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BRIEF COMMUNICATION

Hemolytic Activity of Membrane-Active Peptides Correlates with the Thermodynamics of Binding to 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine Bilayers

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Abstract Understanding the mechanisms of antimicrobial, cytolytic and cell-penetrating peptides is important for the design of new peptides to be used as cargo-delivery systems or antimicrobials. But these peptides should not be hemolytic. Recently, we designed a series of such membrane-active peptides and tested several hypotheses about their mechanisms on model membranes. To that end, the Gibbs free energy of binding to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles was determined experimentally. Because the main lipid components of the outermost monolayer of erythrocyte membranes are zwitterionic, like POPC, we hypothesized that the Gibbs free energy of binding of these peptides to POPC would also be a good indicator of their hemolytic activity. Now, the hemolytic activity of those synthetic peptides was examined by measuring the lysis of sheep erythrocyte suspensions after peptide addition. Indeed, the Gibbs free energy of binding was in good correlation with the hemolytic activity, which was represented by the concentration of peptide in solution that produced 50 % hemolysis. Furthermore, with two exceptions, those peptides that caused graded dye release from POPC vesicles were also hemolytic, while most of those that caused all-or-none release were not.

Keywords Antimicrobial peptides · Hemolytic peptides · Dye release · Graded release · All-or-none release · POPC · Binding

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Introduction

Recent studies have been conducted on the cell selectivity of antimicrobial and cytolytic peptides to bacterial or eukaryotic membranes. Understanding this cell selectivity is important to the drug-development process of antimicrobial peptides (Toke 2005). Different methods have been used for selecting peptides for use as antibiotics with good binding affinities, such as high-throughput methods of broad-spectrum peptides (Rathinakumar et al. 2010; Rathinakumar and Wimley 2008). For the most part, electrostatic interactions of peptides with gram-negative and gram-positive bacteria increase antibacterial activity, while hydrophobic interactions with eukaryotic membranes increase hemolytic activity (Toke 2005; Blondelle et al. 1999; Rathinakumar et al. 2009). Replicating natural membrane properties in model systems is a difficult task because of the complexity of membrane composition. Therefore, this relationship is not completely understood (Blondelle et al. 1999). Some studies have been conducted on erythrocytes themselves, comparing peptide hemolytic activity to dye-release experiments on model membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Kiyota et al. 1996). As the hydrophobicity of the peptides increases, so does the hemolytic activity and the dye release from model membranes (Kiyota et al. 1996).

Here, we examined the hemolytic activity of a large set of membrane-active peptides and their synthetic variants. We sought to predict, from the knowledge of the peptide interactions on model membranes, their cell specificity and their effects on cell membranes. This knowledge is necessary to design new peptides that are to be used as cargo-delivery systems or antimicrobials because in order to be useful they cannot be hemolytic. Previously, we examined the kinetics and thermodynamics of interaction of a series of these

peptides with model membranes (Clark et al. 2011; McKeown et al. 2011). The Gibbs free energy of binding (ΔG°_{bind}) to a zwitterionic lipid model membrane (POPC) was determined experimentally. This correlated strongly with dye-release efficiency from lipid vesicles, which is quantitatively represented by the average time constant of dye release, τ (Clark et al. 2011). Most of the polar lipids of the outer monolayer of erythrocytes are zwitterionic (phosphatidylcholine and sphingomyelin). Therefore, the Gibbs free energy of binding to POPC should be a good indicator of hemolytic activity. This is the hypothesis tested here.

The peptides examined and their sequences are listed in Table 1. TPW-1, TPW-2 and TPW-3 are all synthetic mutants of transportan 10 Y3W (TP10W) (McKeown et al. 2011). DL-1, DL-2a and DL-2b are synthetic mutants of δ-lysin; CE-1 and CE-2, of cecropin A; and MG-1 and MG-2, of magainin 2 (Clark et al. 2011). The hemolytic activity of these peptides was determined by the change in absorbance of erythrocyte suspensions upon addition of the peptide. This reflects a decrease in turbidity, proportional to the decrease in cell population, which indicates the hemolytic behavior of the peptide. The peptide concentration that caused 50 % hemolysis (P_{50}) was determined and compared to the binding Gibbs energy and the rate of dye release from POPC vesicles previously determined.

