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Cell selectivity and interaction with model membranes of Val/Arg-rich peptides

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Antimicrobial peptides are major components of the innate self-defence system and a large number of peptides have been designed to study the mechanism of action. In the present study, a small combinatorial library was designed to study whether the biological activity of Val/Arg-rich peptides is associated with targeted cell membranes. The peptides were produced by segregating hydrophilic residues on the polar side and hydrophobic residues on the opposite side. The peptides displayed strong antimicrobial activity against Gram-negative and Gram-positive bacteria, but weak haemolysis even at a concentration of 256 μM. CD spectra showed that the peptides formed α-helical-rich structure in the presence of negatively charged membranes. The tryptophan fluorescence and quenching experiments indicated that the peptides bound preferentially to negatively charged phospholipids over zwitterionic phospholipids, which corresponds well with the biological activity data. In the *in vivo* experiment, the peptide G6 decreased the bacterial counts in the mouse peritoneum and increased survival after 7 days. Overall, a high binding affinity with negatively charged phospholipids correlated closely with the cell selectivity of the peptides and some peptides in this study may be likely candidates for the development of antibacterial agents. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptides; cell selectivity; haemolysis; liposomes; *in vivo*

Introduction

Antimicrobial peptides (AMPs) are major components of the innate self-defence system [1] and their unique mechanism of action, which is different from antibiotics, has attracted increased attention. Many mechanisms were proposed to explain how these AMPs work, such as the membrane disaggregation as a result of hydrophobic and electrostatic interactions, and the formation of transmembrane pores [2–5]. Recently, many AMPs have been designed based upon a comparison to naturally occurring sequences of known peptides in order to construct synthetic combinatorial libraries or to construct a sequence template to discover new antimicrobial compounds [4,6]. De novo generation of cationic and amphipathic AMPs was designed to study the structure–activity relationships of AMPs [5,7–11].

According to the statistical information provided by the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.html), Arg and Val amino acid residues occur frequently in naturally occurring AMPs. The hydrophobic residues (Val) are permitted to insert into the hydrophobic core of the membrane [12]. The positively charged residues (Arg) may mediate peptide interactions with bacterial cell walls or negatively charged membranes [13].

Lys and Leu are commonly found in AMPs. However, the antimicrobial activity of peptides containing Arg is higher than those of peptides containing Lys [14,15]. The ideally amphipathic peptides containing Leu and Lys had haemolytic activity higher than that of melittin [16], thus Arg and Val were selected. Although the peptides composed of Val and Arg [8,17,18] residues have been synthesised, little is known about whether the different biological activities of these peptides result from the different compositions of targeted cell membranes. In this study, we synthesised a small combinatorial library, mainly composed of Val and Arg residues, according to the helical-wheel projections [19]. Then, we used this model as the structural framework to produce a series of peptides by residue replacement (Table 1). The rationale to use a 16-residue motif was based on the following considerations: (i) the net charge and percentage of hydrophobic residues were fixed to +5 and 50% according to the statistical information of naturally occurring peptides [11]; (ii) the C-terminus was aminated to increase the stability and eliminate potential electrostatic attractions [20] and Gly was positioned at N-terminus to improve the stability of the peptides; and (iii) the distribution of the residues was designed to preserve hydrophobic residues on the nonpolar sides and charged residues on the polar sides using the helical-wheel model. The antimicrobial and haemolytic activities of the peptides were determined. A tryptophan fluorescence experiment was performed to evaluate whether targeted cell membranes are associated with the biological activities of the peptides.

Materials and Methods

Peptide Synthesis

The peptides listed in Table 1 were purchased from GL Biochem Corporation (Shanghai, China), where they were synthesised

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Table 1. The sequence and molecular weight of the peptides in this study

Peptide	Sequence	Mr	H[a]	H[b]
G1	GVVVRVGRVVVRGVRR-NH_2	1762.20	0.30	−1.52
G2	GVVVRIGRVVVRGVRR-NH_2	1776.23	0.33	−1.23
G3	GVVVRWGRVVVRGVRR-NH_2	1849.28	0.36	−1.17
G4	GVVVRIGRVIVRGVRR-NH_2	1790.25	0.37	−0.95
G5	GVVVRWGRVIVRGVRR-NH_2	1863.31	0.40	−0.88
G6	GVVVRWGRVWVRGVRR-NH_2	1936.36	0.42	−0.82

