Temporin L: antimicrobial, haemolytic and cytotoxic activities, and effects on membrane permeabilization in lipid vesicles

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The temporins are a family of small, linear antibiotic peptides with intriguing biological properties. We investigated the antibacterial, haemolytic and cytotoxic activities of temporin L (FVQWFSKFLGRIL-NH_2), isolated from the skin of the European red frog *Rana temporaria*. The peptide displayed the highest activity of temporins studied to date, against both human erythrocytes and bacterial and fungal strains. At variance with other known temporins, which are mainly active against Gram-positive bacteria, temporin L was also active against Gram-negative strains such as *Pseudomonas aeruginosa* A.T.C.C. 15692 and *Escherichia coli* D21 at concentrations comparable with those that are microbiocidal to Gram-positive bacteria. In addition, temporin L was cytotoxic to three different human tumour cell lines (Hut-78, K-562 and U-937), causing a necrosis-like cell death, although sensitivity to the peptide varied markedly with the specific cell line tested. A study of the interaction of temporin L with liposomes of different lipid compositions revealed that the peptide causes perturbation of bilayer integrity of both neutral and negatively charged membranes, as revealed by the release of a vesicle-encapsulated fluorescent marker, and that the action of the peptide is modulated to some extent by membrane lipid composition. In particular, the presence of negatively charged lipids in the model bilayer inhibits the lytic power of temporin L. We also show that the release of fluorescent markers caused by temporin L is size-dependent and that the peptide does not have a detergent-like effect on the membrane, suggesting that perturbation of bilayer organization takes place on a local scale, i.e. through the formation of pore-like openings.

Key words: cationic peptides, fluorescent markers, innate immunity, liposomes, *Rana temporaria*.

INTRODUCTION

Antimicrobial peptides are small gene-encoded peptides which show a broad range of activity against Gram-negative and Gram-positive bacteria, fungi, mycobacteria and some enveloped viruses [1]. Originally identified in insects, they have subsequently been extracted from plants, crustaceans, ascidians and vertebrates [2–6], and it is now clear that these molecules constitute a key component of the innate immune system in multicellular organisms across the evolutionary scale. Amphibian skin has proved to be an especially rich source of such peptides, which form a remarkably heterogeneous ensemble with a broad spectrum of antimicrobial activity and little sequence similarity [7]. The peptides are normally stored in the dermal glands of Anurans (frogs and toads), and are released into skin secretions in a holocrine fashion upon stress or injury, acting as the first line of defence against invading pathogens. Bombinins and magainins, isolated from skin secretions of *Bombina* species [8,9] and *Xenopus laevis* [10] respectively, are well-known examples of amphibian antimicrobial peptides. In most cases, several peptides of the same family, with overlapping sequence and structural features but distinct spectra of antimicrobial activity, are present simultaneously on a single specimen, protecting the animal from a wider range of pathogens. Recently, the synthesis of antimicrobial peptides in the skin of *Rana esculenta* was demonstrated to be stimulated by micro-organisms, providing *in vivo* evidence for the induction of defence peptides in a vertebrate [11].

Spurred by the potential use of antimicrobial peptides in the treatment of infectious diseases that have become resistant to conventional antibiotics, an increasing public health problem, much research work has been conducted and is currently under way in order to understand the principles underlying the modes of interaction of these peptides with their target microbes. Although the exact mechanism(s) by which antimicrobial peptides exert their killing actions is not clearly understood, it is generally accepted that it involves interaction of the peptides with the cytoplasmic membrane of the target microbe, leading to membrane permeabilization and cell lysis and death [12,13]. However, it should be underlined that alternative and/or coexistent antimicrobial mechanisms cannot be ruled out at this stage, and evidence is accumulating that some peptides might actually act by binding to intracellular targets, or by stimulating host defence mechanisms [14]. Focusing on membrane activity, a key problem is understanding the basis of the membrane-selective specificity of antimicrobial peptides, i.e. their ability to distinguish between prokaryotic versus eukaryotic cells, Gram-positive versus Gram-negative bacteria, among the various bacterial strains, etc. Neither the impact of membrane composition nor the structural features of the peptides required for this specificity are as yet fully understood. The long-term goal in the field is to use

Abbreviations used: BMAP, bovine myeloid antimicrobial peptide; CFU, colony-forming units; FITC-D 4/20/70, fluorescein isothiocyanate dextrans of 4, 20 and 70 kDa average molecular mass respectively; Gal-ONp, 2-nitrophenyl β-D-galactoside; LB, Luria Bertani broth; LC, lethal concentration; LPS, lipopolysaccharide; PC, egg yolk L-α-phosphatidylcholine; PEG, poly(ethylene glycol); POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol.

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this knowledge to assist the design of novel anti-infective drugs and human therapeutic agents.

We and others have reported the structures and properties of skin peptides originally isolated from the European red frog *Rana temporaria* [15–18]. These peptides, collectively known as temporins, form a group of linear short (10–14 amino acid residues) peptides with a net cationic charge and an amidated C-terminus. Temporins are among the smallest antimicrobial peptides isolated to date from animal sources. Like many other antimicrobial peptides, temporins show a marked propensity to adopt an amphipathic α-helical conformation in apolar environments, a property believed to play an important role in their mechanism of antimicrobial action. Recent investigations led to the discovery of a number of temporin-like peptides in several North American *Rana* species and in *R. esculenta* (for a recent review, see [19]). The derived composite consensus sequence for temporins is FLP(I/L)IASLL(S/G)KLL-NH_2 [19]. These peptides have been reported in general to be most active against Gram-positive bacteria and *Candida albicans*, although they also have some activity against Gram-negative strains and variable haemolytic activity. Despite the 30 or so peptides known so far to belong to this group, detailed information about their structure–function relationships is available only in very few cases, including some synthetic analogues [15,17,20,21]. Furthermore, little is known about their modes of interaction with membranes and their mechanism of antimicrobial action, and temporins might well serve as models to understand the behaviour of other short naturally occurring peptides.

The purpose of the present study was to contribute to our understanding of the membrane activity and specificity of temporins, selecting temporin L (FVQWFSKFLGRIL-NH_2) as a model. We investigated the antibacterial, antifungal and haemolytic properties of this peptide, and also collected data on its ability to cause the permeabilization of artificial membranes of different lipid compositions. Since cancer cells display an altered membrane lipid composition with respect to normal eukaryotic cells, and some peptides have been shown previously to be selectively toxic towards certain tumours (e.g. [22–24]), we also evaluated the effects of temporin L on several cancer cell lines. The choice of temporin L is motivated by the fact that this peptide is the only member of this group to possess a tryptophan residue in its sequence, which represents a sensitive probe with which to obtain information on a membrane-bound peptide. The information gathered in the present study will therefore be integrated with results provided by ongoing biophysical investigations of the interactions of temporin L with lipid membranes, offering a more in-depth view of its properties and modes of action.

