

Susceptibility of sheep, human, and pig erythrocytes to haemolysis by the antimicrobial peptide Modelin 5

Sarah R. Dennison · David A. Phoenix

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Abstract Modelin-5-CONH₂, a synthetic antimicrobial peptide, was used to gain an insight into species-selective haemolytic activity. The peptide displayed limited haemolytic activity against sheep (12 %), human (2 %), and pig (2 %) erythrocytes. Our results show that Modelin-5-CONH₂ had a disordered structure in the presence of vesicles formed from sheep, human, and pig erythrocyte lipid extract (<26 % helical) yet folded to form helices in the presence of a phosphatidylcholine (PC) membrane interface (e.g. >42 % in the presence of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine). Monolayer studies showed a strong correlation between anionic lipid content and monolayer insertion and lysis inducing surface pressure changes of 9.17 mN m⁻¹ for 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine compared with PC monolayers, which induced pressure changes of ca. 3 mN m⁻¹. The presence of cholesterol in the membrane is shown to increase the packing density as the PC:spingomyelin (SM) ratio increases so preventing the peptide from forming a stable association with the membrane. The data suggests that the key driver for membrane interaction for Modelin-5-CONH₂ is the anionic lipid attraction. However, the key factors in the species-specific haemolysis level for this peptide are the differing packing densities which are influenced by the SM:PC:cholesterol ratio.

Keywords Antimicrobial peptide · α -Helical · Haemolysis · Langmuir monolayer · Membrane interactive · Thermodynamic analysis

Introduction

The overuse of antibiotics in the agriculture industry has led to drug-resistant bacteria that have been postulated to enter the human food chain (Bischoff et al. 2002), and as a consequence it has been suggested that this could lead to foodborne bacterial infections in humans which are less responsive to treatment with conventional antibiotics. For example, the antibiotic chloramphenicol (CHL) was used extensively until veterinary medicine links were made with human illnesses such as aplastic anaemia (Settepani 1984; Bischoff et al. 2002). The detection of CHL in human food products led to a ban by the US Food and Drug Administration on its use in food animals (Gilmore 1986). Similarly, since their discovery in 1945, tetracyclines have been extensively used in veterinary medicine for the treatment of diseases such as respiratory, gastrointestinal, and skin infections (Michalova et al. 2004), but resistance to this antibiotic has now been widely reported. Increased resistance to bacterial infections due to the overuse of antibiotics in veterinary practice has been a driving force for the development of newer antimicrobial agents (Amaral et al. 2007; Courvalin 2005; Tredan et al. 2007). Consequently, veterinary science has turned to other sources of potential new antibiotics such as antimicrobial peptides (AMPs) (Dennison and Phoenix 2011b).

Despite possessing many attractive properties, one of the key challenges of developing AMPs for wider use remains the production of peptides that are able to target microorganism without causing haemolysis and damage

S. R. Dennison
School of Pharmacy and Biomedical Sciences, University
of Central Lancashire, Preston PR1 2HE, UK

D. A. Phoenix (✉)
London South Bank University, 103 Borough Road,
London SE1 0AA, UK
e-mail: phoenixd@lsbu.ac.uk

to eukaryotic cells (Wainwright 2000; Rice et al. 2000). A high therapeutic index, which is the ratio of antimicrobial activity to haemolytic activity, is necessary if AMPs are to have medical relevance, and new AMPs with low haemolytic activity have been used as a base template to develop other peptides. Indeed, Bessalle et al. (1993) have tried to overcome these challenges of haemolysis using quantitative structure–activity relationships (QSAR) of AMPs to develop a family of novel synthetic peptides called modelins. The synthetic modelin peptide family displays a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria yet have low haemolytic activity in human erythrocytes.

It is thought that AMPs work primarily through a membranolytic mechanism of action, and it is known that their efficacy depends not only on the peptide sequence but also the membrane phospholipid composition. However, the lipid composition not only varies between cell types but also between species, adding to the complexity of using AMPs in veterinary practice. Subtle differences in the lipid composition of mammalian erythrocytes have been shown to result in different membrane interaction with AMPs with resultant variations in susceptibility and changes to the therapeutic index (Zhu et al. 2007; Dennison and Phoenix 2011a). Selectivity for a membrane may be affected by the abundance of a specific lipid or by fluctuations in sterol level, which are known to show species-specific variations. For example, the phospholipid content of sheep erythrocytes differs considerably from human red blood cells and pig red blood cells. Sheep erythrocytes contains 1.6 % phosphatidylcholine (PC) whereas human and pig erythrocytes contain 39.5 and 30.3 %, respectively (Salvioli et al. 1993). In contrast, other phospholipids such as phosphatidylserine (PS) (12.9–16.1 %) and phosphatidylethanolamine (PE) (24.4–28.2 %) are present at comparable levels across all three species. Such variations in the overall lipid composition are known to affect membrane-packing characteristics and, therefore, their susceptibility to lysis (Salvioli et al. 1993). Hence, such changes in susceptibility to lysis can be influenced by the presence of sterols such as cholesterol, which is a major component of erythrocyte membrane at an average content of 28 % (Nelson 1967). Cholesterol has been shown to provide a protective effect against AMP induced lysis, but the impact of cholesterol on AMP efficacy is known to be effected by the overall lipid composition of the membrane (Dennison and Phoenix 2011a). Therefore, the mode of action of AMPs is being investigated as is their potential for use in veterinary medicine. There are few studies on species specificity. Here, we use the erythrocyte cell membrane as a model to investigate species-specific variation in Modelin-5-CONH₂ activity for sheep, human, and pig.

