | 21.4 ± 0.8 | 64.7 ± 0.1 |
| AP-2+ DPPE | 1:100 | 21.3 ± 0.8 | 64.5 ± 0.1 |
| AP-2+ DPPE | 1:50 | 24.1 ± 0.8 | 64.5 ± 0.1 |
| AP-2+ DPPE | 1:10 | 26.4 ± 0.8 | 64.7 ± 0.1 |
| Cecropin+ DPPE | 1:100 | 24.6 ± 0.8 | 64.6 ± 0.1 |
| Cecropin+ DPPE | 1:50 | 26.6 ± 0.8 | 64.5 ± 0.1 |
| Cecropin+ DPPE | 1:10 | 26.5 ± 0.8 | 64.5 ± 0.1 |

transition temperature ($L_{\beta'}$−$P_{\beta'}$) by increasing (0.6 °C), with the minor molar ratio (1:100) (Fig. 1a and Table 1). The results obtained for DPPC/cecropin D-like peptide were very similar, increasing the $T_m$ only at 0.2 °C (for 1:10) and pretransition peak 0.5 °C (for 1:100) (Fig. 1b and Table 1). In summary, the experiments showed that there is only a weak level of interaction between the two peptides evaluated and the zwitterionic PC lipid.

The DSC results obtained for the DPPE lipid show a single peak, with a transition temperature of 64.7 ± 0.1 °C and a transition enthalpy $\Delta H$ of 21.4 kJ mol$^{-1}$ (Fig. 1c, d and Table 2) [24]. The presence of the peptides AP-2 and cecropin D-like did not cause the lipid structure to destabilize, because changes were not observed in the thermodynamic parameters of the phase transition with any of the molar ratios evaluated (Table 2). Once again, these results show a very low level of interaction between AP-2 and cecropin D-like peptides from *G. mellonella* with zwitterionic lipids.

The presence of negative charged lipids (phosphatidylserine and phosphatidylglycerol) has been reported in the *Leishmania* membrane [14]. In accordance with this, the negatively charged lipids DMPS and DMPG were also used for the experiments. First, the measurements were made for pure lipid DMPS and mixtures for DMPS and the peptides at three different molar ratios. Between 15 and 55 °C, hydrated DMPS liposomes dispersed in the buffer HEPES at pH 7.4 showed a phase transition temperature ($L_{\beta'}$−$L_{\alpha'}$) at 36.4 ± 0.1 °C and a transition enthalpy $\Delta H$ of 19.3 kJ mol$^{-1}$ [25] (Fig. 2a, b and Table 3). In the presence of increasing concentrations of the peptide AP-2 it can be seen that there was a broadening and height decrease of the main phase transition peak, with the strongest effect at the highest molar ratio (1:10) (Fig. 2a). The thermograms also show the effect of increasing the concentration of the peptide on the transition enthalpy, which dropped from 19.3 to 3.65 kJ mol$^{-1}$ (Table 3). Peptide AP-2 did not cause significant changes in the main transition temperature, which indicates a lesser effect on the bilayer fluidity. Also, in the evaluation of the interaction between DMPS and cecropin D-like peptide, a similar effect in the transition enthalpy was minimized to a value of 4.91 kJ mol$^{-1}$ (Fig. 2d and Table 3), with almost total height decrease of the main transition peak. Furthermore, the transition temperature dropped up to 32.4 °C, showing a higher level of membrane fluidity in the presence of cecropin D-like peptide.

Finally, the effect exerted by the peptides investigated on PG liposomes was evaluated. Between 10 and 40 °C, fully hydrated DMPG liposomes dispersed in HEPES buffer of pH 7.4 showed a main transition temperature of 24.2 ± 0.1 °C, with a transition enthalpy of 14.2 kJ mol$^{-1}$ (Table 4) [26]. The results showed that increasing the concentration of both peptides produced a progressive broadening of the main transition peak (Fig. 2c, d). The presence of AP-2 or cecropin D-like did not cause any shift in the main transition temperature of DMPG (Fig. 2c, d and Table 4). Furthermore, apart from changes in the shape of the main transition peak shape (broadness and height), changes were also observed in the transition enthalpy in the presence of the both peptides, as $\Delta H$ decreased to 7.33 kJ mol$^{-1}$ and 0.89 kJ mol$^{-1}$ for the highest molar ratio (1:10) for AP-2 and cecropin D-like, respectively (Table 4). The thermotropic behavior of the DMPG also displays a pretransition peak with a $T_m$ of 17.4 °C and a $\Delta H$ of 0.87 kJ mol$^{-1}$ [27]. The pretransition was lost with the highest molar ratio of AP-2 (1:10), indicating an effect in the phase gel packing of DMPG (Fig. 2c). Cecropin D-like appears to have a greater effect than AP-2 on DMPG liposomes, since the main transition peak almost disappeared with the highest concentration at a molar ration of 1:10 (Fig. 2d), which indicates that the membrane is solubilized in the presence of this concentration of peptide. Another effect on DMPG liposomes caused by AP-2 and cecropin D-like peptides was the induction of a shoulder in the main transition peak (Fig. 2c, d), which suggests a direct effect on cooperativity in the transition phase of the lipid.