Materials and Methods

Chemicals

DL-1 (93 % pure) was purchased from Bachem (Torrance, CA). DL-2a (95 %), CE-2 (95 %) and MG-2 (88 %) were from New England Peptide (Gardner, MA). DL-2b (99 %), CE-1 (98 %) and MG-1 (95 %) were from Genscript (Scotch Plains, NJ). TPW-1 (98 % pure) was purchased from Genscript, TPW-2 (95 % pure) from New England Peptide and TPW-3 (95 % pure) from Bachem. δ-Lysin was a gift from Dr. H. Birkbeck (University of Glasgow, Glasgow, UK); cecropin A and magainin 2 were purchased from American Peptide (Sunnyvale, CA). Their identity was ascertained by mass spectrometry, and the purity was determined by high-performance liquid chromatography, provided by the manufacturer. Stock solutions were prepared by dissolving lyophilized peptide in deionized water or a 1:1 (v/v) water/ethyl alcohol mixture (AAPER Alcohol and Chemical, Shelbyville, KY) or pure water (pH 3) for δ-lysin (Pokorny et al. 2002). Stock peptide solutions were stored at –80 °C and kept on ice during experiments. Peptide concentrations were determined by Trp absorbance at 280 nm. A phosphate buffer–sodium chloride solution was prepared with 150 mM sodium chloride and 10 mM phosphate buffer with a pH of 7.5. Triton X-100 was purchased from Acros (Fair Lawn, NJ).

Preparation of Erythrocyte Suspensions

Erythrocytes were isolated from fresh sheep blood (Carolina Biological, Burlington, NC) by centrifugation at 1,000×g for 20 min (6,700 rpm in centrifuge 5430; Eppendorf, Hamburg, Germany). The supernatant was removed and replaced with 10 mM phosphate buffer (pH 7.5), 150 mM NaCl. The process was repeated three times before use, and the erythrocyte suspension was kept on ice during experimentation. The erythrocyte suspension sample was diluted 1:10 for experiments.

Table 1 Peptide sequences of TP10W, δ-lysin, magainin 2, cecropin A and their synthetic mutants

Peptide	Charge (pH 7)	Length	Sequence
TP10W	+5	21	AGWLLGKINLKALAALAKKIL-amide
TPW-1	+1	21	AGWLLGDINLDALAALAKKIL-amide
TPW-2	+5	21	AGWLLGKLALKALAALAKKLL-amide
TPW-3	+3	21	AGWLLGDINLKALAALAKKIL-amide
δ-Lysin	0	26	formyl-MAQDIISTIGDLVKWIIDTVNKFTKK
DL-1	+6	26	formyl-MAQKIISTIGKLVKWIIKTVNKFTKK
DL-2a	0	26	formyl-LAADLLAALGDLAKWLLDALAKAAKK
DL-2b	0	26	formyl-LAADLLAALGDLLKWLLDALAKLAKK
Cecropin A	+7	37	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-amide
CE-1	+1	37	EWKLFEKIEKLGQNILDGIIKLGPLLALLGQLTQIAL-amide
CE-2	+7	37	KWKLLKKLEKAGAALKEGLLKAGPALALLGAAAALAK-amide
Magainin 2	+3	23	GIGKFLHSAKKWGKAFVGEIMNS
MG-1	0	23	GILKFLESAKKWLEAFLAEIMNS
MG-2	+3	23	GLGKLLHAAKKLGKAWLGELLAA

Hemolysis Assay

Absorbance readings were taken at 540 nm in a spectrophotometer (Cary 1E UV-Visible; Agilent Technologies, Santa Clara, CA). Readings at 280 nm gave similar results. 10 µl of the diluted erythrocyte suspensions were diluted 1:100 with phosphate buffer and placed into one 1-ml quartz cuvette, and an absorbance reading was taken. Absorbance before addition of peptide was in the range 0.4–0.6. Aliquots of peptides were added until maximal hemolysis occurred. 50 µl of 20 % (v/v) Triton X-100 solutions were then added to the sample, and an absorbance reading of complete hemolysis was taken (found to be approximately 0.05–0.1). Absorbance readings were corrected for the dilution caused by the added volumes of peptide solution.