Materials and methods

Materials

All phospholipids: 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (DMPS), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. Modelin-5-CONH₂ (KLAKKLAK-LAKLAKAL-CONH₂) was synthesized by Pepceuticals (Leicestershire, UK) to purity >95 %. All buffers were prepared using ultrapure water (resistivity 18 MΩ cm). Solvents were obtained from VWR (HPLC grade) and all other reagents were purchased from Sigma-Aldrich (UK).

Haemolysis assay

Three millilitres of fresh sheep, human, and pig red blood cells were washed three times with PBS (35 mM phosphate buffered saline, 0.15 M NaCl, pH 7.4) by centrifugation for 5 min at 1,200×*g* until the supernatant was clear. Washed red blood cells were resuspended in PBS to a final volume of 20 ml. The peptide solutions (10 μl) were added to 190 μl suspension of washed red blood cells and were incubated for 1 h at 37 °C. The samples were then centrifuged at 12,000×*g* for 5 min. The release of haemoglobin was monitored by diluting 100 μl of supernatant with 900 μl PBS and absorbance measured at 576 nm. For negative and positive controls, PBS buffer [*A*_{PBS}] and 0.1 % Triton X-100 [*A*_{Triton}] were used. The percentage haemolysis was calculated according to the following equation (Oh et al. 2000; Song et al. 2005):

$$\text{Percentage haemolysis} = \frac{[A_{\text{Peptide}}] - [A_{\text{PBS}}]}{[A_{\text{Triton}}] - [A_{\text{PBS}}]} \times 100.$$

Circular dichroism measurements

Modelin-5-CONH₂ (0.1 mg ml⁻¹) was either dissolved in PBS pH 7.4 or small unilamellar vesicle (SUVs) suspensions to maintain a peptide to lipid ratio of 1:100. To obtain the SUVs, 5 mg ml⁻¹ DMPS, DOPC, DMPC, and SM were dissolved in chloroform, evaporated under a stream of nitrogen, placed under vacuum for 4 h and rehydrated using PBS pH 7.5. The suspension was vortexed for 5 min before being sonicated for 30 min. The resultant solution was then subjected to three cycles of freezing and thawing. The CD spectra were obtained using a J-815 spectropolarimeter (Jasco, UK) utilizing a 10 mm path length cell over a wavelength range 260–180 nm at 100 nm min⁻¹, 1 nm band width, data pitch 0.5 nm and samples maintained at 20 °C. For all spectra acquired, ten scans were automatically averaged and the

baseline acquired in the absence of peptide was subtracted (Henzler Wildman et al. 2003). The percentage helical content was estimated using a method previously described by Forood et al. (1993). The secondary structure of Modelin-5-CONH₂ was also investigated in the presence of sheep, human, and pig red cell lipid extract using the methodology previously described by Dennison and Phoenix (2011a). In summary, fresh sheep, human, and pig blood was washed four times in cold 145 mM NaCl, 5 mM KCl, 5 mM Hepes, pH 7.4 and centrifuged at 2,000×*g* for 10 min. Cells were then lysed using a cold buffer containing 15 mM KCl, 0.01 mM EDTA, 1 mM EGTA, 5 mM Hepes, at pH 6.0. The cell suspension was centrifuged for 10 min at 4 °C and 12,000×*g*. The cell pellet was washed with 15 mM KCl, 0.01 mM EDTA, 5 mM Hepes, and then centrifuged for a further 10 min at 4 °C and 12,000×*g*. The erythrocyte cell pellets were resuspended in PBS pH 7.4 and to a 0.4 ml aliquot of this cell suspension, 1.5 ml of a 1:2 (v/v) chloroform–methanol mixture was added and then vortexed. Then 0.5 ml water was added and vortexed for 5 min before being centrifuged at low speed (660 g, 5 min) to produce two phases. The lower organic layer was concentrated and dried under N₂ gas.

Peptide insertion into lipid monolayers

To quantify the interaction of the peptide isoforms with phospholipid monolayers, constant area insertion studies were undertaken at 21 °C using a 601M Langmuir Teflon trough (NIMA Technology) equipped with moveable barriers at room temperature. Then 8.5 × 10¹⁵ phospholipid molecules of DMPS, DMPC, DOPC, and SM in chloroform were spread separately onto an air-buffer (10 mM Tris, pH 7.4) interface using a Hamilton microsyringe. The solvent was allowed to evaporate for 15 min before compression of the monolayer to the surface pressure of 30 mN m⁻¹, which corresponds to the packing density of a cell membrane (Seeling 1987; Gennaro and Zanetti 2000) and the liquid condensed phase of the lipids. Once equilibrated, the surface area of the monolayer (0.9 nm² molecule⁻¹) was kept constant throughout the experiment. The Modelin-5-CONH₂ peptide solution was injected underneath the monolayer using an L-shaped needle syringe to give a final peptide concentration of 6 μM in the subphase. Surface pressure increases were monitored by the Wilhelmy method using a Whatman's CH1 filter paper plate and microbalance (Demel 1974). Monolayers were also repeated by varying the DMPC:DMPS ratio and maintaining the DMPS level (15 %) whilst varying the DMPC:SM ratio and also maintaining the cholesterol content.