## Isothermal titration calorimetry (ITC)

To evaluate if there are binding interactions between the PC lipid molecules and the antimicrobial peptides AP-2 and cecropin D-like, the ITC experiments were carried out. The results are shown in Fig. 3, where heat production was recorded in each of the titrations (peptide/DMPC). As is evident from the titration profile, endothermic heat flow decreases with an increase in the number of peptide injections as the free lipid concentration in the vessel progressively decreases. As can be seen from Fig. 3, there is a saturation in the fifth and third injections, to AP-2 and cecropin D-like, respectively. The thermodynamic

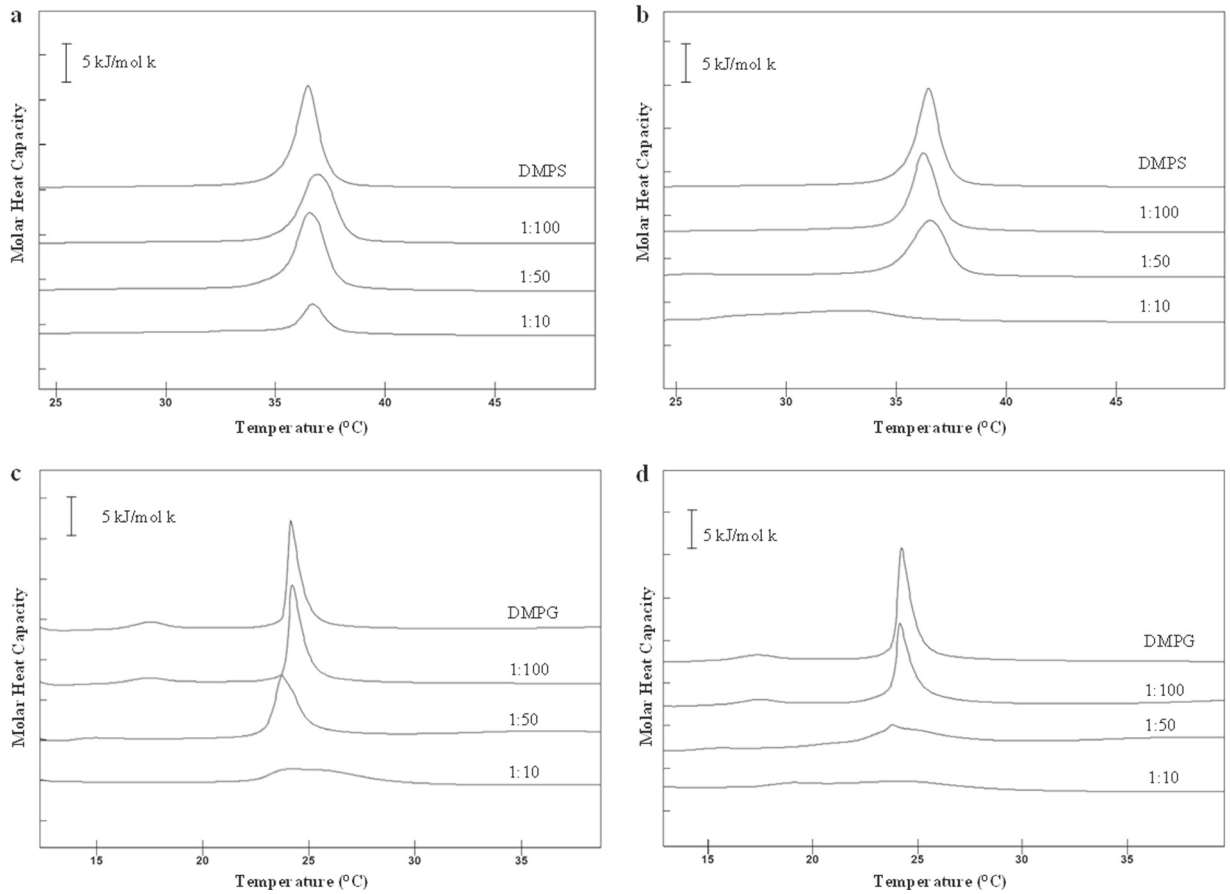


**Fig. 2** Measurement of differential scanning calorimetry obtained for multilamellar DMPS and DMPG liposomes in the presence of anionic peptide 2 (AP-2) (**a**, **c**) and cecropin D-like (**b**, **d**)