Mr, molecular weight (in Da).
[a] H: The hydrophobicity per residue of peptides calculated by the method of Fauchere *et al*.
[b] H: The hydrophobicity per residue of peptides calculated by the method of Tossi *et al*.

by solid-phase methods using N-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry. The peptides were amidated at the C-terminus. The peptides were purified to >95% purity by reverse-phase high-performance liquid chromatography and their identities were confirmed by electrospray mass spectrometry.

Antimicrobial Assays

Minimum inhibitory concentrations (MICs) of the peptides were measured by a modified version of the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method to determine *in vitro* antimicrobial activities of peptides, as described previously [21]. Briefly, bacterial cells were cultured in Mueller–Hinton (MH) broth to mid-log phase and were then diluted to $\sim 1 \times 10^5$ CFU/ml. Serially diluted (two-fold) peptides, dissolved in 0.01% (v/v) acetic acid and 0.2% (w/v) bovine serum albumin (Sigma), were added to each well of the 96-well plates in a volume of 50 µl, followed by 50 µl of inoculum. Plates were incubated at 37 °C for 24 h and the MICs were determined as the lowest concentration of peptide that prevented visible turbidity. Cultures containing no peptide served as positive controls. Uninoculated MH broth was used as a negative control.

Measurement of Haemolytic Activity

The haemolytic activity of the peptides was determined by a previously described method [22]. Briefly, fresh human red blood cells (hRBCs) were collected in a sterile, heparinised borosilicate tube and then centrifuged at 1000 × *g* for 5 min. The erythrocytes obtained were washed three times with phosphate-buffered saline (PBS), centrifuged for 5 min at 1000 × *g*, and resuspended in PBS to attain a dilution of about 1% (v/v) of the erythrocyte volume initially collected. Fifty microliter of the hRBCs solution was incubated with 50 µl of serially different peptide dissolved in PBS for 1 h at 37 °C. Intact erythrocytes were pelleted by centrifugation at 1000 × *g* for 5 min at 4 °C and the supernatant was transferred to a new 96-well microtiter plate. Release of haemoglobin was monitored by measurement of the absorbance at 492 nm. As negative and positive controls, hRBCs in PBS and 0.1% Triton X-100 were employed, respectively. MHC is defined as the peptide concentration causing 5% haemolysis.

Preparation of Liposomes

Small unilamellar vesicles (SUVs) for fluorescence spectroscopy were prepared as described previously [23,24]. Egg yolk L-α-phosphatidylcholine (PC), egg yolk L-α-phosphatidyl-DL-glycerol (PG), egg yolk L-α-phosphatidylethanolamine (PE), cholesterol, acrylamide and calcein were supplied from Sigma-Aldrich Corporation (St. Louis, MO, USA). Following the evaporation of chloroform, the PE/PG (7 : 3, w/w) or PC/cholesterol (10 : 1, w/w) lipids were resuspended in 10 mM Tris–HCl buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mM EDTA) by vortex mixing. The lipid dispersions were sonicated in ice water for 20 min using an ultrasonic cleaner until the solutions clarified.

CD Spectra

Circular dichroism spectra of the peptides were measured at 25 °C with a J-720 spectropolarimeter (Jasco, Tokyo, Japan). The peptides were dissolved in 10 mM sodium phosphate buffer, pH 7.4 or 1 mM PE/PG (7 : 3, w/w) lipids. The solutions were loaded into a 0.1-cm-path-length rectangular quartz cell and spectra were recorded between 190 and 250 nm every 0.5 nm. The average mean residue ellipticities ($[\theta]/10\,000\times$ [degree × square centimetres/decimole]) were plotted against wavelength (in nm).

Tryptophan Fluorescence

Peptide–liposome interaction experiments were performed by measuring tryptophan fluorescence spectra in two lipid systems. The tryptophan fluorescence spectra were measured using an F-4500 fluorescence spectrophotometer (Hitachi, Japan). The fluorescence was excited at 280 nm and the emission was scanned from 300 to 400 nm. Spectra were baseline corrected by subtracting blank spectra of the corresponding solutions without peptide. Measurements were made for each peptide in 10 mM Tris–HCl buffer and in the presence of 500 µM PE/PG or PC/cholesterol lipids and the peptide/lipid molar ratio was 1 : 50.