To investigate the packing effect of SM/PC, compression isotherms of SM/PC 25, 50 and 75 % were performed by spreading 2.5 × 10¹⁵ phospholipid molecules onto a

10 mM Tris, pH 7.4 buffer subphase. The solvent was allowed to evaporate off before compression of the barriers at a speed of 2.08 nm² min⁻¹ until a monolayer collapse pressure was achieved. These experiments were repeated in the presence of 6 μM Modelin-5-CONH₂. The compressibility modulus (*C*_s⁻¹) for each SM percentage was calculated applying the following equation (Davies and Rideal 1963):

$$C_s^{-1} = -A \left(\frac{\delta\pi}{\delta A} \right),$$

where *A* is the area per molecule at a given surface pressure (*π*).

Using the determined isotherms, the thermodynamic stability of monolayers was investigated by applying the Gibbs equation:

$$\Delta G_{\text{mix}} = \int [A_{12} - (X_1 A_1 + X_2 + A_2)] d\pi,$$

where *A*_{1,2,...*n*} is the molecular area occupied by the mixed monolayer, *A*₁, *A*₂...*A*_{*n*} is the area per molecule in the pure monolayers of component, 1, 2, ... *n*, *X*₁, *X*₂...*X*_{*n*} are the molar fractions of the components, and *π* is the surface pressure. Numerical data were calculated from the compression isotherms according to the mathematical method of Simpson (Todd 1963).

These experiments were repeated in the presence of 0.87 cholesterol molar ratio.

Results

Haemolytic activities of Modelin-5-CONH₂

An investigation into the haemolytic properties of Modelin-5-CONH₂ with respect to a range of mammalian species was undertaken. The haemolytic effects of Modelin-5-CONH₂ towards sheep, pig, and human erythrocytes was observed (Fig. 1). Figure 1a shows that overall Modelin-5-CONH₂ has relatively low haemolytic activity (<14 %). ANOVA and post hoc multiple comparison showed a significant difference between sheep, human, and pig erythrocytes with the former showing 12 % lysis and the latter two species showing 2 %. There was no significant difference in the percentage haemolysis between human and pig erythrocytes (*p* > 0.05). The haemolysis kinetics of Modelin-5-CONH₂ at a peptide concentration of 300 μM, which was the concentration at maximum lysis, was investigated over a time course of 6 h (Fig. 1b). Figure 1b shows that the haemolysis versus time follows a standard sigmoidal curve for each of the erythrocytes studied. A lag phase where there is relatively little or no haemolysis was observed for human and pig erythrocytes,

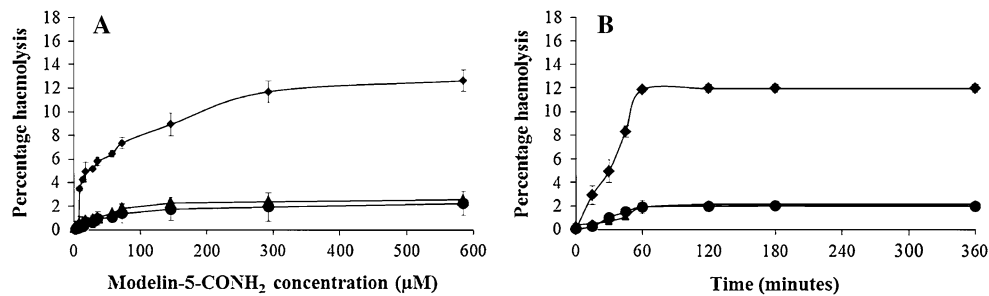
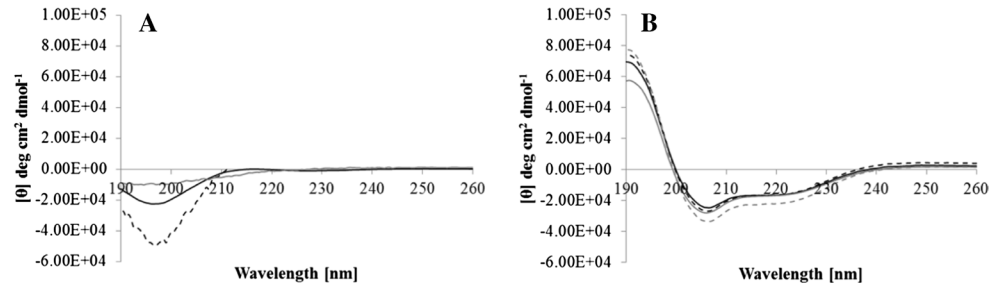


Fig. 1 Percentage haemolysis of Modelin-5-CONH₂ against sheep (filled diamond), human (filled triangle), and pig (filled circle) red blood cells. **a** Percentage haemolysis of Modelin-5-CONH₂ at

concentrations 0–600 μM. **b** Kinetics of haemolysis of 300 μM Modelin-5-CONH₂. Standard error bars are based on four replicates

Fig. 2 CD spectra of Modelin-5-CONH₂. **a** Modelin-5-CONH₂ in the presence pig (solid black), sheep (dotted black), and human (grey) total lipid extract. **b** Modelin-5-CONH₂ in the presence of DMPS (dotted grey), DMPC (black), DOPC (dotted black), and SM (dark grey)



which was followed by a period of rapid haemolysis up to 60 min. However, for sheep erythrocytes the length of the lag phase was not detectable, suggesting that the lysis was more rapid up to 60 min. After 60 min, a static phase was observed indicating that there was no further lysis for sheep, human, and pig erythrocytes.