**Table 3** Thermodynamic parameters of the pretransition and main phase transition of pure DMPS multilamellar liposomes and peptide/DMPS mixtures determined from heating scans

| DMPS + peptide | | Main transition heating | |
|---|---|---|---|
| | Molar ratio (peptide/lipid) | $\Delta H$ (kJ mol$^{-1}$) | $T_m$ (°C) |
| DMPS | | 19.3 ± 0.8 | 36.4 ± 0.1 |
| AP-2+ DMPS | 1:100 | 18.8 ± 0.8 | 36.9 ± 0.1 |
| AP-2+ DMPS | 1:50 | 17.6 ± 0.8 | 36.5 ± 0.1 |
| AP-2+ DMPS | 1:10 | 3.6 ± 0.8 | 36.6 ± 0.1 |
| Cecropin+ DMPS | 1:100 | 16.4 ± 0.8 | 36.2 ± 0.1 |
| Cecropin+ DMPS | 1:50 | 14.7 ± 0.8 | 36.5 ± 0.1 |
| Cecropin+ DMPS | 1:10 | 4.9 ± 0.8 | 32.4 ± 0.1 |

parameters are shown in Table 5. This indicates a low level of interaction between the peptides and the PC liposomes. Similar results were obtained by varying the lipid concentrations (MLVs) to 15 mM and peptides (up to 0.175 mM) (data not shown).

## Fluorescence anisotropy of DPH

It is well known that DPH fluorophore resides at the hydrophobic core of phospholipid liposomes, which allows one to detect changes in the lipid packaging of the bilayer. The effect of AP-2 and cecropin D-like peptide on the internal lipid-hydrophobic side of PC liposomes was determined by estimating the changes that occurred in the fluorescence anisotropy of DPH ($r$). For the fluorescence anisotropy of DPH experiments, three molar ratios (1:100; 1:50, and 1:10 of peptide:lipid) of each peptide were evaluated, and the anisotropy measurements were made at different temperatures (20, 30, 35, 37, 40, 42, 45, 50, and 55 °C). Figure 4 shows that peptide-free DPPC (control) shows a drastic change in the anisotropy value near the DPPC main transition temperature (41 °C). When comparing the result in the presence of the peptide AP-2 (1:10), it was observed that there is a slight decrease in the anisotropy

**Table 4** Thermodynamic parameters of the pretransition and main phase transition of pure DMPG multilamellar liposomes and peptide/DMPG mixtures determined from heating scans

| DMPG + peptide | | Pretransition heating | | Main transition heating | |
|---|---|---|---|---|---|
| | Molar ratio (peptide/lipid) | $\Delta H$ (kJ mol$^{-1}$) | $T_p$ (°C) | $\Delta H$ (kJ mol$^{-1}$) | $T_m$ (°C) |
| DMPG | | 0.872 | 17.4 ± 0.1 | 14.2 | 24.2 ± 0.1 |
| AP-2+ DMPG | 1:100 | 0.927 | 17.5 ± 0.1 | 11.4 | 24.1 ± 0.1 |
| AP-2+ DMPG | 1:50 | 0.282 | 14.9 ± 0.1 | 10.1 | 23.7 ± 0.1 |
| AP-2+ DMPG | 1:10 | – | – | 7.33 | 24.2 ± 0.1 |
| Cecropin+ DMPG | 1:100 | 0.872 | 17.4 ± 0.1 | 11.0 | 24.2 ± 0.1 |
| Cecropin+ DMPG | 1:50 | 0.106 | 17.5 ± 0.1 | 5.93 | 23.7 ± 0.1 |
| Cecropin+ DMPG | 1:10 | 0.0105 | 14.2 ± 0.1 | 0.89 | 24.8 ± 0.1 |

values ($r$) recorded below the main transition temperature of the DPPC (below 41 °C) (Fig. 4a). This difference could indicate a small destabilizing effect on the bilayer exerted by the peptide in the gel phase. As regards, the results obtained for cecropin D-like, a very low effect of the peptide was observed on the DPPC, since the anisotropy values were very similar for the three molar ratios evaluated (Fig. 4b). Besides the anisotropy changes before the main transition, the anisotropy values recorded between 40 and 42 °C were slightly above the values of the pure DPPC. This could indicate a possible stabilizing effect on the bilayer.

## Discussion

In this research, the effect of the PA-2 and cecropin D-like peptides derived from *G. mellonella* on synthetic membrane models was evaluated. These peptides were initially reported by Cytrynska et al. [11], in response to immune-challenge with *E. coli* D31 on *G. mellonella* larvae. In order to understand the possible interactions between the peptides and the *Leishmania* parasite, we employed liposomes that mimic the composition of the parasite membrane. Around 70% of the plasma membrane of *Leishmania* is composed of phospholipids; of these, the lipids phosphatidylcholine and phosphatidylethanolamine constitute 40% and 10%, respectively [14, 28]. Accordingly, liposomes were constructed from the synthetic lipids DPPC and DPPE.