Tryptophan Quenching

Fluorescence quenching experiments were conducted using acrylamide to quench the reaction. To reduce the absorbance of acrylamide, the fluorescence of Trp was excited at 295 nm instead of 280 nm [25,26]. The final concentration of acrylamide was 0.4 M, which was achieved by the titration of a 4 M stock solution in the presence of liposome at a lipid/peptide molar ratio of 50 : 1. The quenching data were analyzed by the Stern–Volmer equation: $F_0/F = 1 + K_{SV}(Q)$, where F_0 and F are the fluorescence values of the peptide in the absence and the presence of acrylamide, K_{SV} represents the Stern–Volmer quenching constant, and Q represents the concentration of acrylamide.

Peptide-induced Leakage of ONPG into *Escherichia coli* Cells

The permeabilisation of *E. coli* was determined by measuring the permeation of ONPG into the cytoplasm across the inner membrane [27,28]. Logarithmic-phase bacteria in LB medium containing 2% lactose were collected, washed and resuspended in 10 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl. The final cell suspension was adjusted to obtain an A600 of 0.4. Bacterial suspension (2.0 ml) was mixed with 30 mM ONPG (100 µl) and then serial dilutions of peptides (10 µl) were added. The production of *o*-nitrophenol over time was determined by monitoring the increase in absorbance at 415 nm using a spectrophotometer.

In vivo Activity

Male KM mice weighing 19–22 g were purchased from the Animal Centre, Harbin Medical University (Harbin, China) and acclimatised for 1 week. Exponential-phase *E. coli* were suspended in sterile PBS to achieve a final concentration of $\sim 1 \times 10^8$ CFU/ml. Infection was induced by intraperitoneal (i.p.) injection with 0.2 ml of the bacterial suspensions. Mice received an i.p. injection of PBS (control group) or 2.5 mg/kg G6 ~60 min after bacterial challenge. The animals (seven mice in each group) were monitored for 7 days for survival. Approximately 2 ml of sterile PBS was injected intraperitoneally into each mouse and peritoneal fluids (approximately 2 ml) were serially diluted. The colony counts of viable bacteria were determined by plating samples of fluids on Mueller–Hinton agar plates. Procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee at the Northeast Agricultural University.

Statistical Analysis

Statistical comparisons were performed by analysis of variance. Quantitative evaluation of intraperitoneal bacteria is presented as the mean ± standard deviation of the mean. Significance was accepted at a *P*-value of less than 0.05.

Results

Design of Peptides

In this study, the 16-residue-long peptides were designed by segregating five hydrophilic residues in the polar face or eight hydrophobic residues in the opposite face based on the helical-wheel projections. Net charge and hydrophobic residue percentage were fixed at +5 and 50% to generate peptides with the same size as the statistical information of naturally occurring peptides (http://aps.unmc.edu/AP/main.html). In actuality, the library possessed peptides with systematically incremental hydrophobicity from 0.33 to 0.42 calculated by the method of Fauchere [29].

The presence of glycine residues seems to be important, especially for selectivity, as recently reported by Juretić *et al*. [30]. The first amino acid residue should be G because most peptides in the SBP data set start with glycine. Position 7 seems to have positional requirements for its effect in increasing selectivity. In addition, position 13 was selected to equally distribute Gly residues to increase the flexibility. It is expected that Val, Ile, and Trp were selected to increase antimicrobial activity. In addition, residues 6 and 10 were changed because they are located in the centre of the nonpolar faces of the amphipathic helix.

Antimicrobial and Haemolytic Activities of the Peptides

MICs of the synthetic peptides against Gram-negative and Gram-positive bacteria are shown in Table 2. The peptides displayed different antimicrobial activities and their MICs against different bacteria ranged from 2 to 16 µM. According to the hydrophobicity scale of Fauchere [29] and Tossi [31], the hydrophobicity decreased in the rank order Trp > Ile > Val. The average *H* values of the peptides varied from 0.3 to 0.42. Figure 1 shows the GM (the geometric mean of the MICs of bacterial strains observed) as a function of the *H* value. Antimicrobial activity increased linearly with the increase in the hydrophobicity of the peptides.