Secondary structure analysis

Previous studies have shown that Modelin-5-CONH₂ adopted an unordered structure in aqueous solution, but in a membrane environment adopted an α -helical structure (Bessalle et al. 1993). The structure of Modelin-5-CONH₂ was investigated in the presence of vesicles formed from different lipid blood extracts. Figure 2a shows that in the presence of vesicles formed from pig, sheep, and human blood lipid extracts, the peptide showed an unordered structure displaying low levels of helicity ca. 20 % (Table 1). However, in the presence of vesicles formed from either DMPS, DMPC, DOPC, or SM, the CD spectra in Fig. 2b showed a characteristic α -helical structure displaying minima at 221–222 and 209–210 nm and a maximum at about 195 nm. Further analysis indicated that the percentage helicity for DMPS was 57 % and for DOPC, DMPC, and SM helicity was observed to be 44, 42 and 44 %, respectively (Table 1). Further statistical analysis showed there were no significant differences between the levels of helicity induced by Modelin-5-CONH₂ in the presence of DOPC, DMPC and SM ($F_{2, 8} = 4.716$; $p = 0.059$).

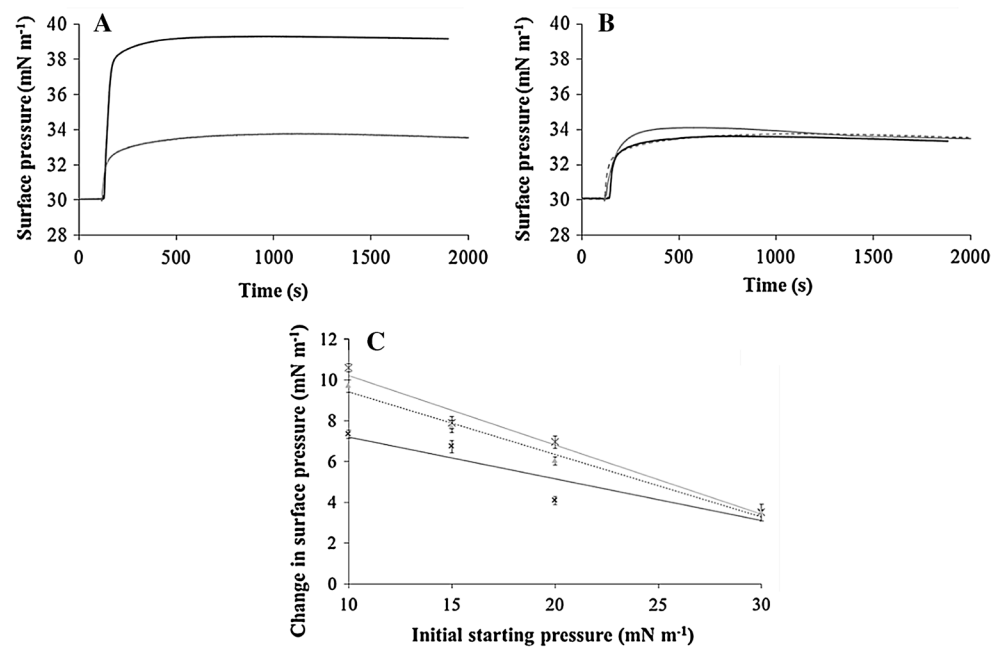
Table 1 Percentage helicity of Modelin-5-CONH₂

Lipid composition	Percentage helicity
Pig	26 ± 0.6
Sheep	21 ± 0.2
Human	24 ± 0.5
DMPS	57 ± 0.8
DOPC	43 ± 0.7
DMPC	42 ± 0.3
SM	44 ± 1.2

Monolayer interactions of Modelin-5-CONH₂

The interactions of Modelin-5-CONH₂ with each of the main phospholipids were investigated (Fig. 3). For each of the phospholipids studied, 8.5×10^{15} molecules was spread onto a buffered subphase and were found to form stable monolayers at a surface pressure of 30 mN m⁻¹, which was taken to represent that of naturally occurring membranes (Seeling 1987). Modelin-5-CONH₂ was found to interact strongly with both anionic and zwitterionic lipid monolayers. Figure 3a shows that Modelin-5-CONH₂ has a high affinity for anionic DMPS membranes, inducing surface pressure changes of 9.17 mN m⁻¹. These levels of interaction are consistent with disruption of the monolayer acyl chain region by the peptide and are comparable to those reported for other membrane active peptides (Dennison et al. 2007). The