MATERIALS AND METHODS

Materials

Synthetic temporins L, B and D were purchased from SYNT:EM (Nîmes, France). The purity of the peptides, their sequences and concentrations were determined as previously described [17]. Culture media and antibiotics for cell cultures were purchased from Sigma (St. Louis, MO, U.S.A.), Gibco Laboratories (Grand Island, NY, U.S.A.) and Labtek-EUROBIO (Les Ulis Cedex, France). 2-Nitrophenyl β-D-galactoside (Gal-ONp), poly(ethylene glycol) (PEG), egg yolk L-α-phosphatidylcholine (PC), calcein, fluorescein isothiocyanate dextrans of 4, 20 and 70 kDa average molecular mass (FITC-D 4/20/70) and melittin were all from Sigma. Cholesterol and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). All other chemicals used were of reagent grade.

Antimicrobial assays

The antibacterial activity of temporin L and, for comparison, of temporin B was tested against the following standard bacterial strains: *Bacillus megaterium* Bm11, *Staphylococcus aureus* Cowan I, *Pseudomonas aeruginosa* A.T.C.C. 15692, *Streptococcus pyogenes* A.T.C.C. 12344, *Escherichia coli* D21, D21 e7, D21 f1, D21 f2 and D22, the clinical isolate *Yersinia pseudotuberculosis* YP III and the ocular pathogens *Staphylococcus capitis*, *Staphylococcus emolyticus*, *Staphylococcus lentus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Micrococcus luteus* and *Pseudomonas aeruginosa*. An inhibition zone assay on Luria Bertani broth (LB)/1% (w/v) agarose plates seeded with 2×10^5 viable bacteria, according to Hultmark et al. [25], was used. The antifungal activity of temporins L and B was tested on *Candida albicans* A.T.C.C. 10261, *Candida guillier-mondii* isolated from frog skin [11] and a *Candida tropicalis* human clinical isolate using the same assay in Winge medium [26]. To study the bactericidal effect and the rate of killing of temporin L, the peptide was added to a bacterial suspension of *E. coli* D21. The number of surviving bacteria was measured at different incubation times at 30 °C. *E. coli* was grown in LB at 37 °C to an D_{590} of 1 and diluted in PBS (2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 136 mM NaCl) to about 1×10^5 colony-forming units (CFU)/ml. Temporin L was added at a final concentration of 10 μM and temporin B, used as control, at 180 μM. Aliquots of 10 μl were withdrawn and spread on a plate for cell counting after an overnight incubation at 30 °C. Permeabilization of bacterial inner membranes was determined by measuring the β-galactosidase activity of an *E. coli* D21 culture as described in [27], using Gal-ONp as substrate. In all antimicrobial assays, temporins were dissolved in distilled water/20% (v/v) ethanol prior to use and added to the relevant medium at the indicated concentrations.

Haemolytic assay

The haemolytic activity of temporin L on human erythrocytes was determined as reported in [28]. Briefly, aliquots of 75 μl of a human erythrocyte suspension in 0.9% (w/v) NaCl were incubated with different concentrations of temporin L (dissolved in 20% ethanol prior to use) for 60 min at 37 °C with gentle mixing. The tubes were centrifuged and the absorbance of the supernatants was measured at 415 nm. Total haemolysis was obtained by suspending erythrocytes in distilled water. To test the effect of osmoprotectants [29], erythrocytes were suspended in 0.9% NaCl containing 25 mM PEG of different molecular sizes (1500, 2000, 3400, 4600 and 6000). Then increasing concentrations of temporin L were added and the haemolytic activity was determined as described above. The molecular diameters of the various PEG species were assumed to be [30]: PEG 1500, $\sim$ 24 Å; PEG 2000, $\sim$ 30 Å; PEG 3500, $\sim$ 38 Å; PEG 4600, $\sim$ 46 Å; PEG 6000, $\sim$ 58 Å. For comparison, PEG experiments were also performed in the presence of increasing concentrations of temporin D [15].

Cancer cell culture and treatment with temporin L

K-562 human erythroleukaemic cells and Hut-78 human cutaneous T lymphoma cells were cultured using an RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal

calf serum, 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. The cells (1×10^6/ml) were exposed for 4 h to temporin L, which was dissolved in distilled water/20 % (v/v) ethanol and added to the wells at the indicated concentrations. Controls contained equivalent quantities of water/ethanol but no peptide. Cells were maintained in exponential growth at 37 °C in a humidified atmosphere with 5 % CO_2. Human monoclonal leukaemia U-937 cells were maintained in RPMI 1640 medium supplemented with 10 % (v/v) foetal bovine serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The cells were grown at 37 °C/5 % CO_2 in a humidified incubator. U-937 cells were adjusted to a density of 1×10^5 cells/ml and cultured in 96-well plates. Temporin L was dissolved in distilled water/20 % (v/v) ethanol and added to the wells at the indicated concentrations. Equivalent quantities of water/ethanol were added to control cells, and samples were incubated at 37 °C/5 % CO_2 for 1 and 48 h. For all cell lines, cell viability was determined by the Trypan Blue exclusion method, and was expressed as a percentage of untreated control cells. Reported values are means of at least three assays.

Preparation of calcein- and dextran-loaded liposomes and leakage experiments

Calcein-entrapping liposomes of different lipid compositions were prepared as previously described [17]. The extent and time course of the release of calcein from liposomes was monitored fluorimetrically on a Perkin-Elmer LS 50 B spectrofluorimeter; excitation and emission wavelengths were 490 nm and 517 nm respectively. The calcein that is entrapped in the vesicles is at high concentration and the fluorescence is self-quenched. Leakage was monitored as a relief of quenching; the maximum fluorescence intensity was determined by the addition of 20 μl of Triton X-100 (10 %, v/v, in water) to the sample, which caused the total destruction of the vesicles, with consequent total relief of the quenching. The apparent percentage leakage value was calculated according to the equation [31]:

$$\text{Leakage } (\%) = 100 \times (F - F_0)/(F_t - F_0)$$

where F and F_t denote the fluorescence intensity before and after the addition of the detergent respectively, and F_0 represents the fluorescence of intact vesicles.

Dextran-loaded vesicles containing the FITC-D of choice (FITC-D 4, 20 or 70) were prepared as reported elsewhere [18]. The release of dextran from loaded vesicles upon interaction with temporin L was examined fluorimetrically; excitation and emission wavelengths were 494 nm and 520 nm respectively. In a typical experiment, an aliquot of the peptide solution in 20 % (v/v) ethanol was incubated with a suspension of dextran-loaded vesicles in buffer, with a final lipid concentration of 50 μM. The mixture (2 ml, final volume) was stirred gently for 10 min in the dark and then centrifuged at 22 500 ***g*** for 30 min. The supernatant was recovered and its fluorescence intensity recorded. The maximum fluorescence intensity was determined by the addition of 20 μl of 10 % (v/v) Triton X-100 to the vesicle suspension. The apparent percentage leakage value was calculated as described above for calcein. All experiments were carried out at room temperature.