The haemolytic activities of the peptides against human erythrocytes were determined by the release of haemoglobin. The results are shown in Table 2. All peptides showed weak or no haemolytic activity even at a concentration of 256 µM, which exceeded the MICs listed in Table 2 by over one or two orders of magnitude.

CD Spectra

Circular dichroism (CD) spectra were measured under an aqueous-mimicking and a membrane-mimicking hydrophobic environment as summarised in Figure 2. In aqueous buffer, the peptides exhibited a strong minimum below 200 nm, parameters characteristic of random coils. Especially for peptides G1 and G4–G6, the CD spectra show a positive band near 192 nm and dichroic minimal values at 208 and 222 nm in the presence of PE/PG lipids, suggesting an α-helical-rich structure. The CD spectra of the compounds G2 and G3 exhibit some beta sheet.

Binding of Peptides to Model Membranes

A tryptophan fluorescence experiment was performed to evaluate the binding ability of peptides with lipid vesicles because the peptides, G3, G5, and G6, contain one or two tryptophan residues. We measured the fluorescence emission spectra of the peptides in Tris–HCl buffer (pH 7.4) as well as in the presence of negatively charged PE/PG (7 : 3, w/w) SUVs or zwitterionic PC/cholesterol (10 : 1, w/w) SUVs (Table 3). In Tris–HCl buffer, all peptides displayed a fluorescence emission maximum at around 350 nm, which is typical for Trp in a water-polar environment.

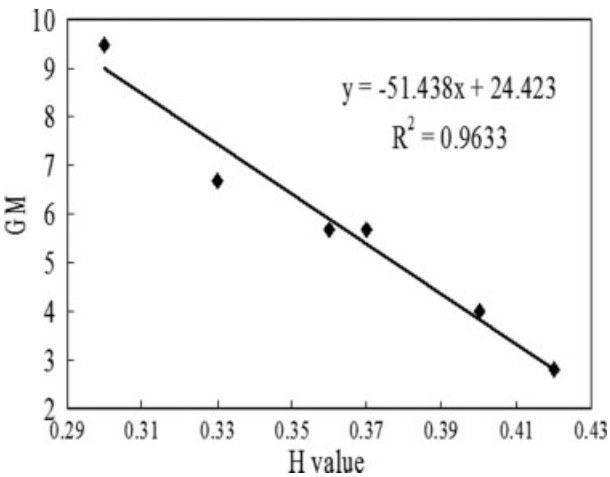

Figure 1. Correlation between the GM of the peptide and *H* values. *H* values were calculated according to the hydrophobicity scales of Fauchere [29]. GM means the geometric mean of the MICs of bacterial strains observed.

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Table 2. MICs and MHCs of the peptides

Peptide	MIC (μM)				GM	MHC (μM)
	E. coli	*Salmonella typhimurium*	*Staphylococcus aureus*	*Bacillus subtilis*		
G1	8	8	16	8	9.5	>256
G2	8	4	8	8	6.7	>256
G3	8	4	8	4	5.7	>256
G4	8	4	8	4	5.7	>256
G5	4	4	4	4	4.0	256
G6	4	2	4	2	2.8	>256