In order to evaluate peptide/lipid interactions, calorimetric analyzes were carried out using DSC. Lipids are characterized by a main phase transition temperature that depends on the length of the acyl chain and the type of polar head group. In the gel phase (below $T_m$), the hydrocarbon chains are tightly packed, whereas the liquid crystalline phase is defined by a low order of acyl chains and an increase in polar head group hydration [29]. Peptide–lipid interaction can be observed based on a change in the phase transition. DSC has provided quantitative information on the effect of peptide interaction on the membrane structure by comparing the thermotropic data of the pure lipid and the sample with the peptide in a concentration-dependent manner [30]. The results obtained when evaluating AP-2 and cecropin D-like peptides with DPPC liposomes indicated a slight effect on the thermotropic behavior of the lipid (Fig. 1a, b). There was a slight increase in $T_m$ with the highest concentration of the peptides evaluated (Table 1). The increase in $T_m$ in the presence of the peptides may be related to the fact that the gel phase is more stable [31]. Since the phospholipids present on the surface of the *Leishmania* parasites may have acyl chains of up to 18 carbons [14], the peptide's effect on DSPC liposomes was evaluated and the effect detected was similar to that obtained by DPPC, with a slight increase in $T_m$ (results not shown). This indicates that the length of the acyl chains does not have any significant influence on the effect of these peptides on the PC liposomes. On the other hand, the results obtained with DPPE did not show any effect of the peptides (Fig. 1c, d), in spite of the fact that DPPC and DPPE lipids have the same length carbon chains. However, these lipids differ in their terminal amino groups, which are $^{+}N(CH_3)_3$ in DPPC and $^{+}NH_3$ in DPPE. This difference means that DPPE lipids pack is tighter than those of DPPC, because terminal amino groups of DPPE are less polar and have reduced hydration. It has been found that due to the extra hydrogen bonding capabilities from $^{+}NH_3$ and $PO_4^{-}$ between separate bilayers, a tight interaction is formed, reducing the hydration levels [32]. Although the DPPC showed a slight change in its thermotropic behavior in the presence of these peptides, the effect of both peptides on zwiterionic lipids was very low. This could be explained by the fact that because apparently some antimicrobial peptides display a preference for negatively charged lipids, whose proportion in the cell membrane of the microorganisms is greater [33].

Similarly to bacterial membranes, the surface of the parasite *Leishmania* has an anionic character because of the presence of negatively charged phospholipids such as