Determination of peptide detergent-like action on model membranes

Void PC liposomes were prepared and lipids quantified as reported [18]. To evaluate the bilayer-disruptive property of temporin L, the change in attenuance at 560 nm of liposome suspensions was measured upon incubation with temporin L or, for comparison, with melittin. Clearing of the turbid vesicle suspension indicates the solubilization of liposomes into smaller particles. Vesicle suspensions in 50 mM potassium phosphate, pH 7.4 (100 μl; 2 mM lipid concentration), were incubated with increasing amounts of either temporin L or melittin at room temperature for 30 min. After this time, samples were diluted to 1 ml with buffer and attenuance at 560 nm was measured on a Perkin-Elmer Lambda 19 spectrophotometer. Controls contained liposomes but no peptides. Measurements were made in triplicate.

RESULTS

Main features of temporin L

The ribbon-like three-dimensional structure, helical wheel diagram and some biophysical data of temporin L are given in Figure 1. The potential for the peptide to be structured as an amphipathic α-helix in an apolar environment is manifest. The mean residue hydrophobicity (H) and hydrophobic moment (μ)

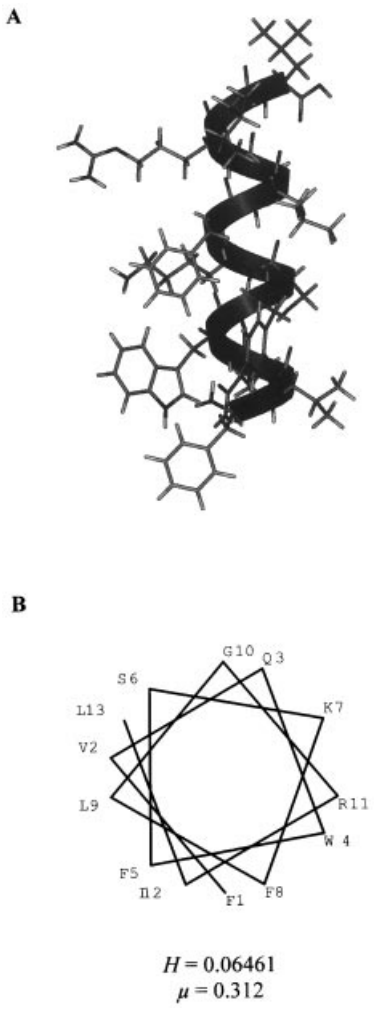

Figure 1 Structure and some biophysical parameters of temporin L

Ribbon-like three-dimensional structure (**A**) and helical wheel diagram (**B**) of temporin L. Hydrophobicity (H) and hydrophobic moment (μ) were calculated using the Eisenberg consensus scale of hydrophobicity [32].

Table 1 Antibacterial activity of temporins L and B against different bacterial strains

LCs were calculated from inhibition zones on agarose plates seeded with the respective organisms. Strains marked with * are clinical isolates kindly provided by the R&D Division of S.I.F.I., Catania, Italy. NA, not active.

	LC (μM)	
Bacterial strain	Temporin L	Temporin B
Bacillus megaterium Bm11	0.3	2.8†
Staphylococcus aureus Cowan I	0.5	6.0†
Escherichia coli D21	1.5	21.0†
Escherichia coli D21 e7	1.2	13.2†
Escherichia coli D21 f1	0.9	10.0†
Escherichia coli D21 f2	0.5	3.3†
Escherichia coli D22	0.7	11.2†
Pseudomonas aeruginosa A.T.C.C. 15692	3.6	> 360†
Yersinia pseudotuberculosis YP III	0.7	7.0†
Streptococcus capitis 1*	0.7	13.0
Streptococcus capitis 3*	0.3	2.8
Streptococcus hemolyticus 1*	0.4	6.3
Streptococcus lentus 1*	0.2	20.0
Streptococcus pyogenes A.T.C.C. 12344	0.6	7.0†
Staphylococcus aureus 7*	0.5	12.2
Staphylococcus aureus 8*	0.6	20.0
Staphylococcus epidermidis 11*	0.6	9.0
Staphylococcus epidermidis 18*	0.3	12.0
Staphylococcus hominis	0.4	13.0
Micrococcus luteus	0.3	9.0
Pseudomonas aeruginosa 2	17.0	NA

† Data taken from Simmaco et al. [15].

Table 2 Antifungal activity of temporins L and B against different *Candida* species

LCs were calculated from inhibition zones on agarose plates seeded with the respective organisms. Amphotericin B has been included as reference. NA, not active.

	LC (μM)		
Organism	Temporin L	Temporin B	Amphotericin B
Candida albicans	2.7	4.0*	0.3
Candida guiller-mondii	1.8	5.2	NA
Candida tropicalis	1.0	1.4	0.5

* Data taken from Simmaco et al. [15].

per residue were calculated using the Eisenberg consensus scale of hydrophobicity [32]. The H and μ values of temporin L are comparably high among temporins and other α-helical naturally occurring peptides, and both values are consistent with a membrane-active compound. Temporin L has an overall cationic charge at neutral pH of +3, the highest value among temporins and temporin-like peptides [19], together with those of temporins 1Lb and 1Lc from *R. luteiventris*.

Antimicrobial activity of temporin L

The antimicrobial activity of temporin L against several bacterial strains and clinical isolates, as well as different *Candida* species, was determined by the inhibition zone assay. The results are given in Tables 1 and 2, where the lethal concentration (LC) values are compared with those obtained with temporin B (LLPIVGNLLKSLL-NH_2) [15]. Temporin L showed at least 10-fold higher activity against all micro-organisms tested. In contrast with other temporins, which are known to be active particularly against Gram-positive bacteria, temporin L also displayed very good activity against Gram-negative bacterial strains such as *E. coli* D21 and *Ps. aeruginosa* A.T.C.C. 15692, with LC values of 1.5 and 3.6 μM respectively. A weak activity was found against the ocular pathogen *Ps. aeruginosa* 2. Temporin L, like temporin B, showed increasing antibacterial potency against cell-wall-defective mutant strains of *E. coli* D21, i.e. D21 e7, D21 f1 and D21 f2, which have lost increasing amounts of sugar residues of their lipopolysaccharide (LPS) chain [33]. Against *Candida* species, the potency of temporin L was of the same order as that reported for temporin B, and approx. 2–9 times lower than that indicated for amphotericin B (with the exception of *C. guillier-mondii*, which is totally resistant to the latter compound).