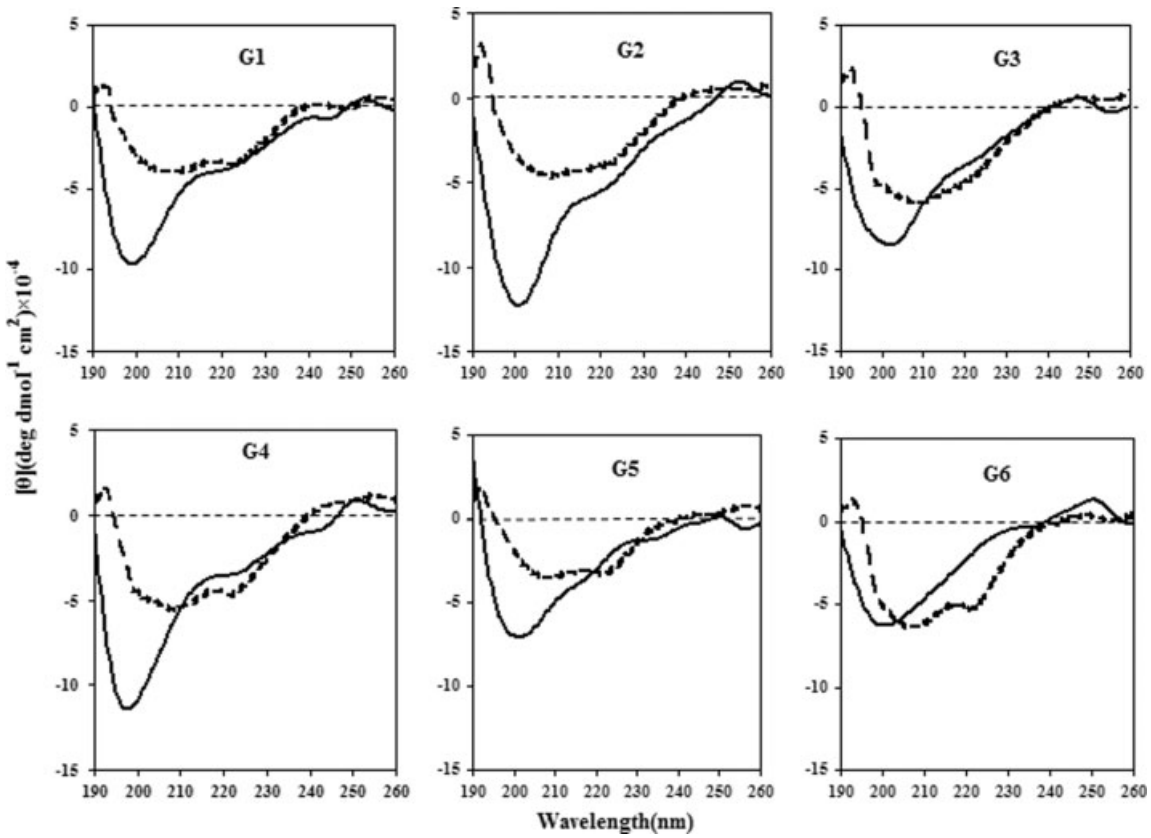

Figure 2. CD spectra of the peptides in the presence of sodium buffer (solid line) or PE/PG (dashed line).

The wavelength of maximum emission shifted from 350 nm in buffer to about 331–339 nm when bound to PE/PG phospholipid vesicles (Table 3). However, little or no shift in wavelength was observed in the presence of PC/cholesterol phospholipid vesicles, which is in accordance with the lack of haemolytic activity of the peptides. The different blue shift indicated that the Trp side chains of these peptides penetrate into a more hydrophobic environment in negatively charged phospholipids compared to zwitterionic phospholipids. Furthermore, three peptides revealed varying degrees of blue shifts in the PE/PG phospholipid vesicles.

Tryptophan Fluorescence Quenching by Acrylamide

To examine the relative extent of burial of the tryptophan residues of the peptides into phospholipids, tryptophan fluorescence quenching was performed with the water-soluble fluorescence quencher, acrylamide. Acrylamide is utilised as a quenching agent because there are no interactions between the head group of negatively charged phospholipids and acrylamide. A decrease in quenching (smaller K_{SV} values) means a more protected Trp residue. As shown in Table 3, the K_{SV} values of the peptides were the smallest in the presence of PE/PG vesicles. In addition, a lower slope is observed when the peptides were added in PE/PG vesicles compared to PC/cholesterol vesicles (Figure 3). Collectively, the tryptophan residues in the peptides were buried more deeply in negatively charged phospholipid vesicles than in zwitterionic phospholipid vesicles, which was consistent with the fact that the antimicrobial activity of the peptides was stronger than their haemolytic activity.