Fig. 3 Monolayer interactions of Modelin-5-CONH₂ with lipid monolayers. **a** Lipid monolayers formed from DMPS (*black*) and DMPC (*grey*). Monolayers were set at an initial surface pressure of 30 mN m⁻¹, mimetic of naturally occurring membranes and after 120 s the peptide was introduced into the Tris buffer subphase. **b** Lipid monolayers, which were formed DMPC (*black*), DOPC (*dotted grey*), and SM (*grey*) at an initial surface pressure of 30 mN m⁻¹. **c** Maximum surface pressure change as a function of different initial surface pressure for DMPC (*black*), DOPC (*dotted grey*), and SM (*grey*)



cationic CONH₂ moieties at the C-termini of Modelin-5-CONH₂ may contribute to membrane binding of the peptide, which facilitates the electrostatic interaction with the head groups of anionic lipid (Fig. 3a). However, although the peptide inserted readily into anionic lipid, the peptide also induced pressure increases ca. 3 mN m⁻¹ for DMPC membranes (Fig. 3a), suggesting that hydrophobic forces may also be important for peptide insertion (Yu et al. 2009). Since both saturated and unsaturated phospholipid tail groups are common components of cell membranes (Ishitsuka et al. 2006), the effect of substituting the tail group of PC lipid on Modelin-5-CONH₂ insertion was investigated using constant area assays. Comparison was made between DMPC, DOPC, and SM membranes (Fig. 3b) and showed that even though the tail group packing density had been altered, the peptide induced surface pressure changes of ca. 3.5 mN m⁻¹ in all cases indicating that there was no effect on insertion. In order to investigate the tail group packing density further, constant pressure monolayer assays were undertaken to evaluate the selectivity of Modelin-5-CONH₂ at various surface pressures. Figure 3c shows insertion of Modelin-5-CONH₂ decreased linearly ($R^2 > 0.9$) as the surface pressure increased regardless of the monolayer type. Furthermore, higher surface pressure increases were observed for SM monolayers, which indicates tighter tail group packing.

To gain insight into the putative role of anionic lipid in the peptide's interactions with erythrocyte cell membranes, synthetic monolayer mimics of erythrocyte lipid membranes were constructed with precisely known

compositions. Monolayer studies were used to investigate the PS:PC ratio of human (1:3), pig (1:2) and sheep (1:0.11) erythrocytes. Increasing the level of PC was seen to reduce the maximal surface pressure changes induced by Modelin-5-CONH₂ and showed a negative correlation with their PS levels decreasing (Fig. 4a). This would indicate that increasing the PC level dilutes the anionic PS level leading to lower levels of binding and decreased insertion of the peptide into the membrane.

The haemolytic susceptibility of various mammalian species has been demonstrated to be related to the PC and SM content (Belokoneva et al. 2003). Researchers have shown that in comparison to human erythrocytes, the PC levels in sheep and cow erythrocytes is replaced with SM although the PS level remains constant (Salvioli et al. 1993). According to Lowe and Coleman (1981), SM rich membranes are less fluid and were less readily lysed than membranes of higher fluidity. However, the interaction of Modelin-5-CONH₂ with PS levels remaining constant, and varying the PC–SM ratio showed no significant changes in surface pressure increases (Fig. 4b). Further ANOVA analysis revealed that there were no statistically significant differences between the surface pressure increases and varying the PC–SM ratio ($F_{4, 14} = 3.6$; $p = 0.046$). Since cholesterol is a major component of erythrocytes, the ratio of PC–SM was investigated further in the presence of a 0.87 cholesterol molar ratio (Fig. 4c), which corresponds to the levels of cholesterol in a mammalian cell membrane (Nelson 1967). Replacing the PC with SM in the presence of a constant level of cholesterol showed an increase in Modelin-5-CONH₂ insertion.

Fig. 4 **a** The effect of varying PC levels in monolayers on the levels of interaction shown by Modelin-5-CONH₂. **b** The effect of varying PC:SM ratio maintaining the PS level at 15 % in monolayers on the levels of interaction shown by Modelin-5-CONH₂. *Error bars are SD.* **c** The effect of varying the PC:SM ratio maintaining 0.87 cholesterol ratio in monolayers on the levels of interaction shown by Modelin-5-CONH₂