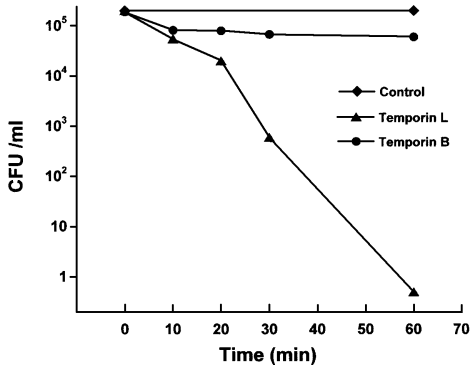

Figure 2 Rate of killing of *E. coli* D21 by temporins L and B

Temporins L and B were present at concentrations of 10 μM and 180 μM respectively in PBS. The number of surviving bacteria is expressed as CFU/ml. The control is bacteria without peptide. Values are means of three independent measurements.

The kinetics of action of temporin L were studied against *E. coli* D21 at high ionic strength in PBS. While a concentration of 10 μM temporin L was necessary to kill approx. 10^5 cells in 60 min, a 20-fold higher temporin B concentration caused only a 70% decrease in the number of CFU (Figure 2).

Since a large number of antibacterial peptides exert their effect by perturbing the permeability properties of the inner membrane in Gram-negative bacteria, we examined the effect of temporin L on the integrity of the cytoplasmic membrane of *E. coli* D21 by monitoring the leakage of cytoplasmic β-galactosidase. The results (Figure 3) demonstrate a progressive release of enzyme at increasing peptide concentrations. Moreover, at 10 μM temporin L, the lowest peptide concentration causing the killing of approx. 10^6 bacterial cells, the β-galactosidase activity detected in the supernatant was approx. 80%, and 100% was reached at 20 μM. This confirms the strong bactericidal activity of temporin L and its ability to greatly permeabilize the inner membrane of *E. coli*.

Haemolytic activity of temporin L

The effects of increasing temporin L concentrations on the haemolysis of human erythrocytes are reported in Figure 4. Beyond 2 μM, considerable haemolysis was observed, with 100% lysis at approx. 55 μM. In order to determine whether the lysis was due to a colloid-osmotic process, and also to measure the size of the membrane lesions, haemolysis was studied in the presence of various osmoprotectants, such as PEGs of different molecular size. Osmotic protection was investigated at different

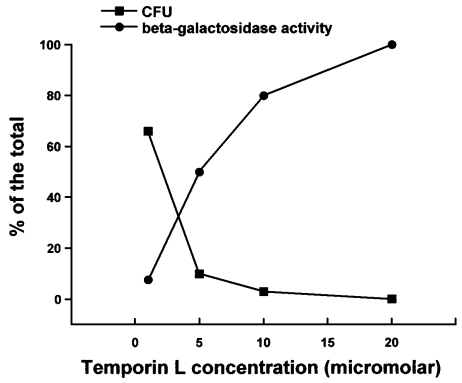

Figure 3 Number of surviving cells and β-galactosidase activity of an *E. coli* D21 culture after incubation with temporin L at various concentrations

Cells were grown in LB at 37 °C, diluted in 10 mM sodium phosphate buffer, pH 7.4, and incubated with temporin L for 60 min at 30 °C. Bacterial viability is expressed as a percentage of the total. β-Galactosidase activity was measured in the culture filtrate by following the hydrolysis of 2 mM Gal-ONp at 420 nm. Total enzyme activity was determined by treating the cells with 0.1% SDS/chloroform. The values, means of three independent determinations, are given as percentages. Enzyme activity detected in the control (bacteria without peptide) was subtracted from all values.

peptide concentrations. Data shown in Figure 5 indicate that, at a temporin L concentration of 8 μM, at which 80% lysis was detected in the absence of osmoprotectants, haemolysis was prevented by PEG of size 3500 and above, suggesting the production of lesions of approx. 36–40 Å in diameter. At 15 and 40 μM temporin L, PEG 4600 and 6000 respectively offered almost complete protection, indicating that the size of the lesions increases with increasing peptide concentration, and that lysis proceeds through a colloid-osmotic mechanism. Virtually no protection was observed at the temporin L concentration (50 μM) that caused 100% haemolysis, suggesting the possibility that a different mechanism is in action at high peptide concentrations. The haemolytic activity of temporin L and temporin D (LLPIVGNLLNSLL-NH_2) [15], which displays the most potent haemolytic activity among temporins tested so far [17], is reported

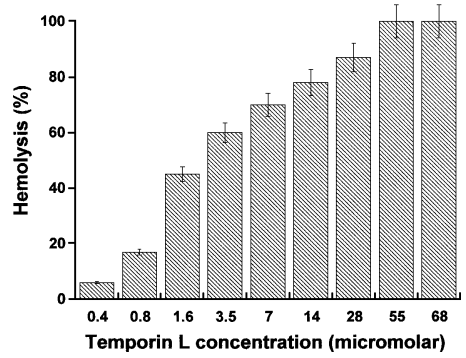

Figure 4 Haemolysis of human erythrocytes as a function of temporin L concentration

Erythrocytes were suspended in 0.9% NaCl and incubated with temporin L at 37 °C for 60 min. The absorbance of the supernatant was recorded at 415 nm. Complete haemolysis (100%) was measured by suspending erythrocytes in distilled water. Values are means ± S.E.M. of four independent measurements.

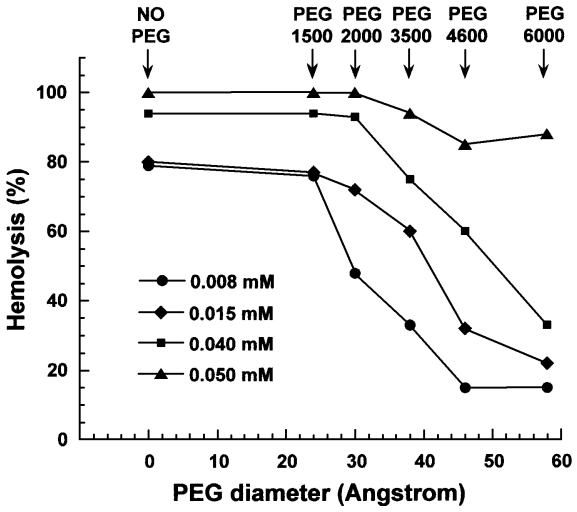

Figure 5 Haemolysis induced by different concentrations of temporin L in the presence of osmoprotectants of different sizes

Erythrocytes were suspended in 0.9% NaCl and 25 mM PEG of different molecular sizes (1500, 2000, 3500, 4600 and 6000). Subsequently, temporin L was added and haemolysis was determined after a 60 min incubation at 37 °C. The absorbance in the supernatant was recorded at 415 nm. Complete haemolysis (100%) was measured by suspending erythrocytes in distilled water. Values are means of five independent measurements. The diameters of the protectants are described in the Materials and methods section.