Inner Membrane Permeabilisation

The permeation of ONPG into the cytoplasm was employed to evaluate the permeabilisation of *E. coli* IM induced by G6. As seen in Figure 4, an immediate and progressive release of β-galactosidase resulted when *E. coli* suspensions were

Table 3. Fluorescence spectroscopy parameters measured for the peptides in the presence and absence of PE/PG and PC/cholesterol vesicles

Peptide	Fluorescence emission maxima (nm)			K_{SV}[a] (M^{-1})		
	Tris buffer	PE/PG	PC/cholesterol	Tris buffer	PE/PG	PC/cholesterol
G3	350	336 (14[b])	350 (0)	23.6	1.5	5.2
G5	349	331 (18)	349 (0)	20.9	1.6	4.5
G6	349	339 (10)	348 (1)	22.2	2.4	8.9

[a] Stern–Vollmer constants, K_{SV} (M^{-1}) were calculated by the Stern–Vollmer equation: $F_0/F = 1 + K_{SV}(Q)$, where Q is the concentration of quencher (acrylamide). Concentrations of the quencher increased from 0.01 to 0.40 M. Smaller K_{SV} values reflect a more protected Trp residue.
[b] Blue shift of emission maximum compared to Tris buffer.

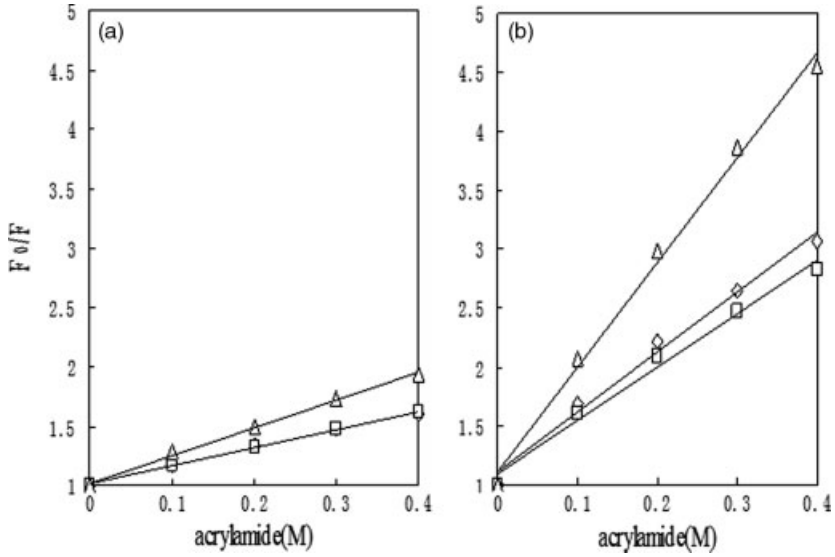

Figure 3. Stern–Volmer plots for the quenching of Trp fluorescence of peptides by an aqueous quencher, acrylamide, in the presence of PE/PG vesicles (a) and PC/cholesterol vesicles (b). The concentrations of the peptides and phospholipid vesicles are 10 and 500 μM, respectively. The designations are as follows: G3(◇), G5(□), and G6(△).

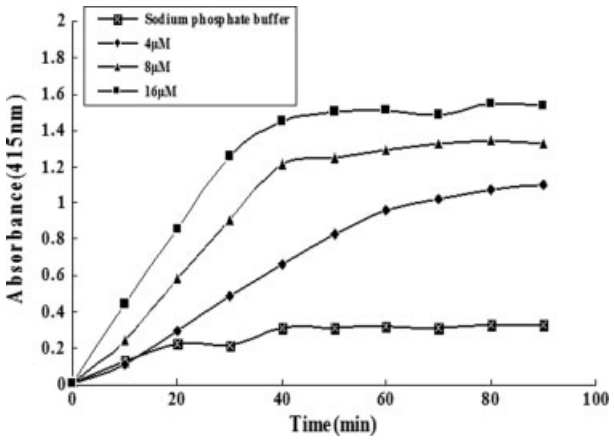

Figure 4. Permeabilization of *Escherichia coli* inner membrane by G6. Permeation was measured at 415 nm by measuring the release of cytoplasmic β-galactosidase activity.

treated with G6 and the release reached the maximum up to 40 min. G6 permeated the IM in a dose-dependent manner at 4–16 μM. In addition, other peptides acted in a dose-dependent manner and increased membrane-damaged kinetics (data not shown).