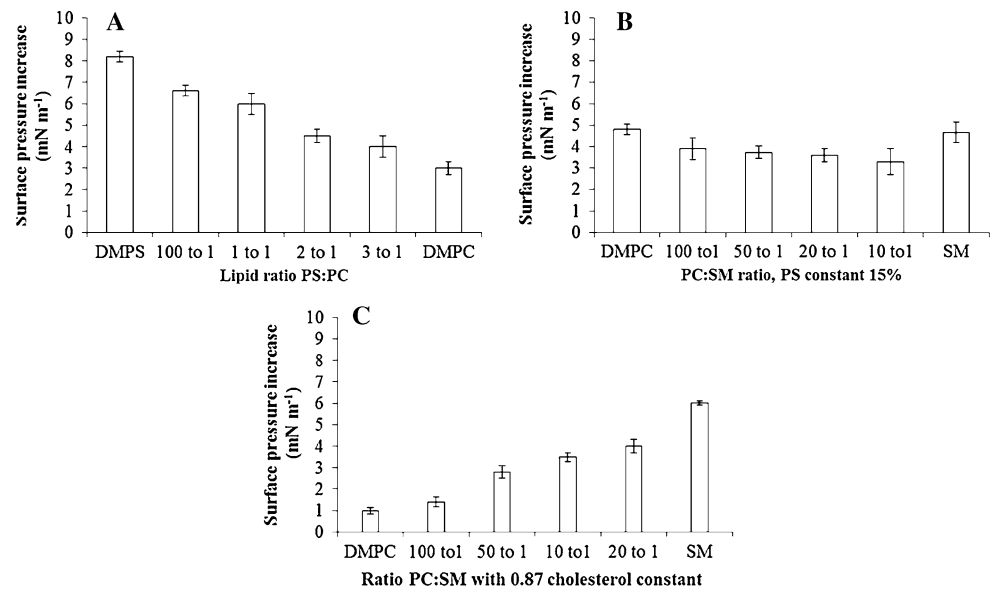


Fig. 5 Compression isotherms derived from lipid mixtures that corresponded to membranes of DMPC (*solid grey*), SM (*solid black*), 25 % SM (*dotted black grey*), 50 % SM (*dotted grey*), and 75 % SM (*solid light grey*) in the absence (**a**) and presence of Modelin-5-CONH₂ (**b**). **c, d** The Gibbs free energy of mixing (ΔG_{mix} , J mol⁻¹) of 25 % SM (*grey*), 50 % SM (*black*), and 75 % SM (*white*) lipid monolayers at varying surface pressure in the absence (**c**) and presence of Modelin 5 (**d**)

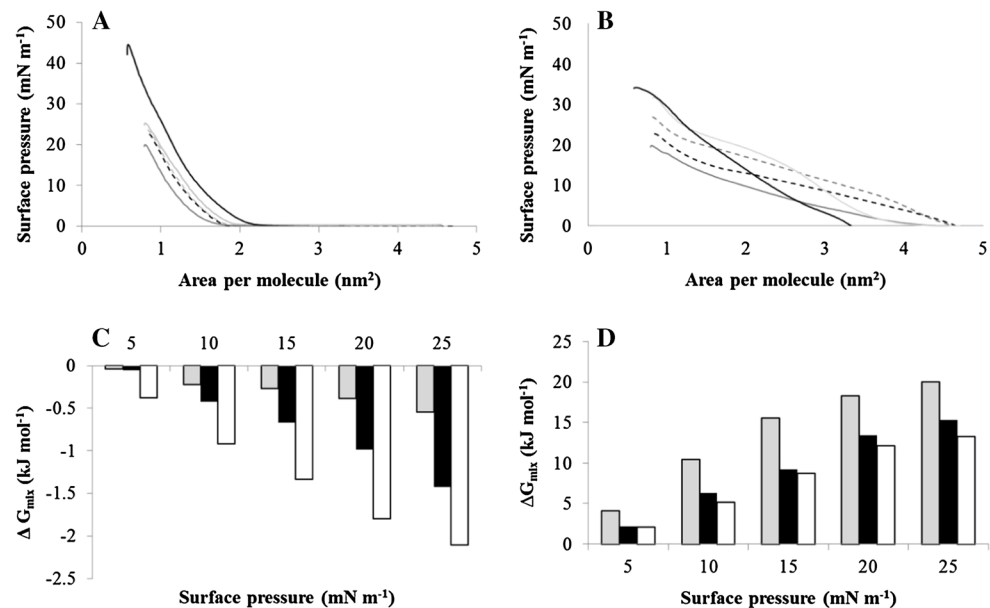


Table 2 Compressibility modulus (C_s^{-1} , mN m⁻¹) for Modelin-5-CONH₂ in the presence of DMPC and SM

Surface pressure (mN m ⁻¹)	Absence of peptide			Modelin-5-CONH ₂		
	25 % SM	50 % SM	70 % SM	25 % SM	50 % SM	70 % SM
10	26.00	26.90	26.37	37.05	21.77	12.60
15	32.82	32.71	30.92	32.34	13.23	6.70
20	38.62	36.38	33.18	14.28	8.83	8.01

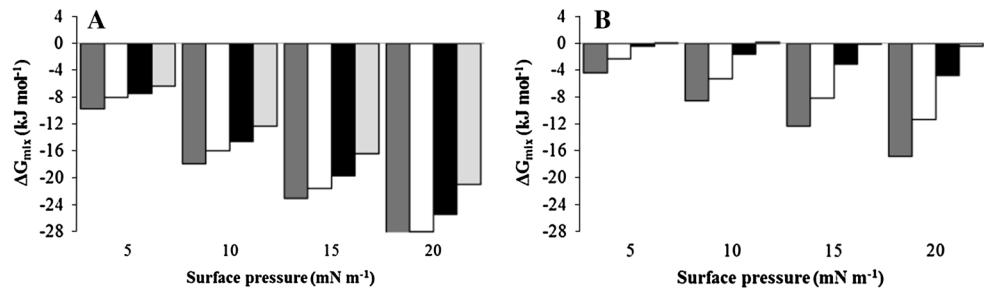
Thermodynamic analysis of monolayers

The packing characteristics of SM:PC monolayers were investigated using the compressibility modulus (C_s^{-1}). Figure 5 shows the compression isotherms of SM:PC at 25, 50 and 75 % SM in the absence (Fig. 5a) and presence

of Modelin-5-CONH₂ (Fig. 5b). The results would suggest that SM increases the area per phospholipid molecule, which is reflected in the C_s^{-1} . This showed that an increase in SM decreased the C_s^{-1} value in the absence of peptide (Table 2). The same trend was observed in the presence of peptide. The Gibbs's free energy of mixing (ΔG_{mix})