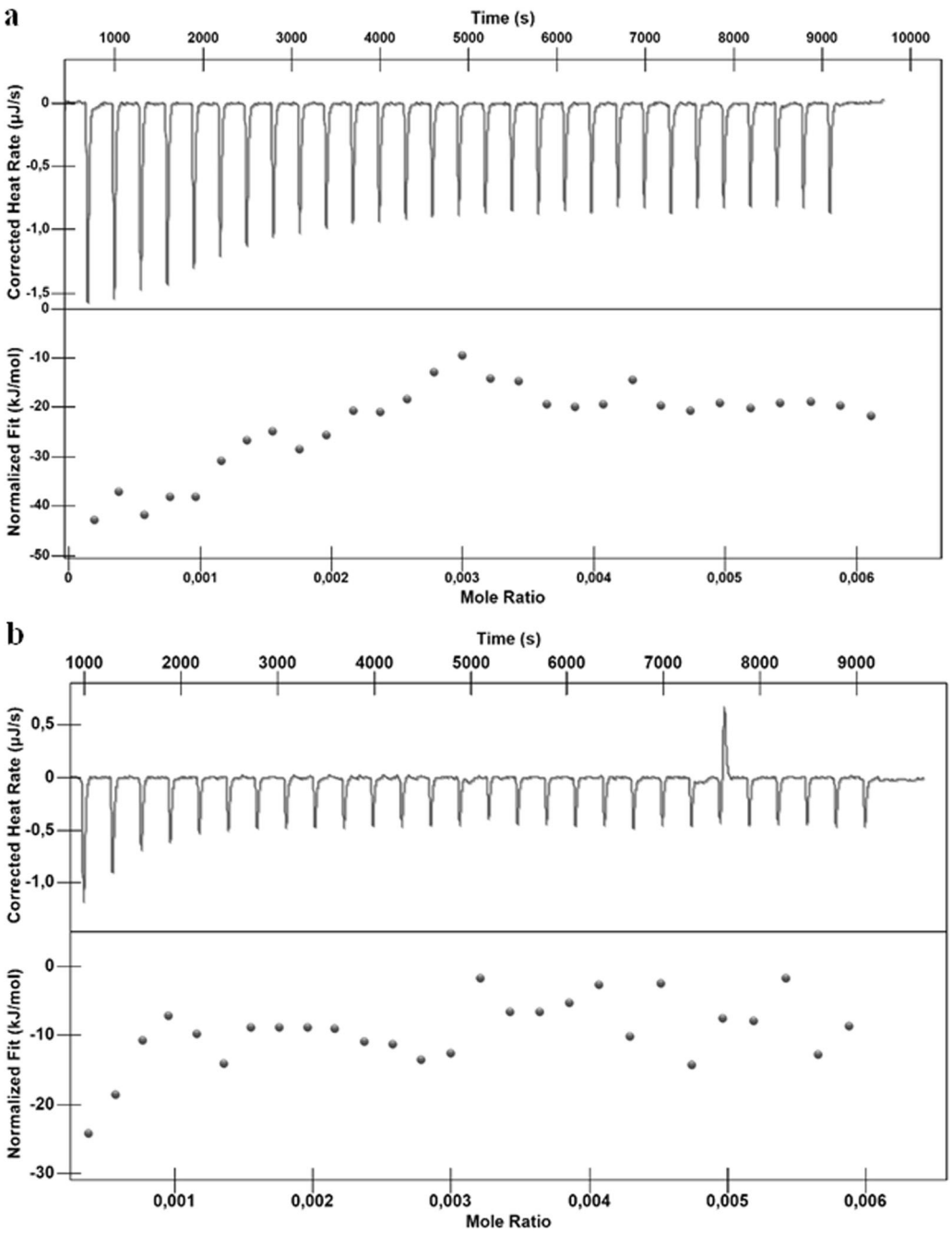


**Fig. 3** Isothermal titration calorimetry of DMPC liposomes (5 mM) with **a** anionic peptide 2 and **b** cecropin D-like, a concentration of 0.125 mM, in 30 injections of 7 µl at 25 °C

phosphatidylserine (1%) and traces of phosphatidylglycerol, which have been identified in different species of *Leishmania* [14]. The models of how AMPs interact on biological membranes are based primarily on electrostatic interaction that occurs between the charges present on the amino acids of the peptide and the negative charges present on the head groups of the phospholipids [6]. Due to the presence of these anionic lipids in the parasite membrane and knowing that AMPs interact electrostatically with lipids, these systems were evaluated using DSC. The results showed a high level of interaction between the peptides and the negative lipids, which produced drastic changes in the thermotropic behavior of PS and PG liposomes. Here, a clear concentration-dependent effect was observed, with a decrease in the transition enthalpy as the peptide concentration increased. This suggests that the peptides bind to

**Table 5** Thermodynamic parameters of DMPC multilamellar liposomes (5 mM) with peptides AP-2 and cecropin D-like(0.125 mM) determined from calorimetric measurements

| Samples | $K_a$ ($M^{-1}$) | $\Delta H$ (kJ $mol^{-1}$) | $n$ | $\Delta S$ (J $mol^{-1}$ $K^{-1}$) | $\Delta G$ (kJ $mol^{-1}$) |
|---|---|---|---|---|---|
| AP-2+ DMPC | $1.000 \times 10^3$ | −33.30 | 0.100 | −52.40 | −17.41 |
| Cecropin+ DMPC | $1.000 \times 10^3$ | −26.39 | 0.100 | −29.61 | −17.41 |

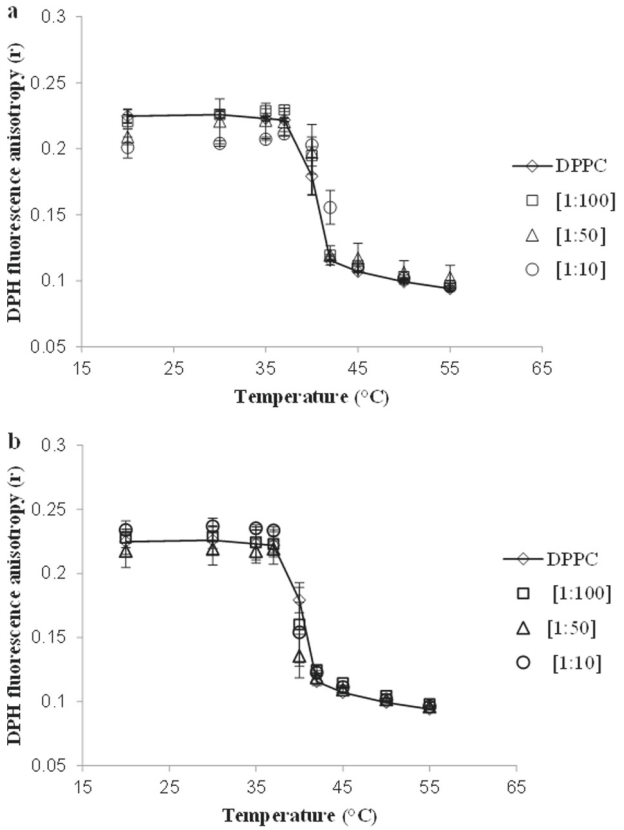


**Fig. 4** DPH fluorescence anisotropy ($r$) values on increasing temperature under the effect of anionic peptide 2 and cecropin D-like. **a** AP-2/DPPC and **b** cecropin/DPPC

the surface of the bilayer [31], with a lower energy requirement for the phase change to occur, which is reflected in $\Delta H$ decrease. Our results show that both peptides had a greater effect on PG than PS liposomes, at a peptide–lipid molar ratio of 1:50 thermotropic changes were more pronounced for PG for both peptides (Fig. 2c, d) as compared to PS at the same molar ratio (Fig. 2a, b). These results agree with those reported by other authors; previous studies showed a preference of some antimicrobial peptides for negatively charged lipids, with a higher affinity for phosphatidylglycerol, followed by phosphatidic acid, phosphatidylserine, and finally cardiolipin [34].