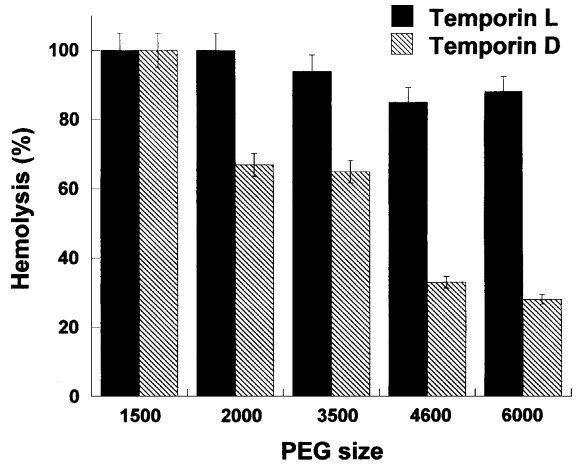

Figure 6 Haemolysis induced by temporin L and temporin D at a peptide concentration causing 100% lysis in the presence of osmoprotectants of different sizes

Temporin L (50 μM) or temporin D (80 μM) was added to a suspension of erythrocytes in 0.9% NaCl and 25 mM PEG of different molecular sizes. Values are means ± S.E.M. of three independent measurements.

in Figure 6. The assay was performed in the presence of PEG at a temporin concentration causing 100% haemolysis.

Effects of temporin L on cancer cell viability

Exposure of cancer cells to increasing concentrations of temporin L caused a significant decrease in cell viability, although the

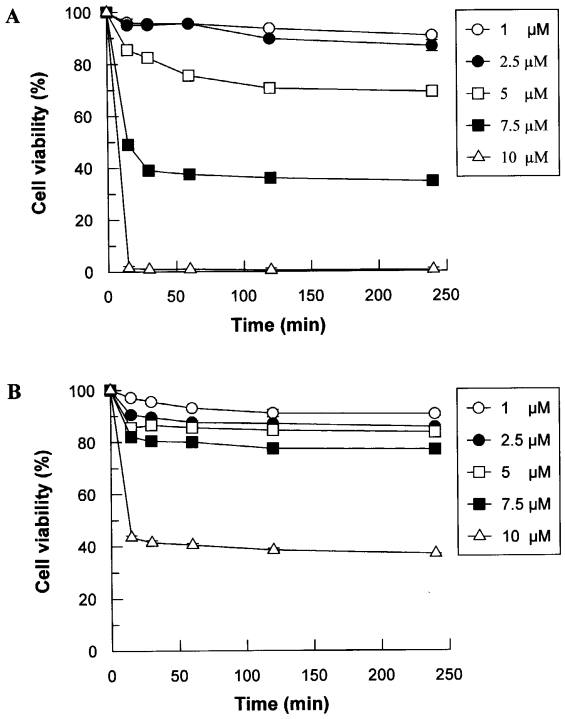

Figure 7 Cytotoxicity of temporin L to Hut-78 and K-562 tumour cells

Hut-78 (**A**) and K-562 (**B**) tumour cells were cultured as described in the Materials and methods section. The cells (1×10^6/ml) were exposed for up to 240 min to different temporin L concentrations, and cell viability was determined by the Trypan Blue exclusion method and expressed as a percentage; untreated control cells were taken as 100% viable. Values are means ± S.E.M. of three independent measurements.

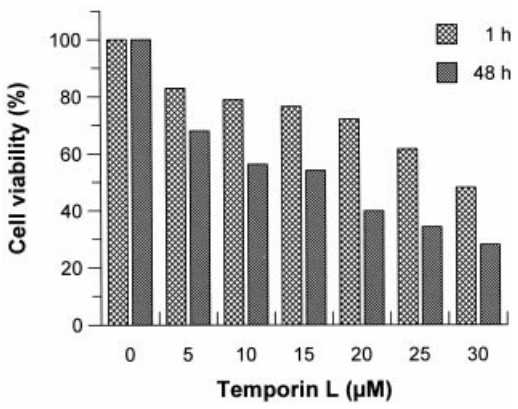

Figure 8 Cytotoxicity of temporin L to U-937 cells

U-937 tumour cells were cultured as described in the Materials and methods section. The cells (1×10^5/ml) were exposed for 1 h or 48 h to different temporin L concentrations, and percentage cell viability was determined by the Trypan Blue exclusion method. Values are means ± S.E.M. of three independent measurements.

sensitivity to the peptide varied markedly with the specific cell line tested. The toxicity of temporin L towards Hut-78 cells is shown in Figure 7(A). Whereas treatment with low peptide concentrations (1–2.5 μM) did not affect viability substantially as compared with control samples, a small fall in viability was evident at 5 μM peptide after a 15 min exposure; this increased, although slightly, upon prolonged treatment (240 min). When exposed to higher concentrations of temporin L (7.5 μM and 10 μM), Hut-78 cells showed a pronounced sensitivity to the treatment, which resulted in cell survival at the end of the experiments as low as 34.5% and 0.5% respectively. It should be noted that a killing effect close to the maximum was visible even after only a 15 min exposure to any concentration of temporin L used in this work, indicating a rapid toxic action of the peptide against Hut-78 cells.

The cell viability profile of K-562 cells (Figure 7B) resembled that of Hut-78 cells when temporin L was tested at relatively low concentrations (1–2.5 μM), but at higher peptide concentration (5–10 μM) the responses of the two cell lines to the treatment diverged significantly. Indeed, K-562 cells displayed marked resistance to temporin L compared with Hut-78 cells, the survival rate being as high as 37% at the highest peptide concentration (10 μM). In accordance with what was seen for Hut-78 cells, the kinetics of temporin L action on K-562 cells were fairly rapid, with a near-maximum killing effect being reached shortly after the beginning of the treatment, followed by little change with increasing exposure time.

To assess the effects of prolonged exposure to temporin L on a resistant cancer cell line, we selected human monoclonal leukaemia U-937 cells, which, in a preliminary attempt, displayed 2-fold increased survival compared with K-562 cells when exposed to 10 μM temporin L for 60 min (78.9% compared with 37%) (Figure 8). Increasing the peptide concentration up to 30 μM resulted in a survival rate over a 60 min exposure time of $\sim$ 48%, confirming the resistance of U-937 cells to the cytotoxic action of temporin L. However, when the exposure time was extended to 48 h, a significant decrease in cell viability relative to control cells was noted (Figure 8), suggesting that the cytotoxic effect of temporin L is both concentration- and time-dependent.