Determination of Peptide Concentrations that Yield 50 % Hemolysis

The peptide concentration that causes 50 % hemolysis (P_{50}) is the amount of peptide found in solution when the absorbance drops to 50 % of its original value minus the final value. These values were calculated by fitting the absorbance (A) data as a function of the total peptide concentration with the equation

$$A = \frac{\beta}{1 + e^{\alpha([P]-P_{50})}} + \gamma \quad (1)$$

where β is the amplitude, α is proportional to the slope of the function at the inflection point, $[P]$ is the peptide concentration in solution and γ is the absorbance at complete hemolysis (Triton X-100 value).

Results and Discussion

The peptide hemolytic activity was evaluated by the change caused in the absorbance of an erythrocyte suspension. This reflects a decrease in turbidity, proportional to the decrease in cell population, which indicates the hemolytic behavior of the peptide. Figure 1 shows plots of the absorbance of the erythrocyte suspension against peptide concentration for δ-lysin and its variants DL-1, DL-2a and DL-2b. For this and all other peptides, gray symbols are used for the original peptide; white symbols, for those of series 1; and black symbols, for series 2. δ-Lysin is known to be hemolytic; DL-1 and DL-2a also show hemolytic activity. Figure 2 shows the plots for TP10W and its variants, TPW-1, TPW-2 and TPW-3. All of these peptides seem to show hemolytic activity, but TPW-2 is clearly more active at low concentrations compared to the others. Figure 3 shows the results for MG-1, MG-2, CE-1 and CE-2. Each of these peptides shows hemolytic activity except for CE-1. This peptide seems to cause an increase in absorbance, which may indicate cell aggregation or fusion. Some of these peptides appear to work cooperatively, in the sense that a certain amount needs to be bound in order to perturb the membrane sufficiently to allow more peptides to bind with higher affinity, eventually lysing the red blood cell. Values of P_{50} and α were calculated by fitting the absorbance data as a function of total peptide concentration. They are listed in Table 2, for each peptide.

We wanted to determine if there was a correlation between the thermodynamics of peptide binding to POPC vesicles and the hemolytic activity of the peptides. Figure 4 shows the plots of the average time constant of dye release (τ) from POPC vesicles and the Gibbs energy of binding to POPC ($\Delta G^{\circ}_{\text{bind}}$) as functions of P_{50}. The symbols used for each peptide are the same as in Figs. 1, 2 and 3. The release time constant τ was used to model dye-release efficiency (Clark et al. 2011; McKeown et al. 2011). The plot of RT ln τ against P_{50} shows a linear correlation (Fig. 4a). The correlation coefficient is only $R = 0.726$, probably because $1/\tau$ is the average specific rate of dye release from POPC vesicles and lysis of cellular membranes is likely to be more complex.

$\Delta G^{\circ}_{\text{bind}}$ is an experimental, quantitative measure of peptide binding (Clark et al. 2011; McKeown et al. 2011). It shows a clearer correlation with P_{50}, with $R = 0.881$ (Fig. 4b). This suggests that the peptides interact with the lipids on the membrane of the erythrocyte in a manner similar to their interactions with POPC vesicles. The better the peptide binds, the more hemolytic it is. One exception to this trend is DL-1. This peptide was predicted to be weakly hemolytic based on its low binding affinity, but it exhibited strong hemolytic activity. However, DL-1 is also an exception in that it is very active in dye release from POPC vesicles (Clark et al. 2011).

Further, peptides with strong binding affinity to POPC, such as TPW-2, show negative deviations from the linear trend on the plot of $\Delta G^{\circ}_{\text{bind}}$ at low P_{50} concentrations. This, however, is unavoidable. Without peptide, no hemolytic activity can occur even if $\Delta G^{\circ}_{\text{bind}} \rightarrow -\infty$. Therefore, since the straight line does not extrapolate to the origin, there must be an asymptote at $P_{50} = 0$. Clearly, the relationship between $\Delta G^{\circ}_{\text{bind}}$ and P_{50} ($R = 0.881$) is stronger than the relationship between ln τ and P_{50} ($R = 0.726$). However, both statistical p values were significant: $p < 0.0001$ and $p = 0.0050$, respectively. Therefore, both correlations support the idea that hemolytic activity reflects peptide binding extent, measured by $\Delta G^{\circ}_{\text{bind}}$.