Table 3 Compressibility modulus (C_s^{-1} , mN m^{-1}) for Modelin-5-CONH₂ in the presence of DMPC and SM containing a 0.87 cholesterol molar ratio

Surface pressure (mN m^{-1})	Absence of peptide				Modelin-5-CONH ₂			
	10 to 1	20 to 1	50 to 1	100 to 1	10 to 1	20 to 1	50 to 1	100 to 1
10	29.61	52.25	61.84	66.05	30.75	25.61	21.79	27.25
15	36.52	65.66	70.07	73.28	32.80	28.04	22.12	22.63
20	51.99	52.65	65.24	77.24	36.10	28.23	32.59	22.28

Fig. 6 The Gibbs free energy of mixing (ΔG_{mix} , J mol^{-1}) of SM:PC ratios 100:1 (grey), 50 to 1 (white), 20:1 (black), and 10:1 (light grey) containing a 0.87 molar ratio of cholesterol in the absence of peptide (a) and presence of peptide (b)

was used to investigate the stability of the monolayers. Figure 5c shows that, in the absence of peptide, negative values of ΔG_{mix} were observed for each of the SM concentrations. Increasing the SM concentration ΔG_{mix} was more negative, indicating that these monolayers were thermodynamically more stable. However, in the presence of peptide (Fig. 5d), the stability of the monolayers was reduced and positive ΔG_{mix} values were observed. The thermodynamic stability of the PC:SM monolayer in the presence of cholesterol was also investigated (Table 3). Figure 6a shows that in the absence of peptide, although at constant cholesterol levels, increasing the SM content increased the stability of the monolayer ($\Delta G_{\text{mix}} < 0$). However, in the presence of peptide, even though the monolayers are thermodynamically stable, the ΔG_{mix} values increased, indicating that Modelin-5-CONH₂ had a slight destabilizing effect on the monolayer compared to the absence of peptide.

Discussion

The ability of an AMP to target a cell membrane has led to a number of proposed models of membrane interaction, which include the barrel stave pore model, the toroidal pore model, and the carpet mechanism (Huang et al. 2010; Jensen et al. 2006; Brogden 2005). More recently, researchers have proposed a two-step mechanism for AMP activity (Dennison and Phoenix 2011b). This mechanism involves initial membrane association during which the presence of a charged lipid headgroup is known to help drive helix formation and supports the initial membrane binding (Dennison and Phoenix 2011b). The second phase involves penetration into the bilayer and stabilisation of the active conformation at the membrane interface. This second phase is driven by the peptide's amphiphilic architecture and, if

membrane partitioning is not achieved, the peptide disassociates from the membrane surface.

Monolayer data showed that Modelin-5-CONH₂ possessed strong ability to partition into anionic membranes ($>9.17 \text{ mN m}^{-1}$; Fig. 2) and lower levels of insertion were seen in the presence of zwitterionic PC, which would indicate that the peptide had a preference for anionic lipid. Since erythrocytes contain only 12.9–16.1 % PS, the ability of Modelin-5-CONH₂ to partition into lipid monolayers formed from varying PS and PC ratios was investigated. Surface pressure changes induced in these monolayers by Modelin-5-CONH₂ showed an increase in surface pressure with increasing levels of DMPS confirming that it had a preference for anionic lipid (Fig. 4a) and this may be a key driver in AMP activity. All three species have comparable anionic lipid content, implying similar binding potential, which is reflected in the helical structure data. This, therefore, implies there are other factors controlling efficacy.

In the presence of lipid extract the peptide shows relatively low levels of helicity (Fig. 2a) in all three cases. This may imply that the peptide is unable to efficiently penetrate the bilayer so preventing the active conformation to be stabilised at the membrane interface. Indeed it is known that the membrane interactions of AMPs, which utilise a pore and/or carpet-type mechanism, are affected by the environment of the lipid bilayer (Yeaman and Yount 2003; Phoenix et al. 2013), and that, if the peptide is unable to achieve insertion, it can dissociate from the membrane interface (Dennison and Phoenix 2011b). This lack of penetration and ability to concentrate at the interface is reflected in the poor monolayer insertion and correlates with low levels of haemolysis (Fig. 1a).

Taken with the fact that PC forms ca. 30 % of the lipid in human and pig erythrocytes membrane and only 1.6 % in sheep erythrocytes membrane (Salvioli et al. 1993), the