The reported effects on cell viability were due to peptide-induced cytotoxicity; no apoptosis-inducing effect, as evaluated by the classic nucleic acid staining method with Acridine Orange and ethidium bromide [34], was observed on the most sensitive Hut-78 cells (results not shown).

Permeabilization of lipid vesicles

The membrane-permeabilizing ability of temporin L was investigated by measuring the release of the fluorescent marker calcein from liposomes of different composition and surface charge density. We employed vesicles composed of electrically neutral PC, PC vesicles with a low content of cholesterol (PC/cholesterol 9:1, mol/mol), and mixed PC vesicles containing different ratios of negatively charged POPG (PC/POPG 9:1, 8:2 and 6:4, mol/mol). The percentage calcein leakage 10 min after exposure to the peptide was used as a measure of the membrane permeability enhancing effect of temporin L (Figure 9A). The peptide caused the release of the entrapped marker from all tested liposomes, although the effectiveness was significantly dependent on vesicle composition. The activity of temporin L was greatest with liposomes consisting of zwitterionic PC, and the peptide caused an almost total disruption of these vesicles at a concentration of 5 μM (lipid/peptide ratio = 10). The presence of 10% (mol/mol) cholesterol in the electrically neutral membrane had no appreciable effect on the permeabilizing activity of temporin L. In contrast, substantial differences in the permeabilizing ability of the peptide were found for vesicles bearing increasing amounts of negatively charged POPG. In this case, an increase in POPG content negatively influenced temporin

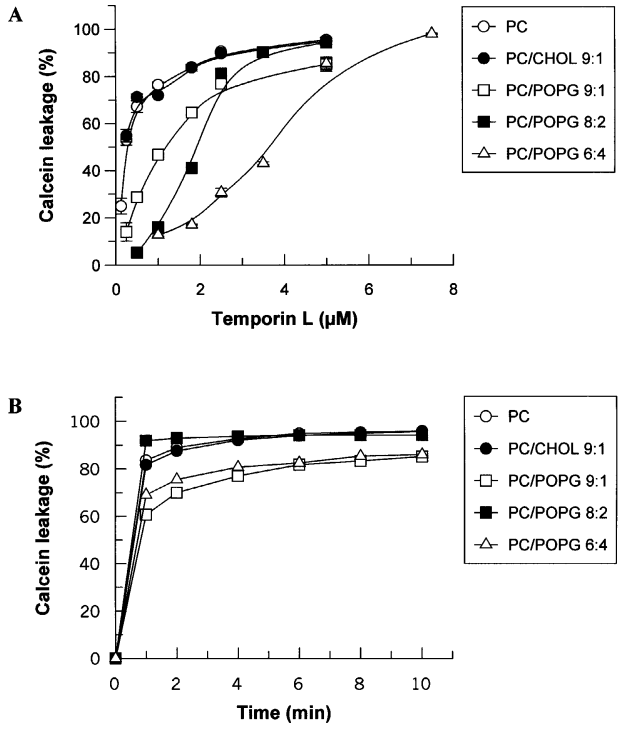

Figure 9 Temporin L-induced calcein release from liposomes

(**A**) Dependence of calcein leakage from lipid vesicles on temporin L concentration. Calcein-containing liposomes of different lipid composition were prepared and quantified as described in the Materials and methods section. Liposomes (final lipid concentration 50 μM in 50 mM potassium phosphate/0.1 mM EDTA, pH 7.4) were incubated in the presence of different concentrations of temporin L for up to 10 min at room temperature. Calcein release was detected fluorimetrically. The apparent percentage leakage after a 10 min incubation was calculated as $100 \times (F - F_0)/(F_t - F_0)$, where F and F_t denote the fluorescence intensity before and after the addition of the detergent respectively, and F_0 represents the fluorescence of intact vesicles. Values are means $\pm$ S.E.M. of three independent measurements. (**B**) Time course of calcein release from liposomes in the presence of 5 μM temporin L. Experimental conditions were as described for (**A**). CHOL, cholesterol. Compositions (e.g. PC/POPG 9:1) are given as mol/mol.

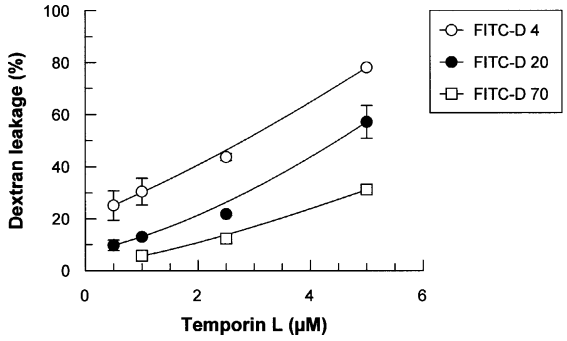

Figure 10 Effect of temporin L on release of FITC-D from PC liposomes

PC liposomes containing FITC-D 4, 20 or 70 were prepared and quantified as described in the Materials and methods section. Liposomes (final lipid concentration 50 μM in 2 ml of 50 mM potassium phosphate/0.1 mM EDTA, pH 7.4) were incubated in the presence of different concentrations of the peptide for 10 min at room temperature. Dextran release was detected fluorimetrically. The apparent percentage leakage was calculated as $100 \times (F - F_0)/(F_t - F_0)$, where F and F_t denote the fluorescence intensity before and after the addition of the detergent respectively, and F_0 represents the fluorescence of intact vesicles. Values are means $\pm$ S.E.M. of three independent measurements.

L activity, particularly at lower peptide concentrations. Total calcein release from PC/POPG 6:4 vesicles, the most resistant liposomes, was attained at a peptide concentration of 7.5 μM. Interestingly, upon doubling the POPG content from 10% to 20%, the percentage calcein leakage exhibited a 'cross-over' point at a peptide concentration of $\approx$ 2.5 μM. Specifically, below that point the permeabilizing activity of temporin L was greater at any peptide concentration for PC/POPG 9:1 vesicles than for PC/POPG 8:2 vesicles, whereas the opposite was observed at peptide concentrations higher than 2.5 μM (Figure 9A). We must point out, however, that the significance of this cross-over should not be overstated, since it might be a consequence of the PC/POPG 9:1 liposomes reaching a slightly lower maximal extent of leakage compared with other systems, a fact for which we do not have an explanation at the moment. However, the variance in the extent of peptide-induced permeabilization of vesicles of any lipid composition at high peptide concentrations was in reality quite low, as shown below, indicating that a threshold peptide/lipid value had been reached.