In addition to the decrease in enthalpy, shifts in $T_m$ were observed at the maximum concentration of cecropin D-like peptide both in the case of PS and PG liposomes. On the other hand, AP-2 did not generate changes in the $T_m$ in PG or PS liposomes. The decrease in $T_m$, enthalpy, and cooperativity was generated as the peptide concentration increased. This indicates that cecropin D-like peptide is located at the bilayer interface, where lipid–peptide are bonded by electrostatic interactions and hydrogen linkages. Peptide molecules are associated with polar head groups and the glycerol region and some hydrophobic interactions can occur with the acyl chains situated close to the polar surface [35]. Besides, after exposure of PG liposomes to a 1:50 molar ratio of AP-2 and cecropin D-like peptide, it was possible to see the presence of two endothermic shoulders in the main transition peak. The presence of multiple peaks in the endothermic phase transition with an increase in $T_m$ in the membrane models in the presence of peptides was previously recorded [36]. Chen et al. attributed these changes to a possible aggregation (or pore formation) occurring within the liposome bilayer [36]. The existence of two endothermic peak would indicate the existence of two domains, one composed of lipid–peptide aggregates and the other of lipids free of peptides.

DSC measurements showed a greater effect of cecropin D-like peptide than AP-2 on the anionic lipids, with a complete disappearance of the main transition peak at the highest molar ratio (1:10) of cecropin D-like peptide is observed (Fig. 2b–d), which indicates a complete disintegration of the vesicles. These results suggest that the way in which both peptides interact on the anionic lipid is different and this could mean a difference in the mode of action of the peptides. This distinction in the behavior of both these peptides could be explained by the difference in their hydrophobicity, the cecropin D-like peptide being more hydrophobic than the AP-2. This greater hydrophobicity is manifested through a higher level of interaction of the cecropin D-like peptide with the PS and PG liposomes. Hydrophobicity is a parameter that is directly related to the antimicrobial activity of the AMPs, variations in this characteristic have shown that it is possible to increase or decrease the activity of a peptide [12]. Although cecropin D-like peptide has a net charge of zero at physiological pH, the positively charged amino acids in its sequence are mostly located toward the amino-terminal, while hydrophobic residues are located at the carboxyl-terminal side. This gives the peptide an amphipathic character, with clear polar and hydrophobic sides, which

is important for interaction with the lipids and it may assist in inserting the peptide into the hydrophobic core of the bilayer through its carboxyl-terminal, which results in further destabilization of the membrane. On the contrary, the AP-2 fragment of 41 amino acids used in this study has a net charge of +1 at physiological pH, and this peptide does not present a clearly segregated charged amino acids, thus its amphipathic character is less pronounced than that of cecropin D-like. Despite AP-2 having a higher net charge than cecropin D-like peptide, this does not seem to have an effect since AP-2 generated perturbations in the PS and PG vesicles to a lesser extent. Another effect on PS and PG anionic phospholipids observed with both peptides was the loss of turbidity of the liposome suspension immediately after the peptides were added. As the amount of peptide in the preparation of the liposome suspension was increased, it became more transparent, being completely transparent at a molar ratio of 1:10 for each peptide. In previous studies, this effect was observed in PG, where the authors attributed optical transparency to a low ionic strength, characterized by a wide phase transition, of approximately 18–35 °C, which is induced by a rearrangement in the packaging of bilayer lipids [37, 38]. The reduction in the turbidity of lipid suspension may indicate that the high level of interaction between the anionic lipids and the peptides leads to a reduction in ionic strength. It causes the formation of vesicles of a smaller size or the disintegration of the vesicles in the presence of high concentrations of peptides, which cause that the suspension is transparent.