Next, we examined whether there is any correlation between hemolytic activity and the mechanism of dye release from lipid vesicles induced by these peptides. The release mechanism is graded if all vesicles behave

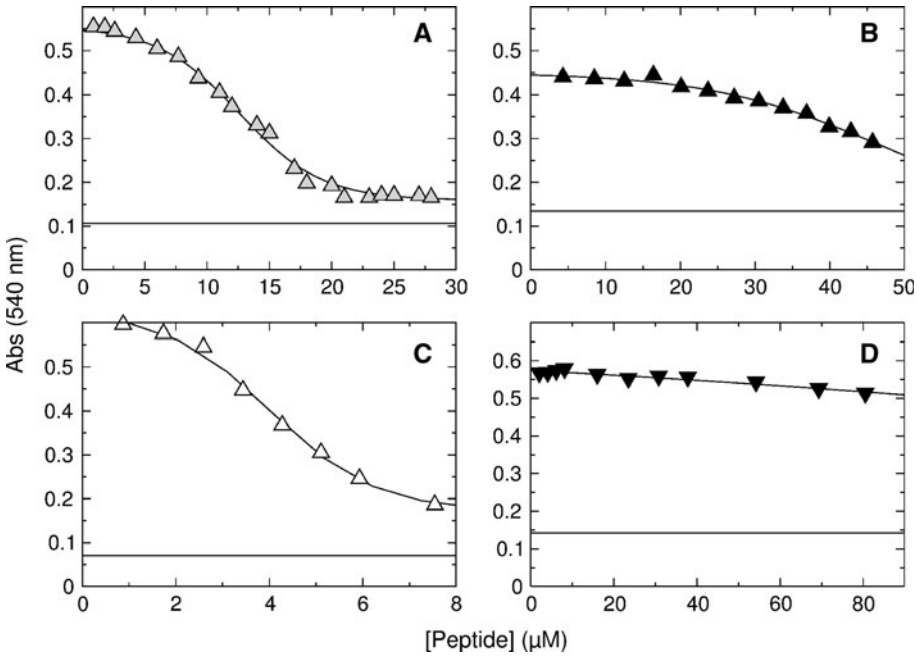

Fig. 1 Representative curves of the absorbance of the erythrocyte sample ($\lambda = 540$ nm) as a function of total peptide concentration for δ-lysin and its variants: **a** δ-lysin, **b** DL-2a, **c** DL-1, **d** DL-2b. *Points* represent experimental absorbance measures. *Lines* are fits of Eq. 1 to the data. *Horizontal lines* indicate absorbance levels after complete hemolysis