The time-dependence of calcein release from lipid vesicles in the presence of a relatively high temporin L concentration (5 μM) is shown in Figure 9(B). In general, temporin L-induced leakage followed reproducible biphasic kinetics, with a fast phase (approx. within the first 1 min) triggered by the initial interaction of the peptide with the membrane, which decayed to a slow steady-state release of the encapsulated marker. It should be stressed, however, that while total calcein release at the end of the incubation time attained a comparable value for every lipid composition ($\approx$ 85–95%), significant differences in the extent of leakage during the first phase were noted, in particular among liposomes containing various amounts of negatively charged POPG. Whereas, with PC/POPG 8:2 vesicles, over 97% of the total leakage occurred during the first 1 min after addition of the peptide, this value was 80% and 71% for PC/POPG 6:4 and PC/POPG 9:1 vesicles respectively.

To gather further clues as to the type and size of membrane damage caused by temporin L, we assessed the peptide-induced release of liposome-encapsulated markers of different sizes. PC vesicles were preloaded with fluorescently labelled dextrans of 4, 20 or 70 kDa average molecular mass (FITC-D 4/20/70) and then incubated with increasing amounts of temporin L. This method allows one to distinguish between the action of a pore-forming peptide, with the possibility to size such pores, and a peptide that acts in a detergent-like manner [35]. In the latter case, no size dependence in the release of encapsulated markers should be noted. The results obtained with temporin L (Figure 10) reveal a marked dependence of peptide-induced leakage on the molecular mass/size of the fluorescent probe. In particular, temporin L was found to release 78% of FITC-D 4, but only 31% of FITC-D 70, from PC vesicles at a peptide concentration of 5 μM (lipid/peptide ratio = 10).

Evaluation of detergent-like effects on membranes

Several α-helical peptides, including melittin and magainins, are known, at high peptide concentrations, to solubilize membranes in a detergent-like manner, causing both bilayer micellization and fusion [36,37]. Evidence for such a detergent-like action can be obtained, in a semi-quantitative way, by analysing changes in the macroscopic appearance of vesicle suspensions [37]. This approach was used here to evaluate whether temporin L was able to cause the breakdown of PC membranes into micelles at temperatures below the gel–liquid-crystalline phase transition. Results were compared with those obtained with melittin, used as a positive reference. PC vesicles were selected because melittin

Table 3 Effects of temporin L and melittin on membrane solubilization

The detergent-like ability of temporin L was evaluated by its capacity to clear a liposome suspension, as compared with that shown by melittin. PC liposomes (2 mM lipid concentration) were incubated at room temperature for 30 min with increasing peptide concentrations. After dilution to 1 ml, attenuance was read at 560 nm. Values are percentages relative to control with no peptides (100%), and are means ± S.E.M. of three independent measurements.

	Turbidity (%)	
Peptide concentration (μM)	Temporin L	Melittin
30	94.50 ± 1.97	82.30 ± 0.85
50	94.21 ± 1.58	76.08 ± 1.98
100	95.67 ± 0.61	65.21 ± 0.56
130	95.96 ± 0.78	61.92 ± 0.80

is particularly active on zwitterionic membranes, and also temporin L was shown in the present study to interact strongly with liposomes of this lipid composition. As shown in Table 3, although the addition of increasing amounts of melittin up to 130 μM (peptide/lipid 6.5%, mol/mol) caused, as expected, progressive clearance of the vesicle suspension, equal concentrations of temporin L had virtually no effect on this parameter.

DISCUSSION

Temporins constitute an emerging class of antibiotic peptides that are secreted in the skin of several members of the Anuran genus *Rana*. About 30 of the 100 or so peptides isolated to date from *Rana* frogs can be classified as temporins or temporin-like peptides [19]. This testifies to the important role played by these peptides in protecting the host animals from pathogens. In addition, this appreciation is an incentive to studies aimed at disclosing the biological activities and modes of action of these peptides, possibly leading to new treatments for nosocomial and multidrug-resistant infections. Previous studies have demonstrated that temporins and temporin analogues have antimicrobial activity against a broad spectrum of bacteria [20,21], including clinically important methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* [16]. In addition, while most reports have focused on the antibacterial and antifungal activities of temporins, recent studies hint at a broader spectrum of bioactivities for these peptides. Four temporins isolated from the skin of the North American pig frog *Rana grylio* were shown to possess vasorelaxant activity in rat thoracic aorta [38]. Also, temporins A and B are active against *Leishmania donovani* promastigotes, with a membrane-permeabilizing effect (L. Rivas, personal communication).

Here we report on the antimicrobial, haemolytic and cytotoxic activities of temporin L, and also on its permeabilization effects on model membranes. Together with previously published results on other peptides of the same class, the present study permits us to appreciate the diversity of specificity displayed by different temporins. In particular, temporin L proved to possess not only the highest bioactivity among temporins studied so far, against both bacterial and fungal strains and human erythrocytes, but also a different spectrum of activity. Temporin L was at least 10 times more active than temporin B against all Gram-positive strains tested. Furthermore, at variance with other temporins, it is also active against Gram-negative strains. The leakage of large molecules such as β-galactosidase from *E. coli* D21 cells caused by the peptide shows that temporin L is able to seriously perturb the bacterial inner membrane. In the case of Gram-negative bacteria, the outer membrane, which acts as a strong permeability barrier to various antibiotics, is built up mainly from polyanionic LPS, to which cationic peptides are expected to bind to some extent before reaching the plasma membrane [31,39]. The assay performed using LPS-defective strains of *E. coli* D21 confirmed that the interaction of the peptide with the negatively charged portions of LPS may be important in limiting or regulating the antibacterial activity of temporin L against Gram-negative bacteria. Electrostatic, rather than hydrophobic, interactions are believed to play a key role in antimicrobial peptide activity against Gram-negative bacteria [40], and in this connection we stress once more that temporin L has the highest cationic charge measured so far among temporins and temporin-like peptides [19].

With regard to haemolytic activity, previous research has shown that temporins A, B and D are able to lyse erythrocytes in liquid media, with temporin D being the most active [17]. When tested under the same conditions, temporin L was found to be significantly more efficient than temporin D in lysing human erythrocytes. It is of interest to note the different levels of osmotic protection provided by PEGs of diverse molecular size against haemolysis caused by temporins L and D at high concentrations (Figure 6). This finding may hint at dynamics and/or aggregation states of the erythrocyte-bound temporins that may differ among peptides, leading to membrane lesions of variable size. It should be stressed that the haemolytic activity of temporins fits well with the high affinity of these peptides for zwitterionic model membranes ([17]; the present study, see below), since, in contrast with bacterial membranes, the plasma membrane of human erythrocytes does not contain negatively charged lipids in the outer leaflet. There is also a general consensus that hydrophobic-peptide–membrane interactions should determine the haemolytic effect [41], and this correlates with the relatively high hydrophobicity and hydrophobic moment displayed by temporin L.