role of this lipid in the resistance of the organism to the action of Modelin-5-CONH₂ was investigated. It is known that the variation in lipid-AMP interaction depends upon the relative composition, stability, and lipid packing characteristics of the target cell membrane (Epand 1997; Lohner and Prenner 1999). For example, the enhanced levels of haemolysis in sheep blood, and the kinetics showing rapid lysis before the static phase (Fig. 1b) may be because the reduced level of PC in sheep blood is compensated for by a large increase in SM levels (Salvioli et al. 1993), which is known to promote positive lipid membrane curvature. The ratio of PC and SM in mammalian membranes varies significantly (Salvioli et al. 1993) and these differences have been shown to affect the efficacy of AMPs and, hence, could be key in the membrane interaction (Sood et al. 2008; Li et al. 2001; Martínez et al. 2007). Although SM and PC are very similar in head group size and shape, the tail group packing density can be altered by the addition of SM to a PC monolayer. The packing characteristics of SM:PC monolayers showed that SM increased the area per phospholipid molecule which decreased the C_s^{-1} value (Table 2) in the absence and presence of Modelin-5-CONH₂. SM increases the rigidity of the membrane; hence, in the absence of peptide, the monolayers were thermodynamically more stable ($\Delta G_{\text{mix}} < 0$). However, increasing the SM content in the presence of the peptide had a destabilizing effect ($\Delta G_{\text{mix}} > 0$) on these membranes. Furthermore, taken with the fact that PS forms ca. 15 % of the lipid mammalian erythrocyte membrane (Salvioli et al. 1993) and this peptide has a preference for anionic lipid, monolayer experiments were undertaken at constant PS levels. This revealed a similar insertion when varying the PC:SM ratio, which overrode the inhibitory effect of a low PC high SM composition (Fig. 4b). Hence, at a starting pressure of 30 mN m⁻¹, the levels of PS drives binding, which indicates that the anionic interaction is key in membrane interaction.

Although the most abundant lipid tail groups in mammalian membranes are saturated chains, there are also unsaturated chains which are common components of the cell membrane. These can affect the packing characteristics. However, the structural characteristics of Modelin-5-CONH₂ had comparable levels of helicity (ca. 42 %) in the presence of DOPC, DMPC, and SM lipid vesicles (Fig. 2b). Whilst it is recognised that erythrocytes membranes are more complex, these data are supported by the insertion results obtained using monolayers at constant area at a starting pressure of 30 mN m⁻¹ showed that altering the tail group did not affect surface pressure changes with all inducing ca. 3.5 mN m⁻¹ (Fig. 3b). The effect of lipid tail group packing is more apparent when decreasing the surface pressure of the monolayer as, for example, when comparison of peptide insertion into DMPC and DOPC

was carried out at lower surface pressures, the effect of the unsaturated lipid tail group on Modelin-5-CONH₂ was found to be enhanced at lower surface pressures (10 mN m⁻¹, Fig. 3c).

Whilst no tail group effects were observed, sterols are known to also alter the packing characteristics. Previous studies have shown that cholesterol exhibits a protective effect on membrane disruption by Modelin-5-CONH₂ (Dennison and Phoenix 2011a) and can play an important role in AMP selectivity over bacterial membranes. The cholesterol content remains approximately constant in red blood cells at an average content of 28 % (Nelson 1967) and plays a key role in the lipid ordering in membranes. Cholesterol is known to influence membrane properties by functioning as a spacer molecule in a phospholipid mixed membrane to compensate for the head-tail size mismatch in the lipid molecules (Dennison and Phoenix 2011a), hence, giving the variation in PC/SM ratios across the species tested. There is a potential for cholesterol to have a differential effect. The increased C_s^{-1} value in the presence of cholesterol indicates tighter packing of the lipids, which may present a barrier for the peptide accessing the hydrophobic interface, and so inhibit the formation of the α -helical structure association required for interaction (Table 3). Table 2 shows that for PC:SM monolayers, and, in the absence of peptide, the C_s^{-1} increases in the presence of a 0.87 cholesterol molar ratio, which are comparable to the ratios found within pig erythrocyte lipid extract (Nelson 1967). However, in the presence of Modelin-5-CONH₂, a C_s^{-1} decrease is observed for PC:SM monolayers containing a 0.87 cholesterol molar ratio. Studies have also shown that the bending rigidity of DMPC increases with cholesterol content (Gracia et al. 2010) and, in the presence of Modelin-5-CONH₂, appeared to prevent stable α -helix formation at the membrane interface, hence, driving the mode of peptide interactions towards dissociation. This is supported by the C_s^{-1} data which indicated that, in the presence of cholesterol at high PC levels, the monolayers were less compressible. Here, the thermodynamic analysis of PC:SM monolayers at constant 0.87 cholesterol molar ratio levels showed that the ΔG_{mix} was negative in the absence of peptide, indicating that these membranes were thermodynamically stable. However, in the presence of peptide, although for these monolayers the ΔG_{mix} remained negative, the peptide had an overall destabilizing effect on the membrane as the SM content increased. Although SM increases the rigidity of membrane in the presence of cholesterol, this membrane rigidity decreases, and, hence, the elasticity of the membrane also decreases (Gracia et al. 2010). Hence, the presence of cholesterol has an effect on the membrane interaction, which is enhanced with increased SM. The data clearly suggests that the key driver for AMP efficacy is the ratio of SM:PC:cholesterol for membrane interaction.

Increasing SM in the presence of cholesterol increases the lipid packing density characteristics of the membrane and, hence, decreases the ability of Modelin-5-CONH₂ to insert into the target membrane.

In summary, it appears that anionic lipid is a key driver of membrane interaction and is instrumental in stabilizing the peptide at the membrane interface. In those species showing greater resistance to lysis it would appear that the varying ratios of PC, SM, and sterol affects the overall packing density and, thus, influences the ability of the peptide to insert. Whilst, therefore, the comparable levels of anionic lipid would be predicted to support similar levels of membrane peptide association with the more densely packed membranes, the peptide is unable to insert so this leads to dissociation from the membrane interface and reduced lysis. The species variation in AMP efficacy is, therefore, due to packing variation caused by sterol:lipid ratios rather than head group effects involved in the initial binding levels.

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