In vivo Activity of the Peptide G6

The *in vivo* activity of G6 was evaluated by the decrease of the mortality and peritoneal bacterial counts in peptide-treated mice in comparison with PBS-treated control mice. In the peritoneum, the bacterial counts at 24 h in the G6-treated group and PBS-treated group were $9.1 \times 10^5 \pm 4.3 \times 10^5$ and $1.9 \times 10^9 \pm 8.7 \times 10^8$ CFU/ml ($P < 0.01$), respectively. G6 decreased the bacterial load by 10^3-fold 24 h after injection compared with the control mice. The group to which G6 was administered showed an increased survival after 7 days compared with the PBS-treated group, with one fatality out of seven mice and 100% lethality, respectively. Further work needs to be done to determine the effectiveness of prophylaxis.

Discussion

It has been demonstrated that peptides with antimicrobial activity could be derived de novo by strategically positioning commonly occurring amino acids to generate cationic, hydrophobic, and amphipathic properties [7,8,32,33]. CD spectra showed that the peptides indeed formed α-helical structure in the presence of negatively charged membranes. Deslouches and co-workers designed a series of amphipathic α-helical peptides and the 24-residue WLBU2 peptide showed potent antimicrobial activity

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[8]. More positively charged residues may result in stronger antimicrobial activity for the LBU series. According to the model described previously [34,35], the positively charged domain of AMPs first interacts with the negatively charged components of target membranes because of the physical attraction. Increasing the hydrophobicity tends to improve antimicrobial activity (Figure 1) and relatively high hydrophobicity of the peptides is required to guarantee that the membrane is damaged by the powerful penetration of the nonpolar face of the α-helix into the hydrophobic component of the lipid bilayer. An initial attraction between the positively charged residues of the peptides and the negatively charged surface of the phospholipid head groups may make the peptides reach the interface, thus orienting them parallel to the membrane surface [36]. A larger fluorescence shift in the presence of membranes can be due to two reasons: a deeper insertion in the bilayer or a higher affinity for the membrane, with a larger portion of the peptides associated to the bilayer.

To determine whether targeted cell membranes correlate with strong antimicrobial activity and weak haemolysis of the peptides, two kinds of vesicles (either PE/PG, a phospholipid composition typical of bacteria, or PC/cholesterol, a phospholipid composition used to mimic the outer leaflet of human erythrocytes) [37] were prepared to compare membrane-binding affinities of the peptides. The results suggest that the peptides preferentially bind to model membranes containing a negatively charged headgroup relative to those containing a zwitterionic headgroup. This selective membrane interaction with the negatively charged phospholipids of the peptides might explain the cell selectivity. Weak haemolysis of the peptides may correlate with three Gly residues because they are expected to decrease the cytotoxicity of the peptides [30,38]. In the *in vivo* experiment, the reduction of bacterial counts and the mortality of infected mice suggest that G6 effectively protected the mice against microbial infection.

G6, which is the more hydrophobic and more active peptide, has the smaller blue-shifted emission and the highest acrylamide accessibility among G3, G5, and G6. G3 and G5 have similar acrylamide accessibilities, but different emission shifts. Given the complexity of the biological system, the interactions with synthetic vesicles are likely to model those with intact bacterial membranes imperfectly [39]. In addition, the presence of multiple Trp residues does not allow for a simple interpretation of some of the spectroscopic data [39]. Furthermore, 0.4 M acrylamide absorbs significantly even at 295 nm. This absorption gives rise to a small but non-negligible inner filter effect, with a fluorescence reduction of about 10%. Addition of 0.4 M acrylamide to the aqueous volume outside the vesicles definitely creates a strong osmotic stress on the vesicles [40]. We cannot exclude the interference of acrylamide, but different interactions in two lipid systems can still be identified.

In conclusion, we have successfully synthesised a series of peptides with strong antimicrobial activity but weak haemolysis. The peptides bound preferentially to negatively charged phospholipids compared to zwitterionic phospholipids. Different membrane-binding affinities led to the cell selectivity of the peptides. The peptide G6 also works as a therapeutic agent to control bacterial infection *in vivo*. Some peptides in this study may be likely candidates for the development of antibacterial agents.

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