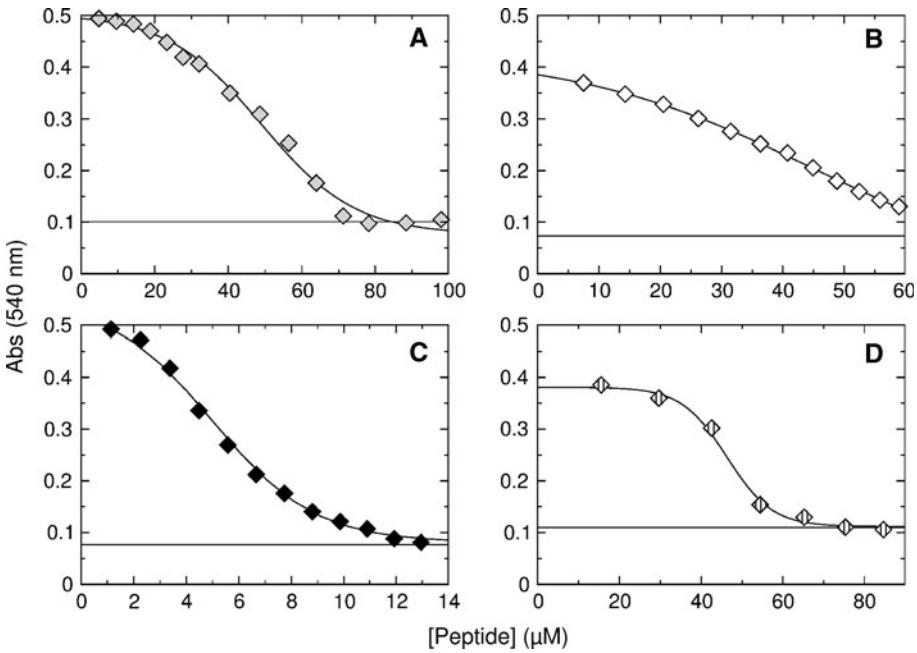

Fig. 2 Representative curves of the absorbance of the erythrocyte sample ($\lambda = 540$ nm) as a function of total peptide concentration for TP10W and its variants: **a** TP10W, **b** TPW-1, **c** TPW-2, **d** TPW-3. *Points* represent experimental absorbance measures. *Lines* are fits of Eq. 1 to the data. *Horizontal lines* indicate absorbance levels after complete hemolysis

similarly; then, the overall release reflects that of individual vesicles. Release is all-or-none if some vesicles release all their contents while others release none. Table 2 summarizes the results of the standard fluorescence requenching assay to determine graded or all-or-none release (using 8-aminonaphthalene-1,3,6-trisulfonic acid and *p*-xylene-bis-pyridinium bromide) (Wimley et al. 1994; Ladokhin et al. 1995, 1997) for this series of peptides (Clark et al. 2011; McKeown et al. 2011). DL-1, CE-1 and MG-1 are all-or-none peptides; DL-2, CE-2 and MG-2 are graded; and TPW-1, TPW-2 and TPW-3 are weakly graded, TPW-3 being the least clear.

CE-2 and MG-2 are extremely graded peptides compared to all the others (Clark et al. 2011; McKeown et al. 2011). These two peptides also have similar hemolysis plots (Fig. 3b, d). This is especially interesting because CE-2 and MG-2 were obtained by conservative mutation of cecropin A and magainin 2, respectively, which are well-known antimicrobial, but not hemolytic, peptides. Further, both cecropin A and magainin 2 cause all-or-none release

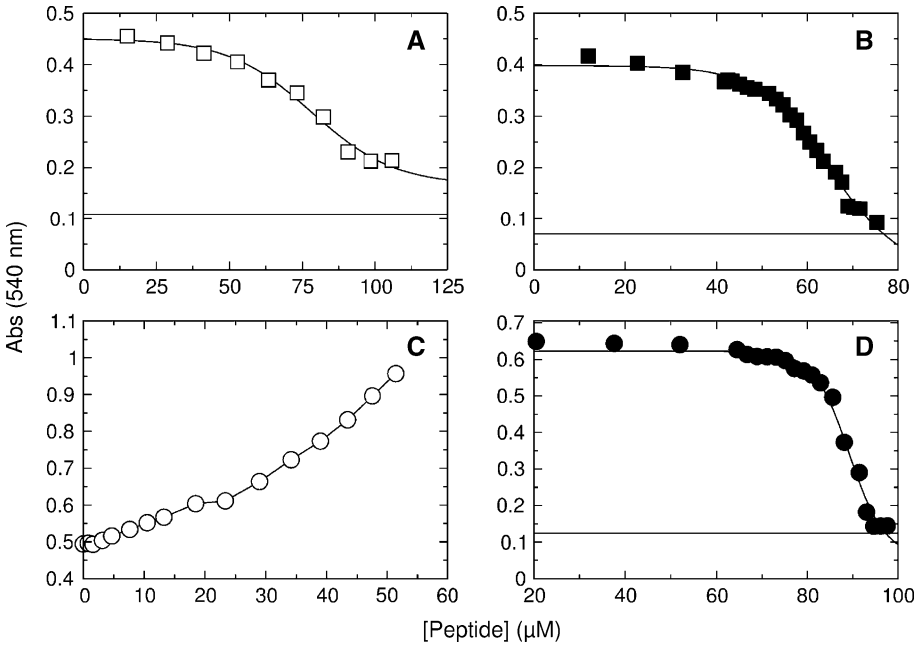

Fig. 3 Representative curves of the absorbance of the erythrocyte sample ($\lambda = 540$ nm) as a function of total peptide concentration for magainin 2 variants and cecropin A variants: **a** MG-1, **b** MG-2, **c** CE-1, **d** CE-2. *Points* represent experimental absorbance measures. *Lines* are fits of Eq. 1 to the data. *Horizontal lines* indicate absorbance levels after complete hemolysis