In addition to measurements using DSC, the effect of these peptides on membrane fluidity was evaluated using a DPH probe. The changes in DPH anisotropy indicate whether or not an exogenous molecule is interacting with the lipid's hydrophobic core. As previously mentioned, phosphatidylcholine is the most abundant lipid in the *Leishmania* membrane, and it is also the most abundant in cells such as erythrocytes. Given this, the effect of the AP-2 and cecropin D-like peptides on the DPPC liposomes was measured as the changes in the anisotropy of fluorescence of DPH. These experiments indicated changes in the DPH anisotropy values at temperatures below the $T_m$ of DPPC (<41 °C), in comparison with pure lipids (Fig. 4). This means that the peptides are exerting an effect on the gel phase of the lipid; lower values in the anisotropy indicate that the DPH probe inserted in the hydrophobic core of the lipid bilayer has a greater rotation capacity. This greater freedom of movement of the DPH is the product of less ordering of the acyl chains [39]. As can be seen in the graph of the anisotropy as a function of temperature (Fig. 4), there is a strong decline in anisotropy values after 40 °C. This is because the bilayer undergoes the transition from the gel phase to the more fluid liquid crystalline phase, which allows the DPH to be more mobile. The anisotropy values recorded between 40 and 42 °C in the presence of AP-2 were slightly above the values of the pure DPPC. The interaction between the peptides and the bilayer could cause the DPH probe to be less mobile, and as a consequence there is a slight increase in anisotropy within this temperature range (40–42 °C). These results agree with a low-level interaction between cecropin D-like peptide and PC lipid previously recorded using DPH probe, where the anisotropy values had a slight increase [40]. Furthermore, these results correlate with those obtained by DSC, where the $T_m$ also rose. As previously mentioned, the peptides because of their interaction with the bilayer induced more stability to liposomes, since there is a slight increase in the $T_m$ of the DPPC. Likewise, the results obtained using ITC showed a very low level of interaction of AP-2 and cecropin D-like peptide with PC liposomes. In these analyses, a rapid saturation was obtained in the first peaks, although the concentration of lipid and peptide varied. Additionally, the effect of liposomes on the peptide contained in the cell was evaluated (results not shown). In both experiments a small change in heat production which is related to the amount of bound ligand was observed. This indicates a low level of bonding between the peptide and lipid molecules [41].

In conclusion, the differential scanning calorimetry studies identified that the AP-2 and cecropin D-like peptides have a preference for some classes of phospholipids. These peptides mainly interacted with negatively charged phospholipids, which are present in the *Leishmania* membrane. On the other hand, these peptides had a lesser effect on the phase transition behavior of zwitterionic PC liposomes and they did not have any effect on PE liposomes. The information obtained in this study is a contribution to the knowledge required to understand the possible action mechanism of AP-2 and cecropin D-like peptide, in order to facilitate the design of new peptides with leishmanicidal activity, which serve as an alternative to the treatment of this disease.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References