Several antimicrobial peptides, e.g. defensins [42], magainins [22] and the bovine myeloid antimicrobial peptides BMAP-27 and BMAP-28 [23] have been shown to be cytotoxic to normal or transformed mammalian cells. Besides adding to the increasing evidence for the multifunctional activity of antimicrobial peptides, this poses intriguing questions regarding the mechanism by which eukaryotic (mammalian) cell death is induced. In particular, it is important to assess whether cytotoxicity is caused by plasma membrane permeabilization, as is generally accepted in the case of prokaryotic cells, and/or by peptide action on other cell compartments and/or metabolic pathways. A detailed investigation of the action of BMAP-28 peptide on two tumour cell lines (U-937 and K-562) and activated normal lymphocytes suggested that cytotoxicity is probably caused by the concurrence of several peptide actions, such as damage to plasma membrane integrity, but also increased mitochondrial membrane permeability [23,43]. In particular, BMAP-28 was found to induce opening of the mitochondrial permeability transition pore and cytochrome *c* release, the latter event being accompanied by initiation of a cell death programme and subsequent DNA fragmentation. In both U-937 cells and activated human lymphocytes, Ca^{2+} influx into the cytosol occurs in the early steps of permeabilization that follow interaction of the peptide with the plasma membrane, and this event is followed by programmed cell death [23]. The authors rationalize this observation by suggesting that the early rise in cytoplasmic Ca^{2+} may be an important additional factor generating signals that synergize with the perturbation of mitochondrial membrane permeability to cause cell death. Unlike BMAP-28, temporin L was found not to possess a clear apoptosis-inducing effect on any cancer cell type tested. The mechanism of cytotoxicity of temporin L may

therefore be plausibly attributed to its plasma-membrane-permeabilizing activity, clearly shown in the present study and also a hallmark of most antimicrobial peptides. The reason for the different degrees of toxicity exerted by temporin L on the three cell lines is not yet clear, and needs further investigation, but is likely to be associated with differences in the composition of the membrane, as was suggested for the selective action of toxic agents on neoplastic cells [44,45]. However, we cannot rule out the possibility that temporin L may also be mitochondrially active and thus affect cell function in more complex manner. Indeed, perturbation of mitochondrial permeability and function may also lead to a necrosis-like cell death rather than apoptosis [46,47]. As several membrane-active antimicrobial peptides, including melittin, cecropins and magainins, have been shown to affect mitochondrial function, it is possible that the coupling of plasma membrane and mitochondrial mechanisms of cytotoxicity may be of general significance ([43] and references therein).

Our investigation of the action of temporin L on model membranes revealed that the peptide interacts strongly with neutral, zwitterionic lipid bilayers. This interaction leads to perturbation of membrane integrity and lysis of the lipid vesicle, as shown by the leakage of fluorescent markers. As previously reported for temporins A and B [18], the extent of leakage was found to be dependent on the size of the entrapped markers, with the release of the larger molecules being less than that of smaller ones. This indicates the formation of a pore or of local breaks in the membrane, rather than its disruption through a detergent-like action [35]. This is also suggested by the fact that relatively high concentrations of the peptide did not provoke the clearance of a turbid lipid suspension, at variance with the well known micellization-inducing action of the bee venom peptide melittin.

Interestingly, the presence of negatively charged POPG in the neutral PC matrix inhibited the lytic power of temporin L. Although this was at first unexpected, since negatively charged peptides are generally believed to be the main target of cationic antimicrobial peptides, a careful reading of the relevant literature reveals that the finding is not unprecedented, and provides useful indications to interpret our data. In fact, inhibition of leakage by negatively charged lipids has been observed previously for the cationic amphipathic peptides melittin [48], nisin [49] and mastoparan [50], and for the synthetic model peptide KLAL [40]. For melittin, Benachir and Lafleur [48] considered a two-step approach in order to rationalize the effect of negative charge density on peptide-induced vesicle permeabilization. The two steps involve binding of melittin to the bilayer surface and its subsequent redistribution and reorganization leading to pore formation. Taking into account the high affinity of melittin for zwitterionic membranes, the authors argue that electrostatic interactions between cationic residues of melittin and the negatively charged head groups of anionic lipids anchor the peptide on the membrane surface, preventing its penetration into the bilayer core and restricting its mobility and thus lytic activity [48]. In keeping with these findings, Dathe and co-workers [40] have shown that the interaction of the model peptide KLAL with lipid bilayers is mainly due to hydrophobic forces that drive the penetration of the amphipathic peptide into the inner membrane region, disturbing the arrangement of lipid acyl chains and causing local disruption. At low negative surface charge, the hydrophobic transfer of the peptides from the bilayer surface into the non-polar acyl chain region is favoured. In contrast, at higher negative surface charge, electrostatic interactions between the positive peptide charges and the acidic phospholipid head groups anchor the peptide in the membrane surface region, causing in addition reorientation of lipid head groups and the formation of densely packed peptide–lipid clusters [40].

Since temporin L was clearly shown to interact spontaneously with PC bilayers, inhibition of the lytic activity of the peptide by negatively charged lipids can be tentatively explained in a manner similar to that proposed for melittin and other cationic peptides. Given the fact that temporin L, like other small peptides, cannot span the membrane and thus form a simple transmembrane pore composed of a helical cluster (such as that formed by alamethicin), it is presumed that it undergoes profound reorganization in the bilayer, involving hydrophobic contacts with lipid acyl chains, to cause membrane disturbance and rupture. It might be of interest here to note that although melittin, like alamethicin, is usually described to induce pores conforming to the so-called barrel-stave model, a recent re-examination of the issue led to the proposal that melittin, by analogy with magainins and other peptides, actually forms pores that are consistent with the toroidal model [51]. This differs from the barrel-stave model in that the peptides are always associated with the lipid head groups even when they are inserted perpendicularly into the lipid bilayer, and that the lipid monolayer bends continously through the pore so that the water core is lined by both the peptides and the lipid head groups [51].

We have recently studied the ability of temporin L to insert into lipid monolayers, and its effects on lipid dynamics in model bilayers and on the membrane topology of giant vesicles [52]. Consistent with the evidence provided by the present work, we found that the presence of acidic phospholipids in the bilayer markedly affects the membrane activity of temporin L. The peptide's capacity to penetrate membranes, and its orientation, aggregation state and dynamics when bilayer-bound, differ quantitatively and qualitatively in the absence and presence of negatively charged lipids. Furthermore, the presence of acidic phosphpolipids induces lipid segregation and the formation of microdomains with clusters of lipid-bound peptide molecules [52].

Although it is not yet possible to reconcile the observations collected so far to provide an unequivocal mechanism of action of temporin L, the available data suggest that an appropriate balance of hydrophobic and electrostatic interactions must regulate, in a complex manner, the interaction of this peptide with lipid membranes, and thus modulate its target cell selectivity and biological properties. Further studies are in progress to describe the membrane activity of temporin L in more detail.

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