Table 2 P_{50} and α values with their standard deviations (SD) of TP10W, δ-lysin, magainin 2, cecropin A and their synthetic mutants as well as the dye-release mechanism (all-or-none or graded) and hemolytic activity

Peptide	P_{50} (μM)	α	Hemolytic	Mechanism
TP10W	47 ± 12	0.13 ± 0.09	Yes	Graded
TPW-1	45 ± 0.05	–	Yes	Graded
TPW-2	5.3 ± 0.8	0.62 ± 0.16	Yes	Graded
TPW-3	50 ± 7	0.90 ± 0.08	Yes	Graded
δ-Lysin	12.7 ± 2.4	0.30 ± 0.04	Yes	Graded
DL-1	4.7 ± 1.0	0.61 ± 0.40	Yes	All-or-none
DL-2a	40	0.03	Yes	Graded
DL-2b	≥80	–	No	–
Cecropin A	≥12	–	No	All-or-none
CE-1	≥50	–	No	All-or-none
CE-2	85 ± 8	0.20 ± 0.07	Yes	Graded
Magainin 2	Not tested		No	All-or-none
MG-1	67 ± 22	0.05 ± 0.03	Yes	All-or-none
MG-2	55 ± 14	0.51 ± 0.53	Yes	Graded

(Gregory et al. 2008, 2009; Silvestro et al. 1997; Tamba and Yamazaki 2005). The mutations in CE-2 and MG-2 involved a drastic simplification of the amino acid sequence diversity of the original peptides but did not significantly change secondary structure, hydrophobic moment, membrane binding affinity or the rate of dye release from POPC vesicles (Clark et al. 2011). Like cecropin A and mangainin 2, CE-2 and MG-2 bind very poorly to POPC and probably to erythrocyte membranes, which is why a large concentration needs to be reached for hemolytic activity to be observed.

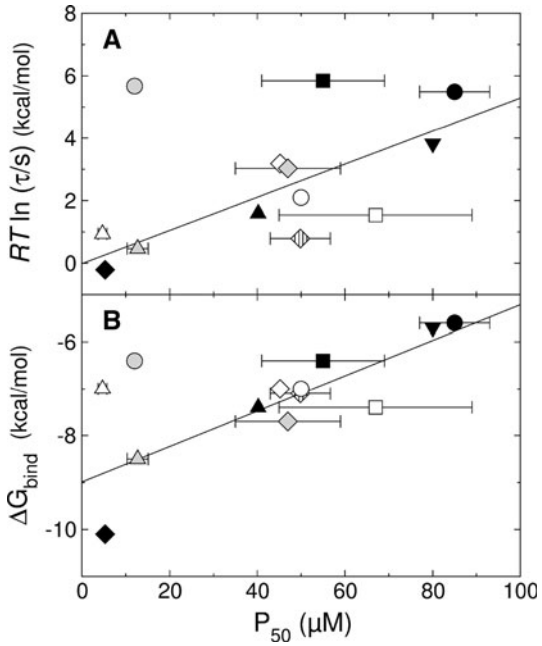

Fig. 4 **a** RT ln τ (time in seconds) as a function of P_{50}. **b** $\Delta G^{\circ}_{\text{bind}}$ (= RT ln K_D—2.4 kcal/mol, where the last term arises from conversion of concentrations from molar to mole fraction units) as a function of P_{50}. Each point corresponds to a different peptide. Symbols are the same as in Figs. 1, 2 and 3. *Gray circle* is cecropin A. For cecropin A, CE-1, and DL-2b, *points* represent lower bounds. *Lines* are best fits with correlation coefficients of $R = 0.726$ in **a** and $R = 0.881$ in **b**

On the other hand, DL-1 is clearly all-or-none. DL-1 was hypothesized to be weakly hemolytic because of its low binding affinity to POPC. However, it proved to be one of the most hemolytic peptides. In Table 2, we grouped the peptides as hemolytic or nonhemolytic and as graded or all-or-none using the data from Clark et al. (2011) and

McKeown et al. (2011). It is apparent that all graded peptides are hemolytic, whereas the nonhemolytic peptides are all-or-none. However, DL-1 and MG-1, which are both all-or-none, are also hemolytic.