1. Torrent M, Pulido D, Rivas L, Andreu D. Antimicrobial peptide action on parasites. Curr Drug Targets. 2012;13:1138–47.
2. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M. Leishmaniasis worldwide and global estimates of its incidence. PLoS ONE. 2012;7:e35671.
3. Sundar S. Drug resistance in Indian visceral leishmaniasis. Trop Med Int Health. 2001;6:849–54.
4. Sundar S, Chakravarty J. Antimony toxicity. Int J Environ Res Public Health. 2010;7:4267–77.
5. Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis. 2001;1:156–64.
6. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev. 2003;55:27–55.
7. Tossi A, Sandri L, Giangaspero A. Amphipathic, α-helical antimicrobial peptides. Biopolymers. 2000;55:4–30.
8. Bulet P, Charlet M, Hetru C. Antimicrobial peptides in insect immunity. In: Ezekowitz RAB, HoVmann JA, editors. Innate immunity. Humana Press Inc: Totowa; 2003. p. 89–107.
9. Bulet P, Stöcklin R. Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept Lett. 2005;12:3–11.
10. Lemaitre B, Reichhart J-M, Hoffmann JA. Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Immunology. 1997;94:14614–14619.
11. Cytryńska M, Mak P, Zdybicka-Barabas A, Suder P, Jakubowicz T. Purification and characterization of eight peptides from Galleria mellonella immune hemolymph. Peptides. 2007;28:533–46.
12. Chen Y, Guarnieri MT, Vasil AI, Vasil ML, Mant CT, Hodges RS. Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides. Antimicrob Agents Chemother. 2007;51:1398–406.
13. Sato H, Feix JB. Peptide–membrane interactions and mechanisms of membrane destruction by amphipathic α-helical antimicrobial peptides. Biochim Biophys Acta. 2006;1758:1245–1256.
14. Zhang K, Beverley SM. Phospholipid and sphingolipid metabolism in Leishmania. Mol Biochem Parasitol. 2010;170:55–64.
15. Ramarao N, Nielsen-Leroux C, Lereclus D. The insect Galleria mellonella as a powerful infection model to investigate bacterial pathogenesis. J Vis Exp. 2012; e4392, https://doi.org/10.3791/4392
16. Cook SM, McArthur JD. Developing Galleria mellonella as a model host for human pathogens. Virulence. 2013;4:350–3.
17. Mak P, Zdybicka-Barabas A, Cytryńska M. A different repertoire of Galleria mellonella antimicrobial peptides in larvae challenged with bacteria and fungi. Dev Comp Immunol. 2010;34:1129–1136.
18. Mak P, Chmiel D, Gacek GJ. Antibacterial peptides of the moth Galleria mellonella. Acta Biochim Pol. 2001;48:1191–5.
19. Brown SE, Howard A, Kasprzak AB, Gordon KH, East PD. A peptidomics study reveals the impressive antimicrobial peptide arsenal of the wax moth Galleria mellonella. Insect Biochem Mol Biol. 2009;39:792–800.
20. Isabel A. Patiño-Márquez, Edwin Patiño-González, Laura Hernández-Villa, Blanca Ortíz-Reyes, Marcela Manrique-Moreno, Identification and evaluation of Galleria mellonella peptides with antileishmanial activity. Anal Biochem. 2018;546:35–42.
21. Pignatello R, Musumeci T, Basile L, Carbone C, Puglisi G. Biomembrane models and drug-biomembrane interaction studies: involvement in drug design and development. J Pharm Bioallied Sci. 2011;3:4–14.
22. Pons M, Foradada M, Estelrich J. Liposomes obtained by the ethanol injection method. Int J Pharm. 1993;95:51–56.
23. Koynova R, Caffrey M. Phases and phase transitions of the phosphatidylcholines. Biochim Biophys Acta. 1998;1376:91–145.
24. Koynova R, Caffrey M. Phases and phase transitions of the hydrated phosphatidylethanolamines. Chem Phys Lipids. 1994;69:1–34.
25. Lewis RN, McElhaney RN. Calorimetric and spectroscopic studies of the thermotropic phase behavior of lipid bilayer model membranes composed of a homologous series of linear saturated phosphatidylserines. Biophys J. 2000;79:2043–55.
26. Koynova R. Liquid crystalline phase metastability of phosphatidylglycerols. Chem Phys Lipids. 1997;89:67–73.
27. Kinoshita M, Kato S, Takahashi H, Kinoshita M. NaCl-dependent formation of the highly crystalline phase in sufficiently hydrated dimyristoylphosphatidylglycerol bilayers. Chem Phys Lipids. 2009;161:1–16.
28. Wassef MK, Fioretti TB, Dwyer DM. Lipid analyses of isolated surface membranes of Leishmania donovani promastigotes. Lipids. 1985;20:108–15.
29. Tristram-Nagle S, Nagle JF. Lipid bilayers: thermodynamics, structure, fluctuations, and interactions. Chem Phys Lipids. 2004;127:3–14.
30. Lohner K, Prenner EJ. Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimetic systems. Biochim Biophys Acta. 1999;1462:141–56.
31. Chiu MH, Prenner EJ. Differential scanning calorimetry: an invaluable tool for a detailed thermodynamic characterization of macromolecules and their interactions. J Pharm Bioallied Sci. 2011;3:39–59.
32. McIntosh TJ. Hydration properties of lamellar and non-lamellar phases of phosphatidylcholine and phosphatidylethanolamine. Chem Phys Lipids. 1996;81:117–31.
33. Teixeira V, Feio MJ, Bastos M. Role of lipids in the interaction of antimicrobial peptides with membranes. Prog Lipid Res. 2012;51:149–177.
34. Arouri A, Dathe M, Blume A. Peptide induced demixing in PG/PE lipid mixtures: a mechanism for the specificity of antimicrobial peptides towards bacterial membranes? Biochim Biophys Acta. 2009;1788:650–659.
35. Papahadjopoulos D, Moscarello M, Eylar EH, Isac T. Effects of proteins on the thermotropic phase transitions of phospholipid membranes. Biochim Biophys Acta. 1975;401:317–335.
36. Chen HM, Leung KW, Thakur NN, Tan A, Jack RW. Distinguishing between different pathways of bilayer disruption by the related antimicrobial peptides cecropin B, B1 and B3. Eur J Biochem. 2003;270:911–20.
37. Heimburg T, Biltonen RL. Thermotropic behavior of dimyristoylphosphatidylglycerol and its interaction with cytochrome c. Biochemistry. 1994;33:9477–88.
38. Lamy-Freund MT, Riske KA. The peculiar thermo-structural behavior of the anionic lipid DMPG. Chem Phys Lipids. 2003;122:19–32.
39. Parasassi T, De Stasio G, Rusch RM, Gratton E. A photophysical model for diphenylhexatriene fluorescence decay in solvents and in phospholipid vesicles. Biophys J. 1991;59:466–475.
40. Oñate-Garzón J, Manrique-Moreno M, Trier S, Leidy C, Torres R, Patiño E. Antimicrobial activity and interactions of cationic peptides derived from Galleria mellonella cecropin D-like peptide with model membranes. J Antibiot. 2016;70:238–45.
41. Pierce MM, Raman CS, Nall BT. Isothermal titration calorimetry of protein–protein interactions. Methods. 1999;19:213–221.