Conclusion

We examined the hemolytic activity of a series of antimicrobial, cytolytic and cell-penetrating peptides whose binding and activity in model membranes we previously studied in detail. Our results support the idea that hemolytic activity reflects the Gibbs free energy of binding to POPC. As the $\Delta G^{\circ}_{\text{bind}}$ for each peptide increases in absolute value, so does its hemolytic activity. Previously, we found that ln τ, where τ is the characteristic time required for dye release from synthetic vesicles, was in good correlation with $\Delta G^{\circ}_{\text{bind}}$ (Clark et al. 2011). Therefore, we predicted that if $\Delta G^{\circ}_{\text{bind}}$ correlated with hemolytic activity, τ would also show a good correlation. The direct correlation of ln τ to hemolytic activity is weaker, but the p values indicate that both correlations are significant. Furthermore, our results suggest that graded peptides are hemolytic, whereas all-or-none peptides tend to be nonhemolytic for the most part.

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References

Blondelle SE, Lohner K, Aguilar M (1999) Lipid-induced conformation and lipid binding properties of cytolytic antimicrobial peptides: determination and biological specificity. Biochim Biophys Acta 1462:89–108

Clark KS, Svetlovics J, McKeown AN, Huskins LJ, Almeida PF (2011) What determines the activity of antimicrobial and cytolytic peptides in model membranes. Biochemistry 50: 7919–7932

Gregory SM, Cavenaugh AC, Journigan V, Pokorny A, Almeida PFF (2008) A quantitative model for the all-or-none permeabilization of phospholipid vesicles by the antimicrobial peptide cecropin A. Biophys J 94:1667–1680

Gregory SM, Pokorny A, Almeida PFF (2009) Magainin 2 revisited: a test of the quantitative model for the all-or-none permeabilization of phospholipid vesicles. Biophys J 96:116–131

Kiyota T, Lee S, Sugihara G (1996) Design and synthesis of amphiphilic α-helical model peptides with systematically varied hydrophobic–hydrophilic balance and their interaction with lipid- and bio-membranes. Biochemistry 35:13196–13204

Ladokhin AS, Wimley WC, White SH (1995) Leakage of membrane vesicle contents: determination of mechanism using fluorescence requenching. Biophys J 69:1964–1971

Ladokhin AS, Wimley WC, Hristova K, White SH (1997) Mechanism of leakage of contents of membrane vesicles determined by fluorescence requenching. Methods Enzymol 278:474–486

McKeown AN, Naro JL, Huskins LJ, Almeida PF (2011) A thermodynamic approach to the mechanism of cell-penetrating peptides in model membranes. Biochemistry 50:654–662

Pokorny A, Birkbeck TH, Almeida PFF (2002) Mechanism and kinetics of δ-lysin interaction with phospholipid vesicles. Biochemistry 41:11044–11056

Rathinakumar R, Wimley WC (2008) Biomolecular engineering by combinatorial design and high-throughput screening: small, soluble peptides that permeabilize membranes. J Am Chem Soc 130:9849–9858

Rathinakumar R, Walkenhorst WF, Wimley WC (2009) Broad-spectrum antimicrobial peptides by rational combinatorial design and throughput screening: the importance of interfacial activity. J Am Chem Soc 131:7609–7617

Rathinakumar R, Walkenhorst WF, Wimley WC (2010) High-throughput discovery of broad-spectrum peptide antibiotics. FASEB J 24:3232–3238

Silvestro L, Gupta K, Weiser JN, Axelsen PH (1997) The concentration-dependent membrane activity of cecropin A. Biochemistry 36:11452–11460

Tamba Y, Yamazaki M (2005) Single giant unilamellar vesicle method reveals effect of antimicrobial peptide magainin 2 on membrane permeability. Biochemistry 44:15823–15833

Toke O (2005) Antimicrobial peptides: new candidates in the fight against bacterial infections. Biopolymers 80:717–735

Wimley WC, Selsted ME, White SH (1994) Interactions between human defensins and lipid bilayers: evidence for formation of multimeric pores. Protein Sci 3:1362–1373