Membrane Binding and Perturbation Studies of the Antimicrobial Peptides Caerin, Citropin, and Maculatin

C. S. Brian Chia,[1] Yujing Gong,[2] John H. Bowie,[3] Johannes Zuegg,[2] Matthew A. Cooper[2]

[1]*Experimental Therapeutics Centre, Agency for Science, Technology and Research, 31 Biopolis Way, #03-01 Nanos, 138669, Singapore*

[2]*Institute for Molecular Bioscience, University of Queensland, 306 Carmody Rd., St Lucia, Qld 4072, Australia*

[3]*Department of Chemistry, The University of Adelaide, North Terrace 5005, South Australia*

Received 21 January 2010; revised 22 February 2010; accepted 6 March 2010
Published online 21 August 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bip.21438

ABSTRACT:

Citropin 1.1, maculatin 1.1, and caerin 1.1 are short antibacterial cationic peptides from the skin glands of the Australian tree frog Litoria *species. Several analogues have been synthesized to give a better insight into the relationship between the structure of the peptides and their antibacterial and haemolytic activity. Binding studies using a surface plasmon resonance (SPR) biosensor together with a vesicle-capture sensor chip have been used to investigate selectivity of the peptides and their analogues for 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) vesicles, as well as for vesicles made from lipid extracts from* Escherichia coli *and bovine brain. Data obtained for membrane selectivity using natural lipid extracts show better correlation with minimum inhibitory concentration (MIC) values against Gram-positive bacteria and haemolytic activity than that obtained using synthetic DMPG and DMPC. Electron microscopy and membrane leakage studies using Gram-positive bacteria gave further insight into the membrane disruption properties of the peptides. For maculatin 1.1, it was found that the central proline residue, which is responsible for a bend in the α-helical structure, is essential not only for the antibacterial activity but also for binding, and perturbation of membranes. The caerin analogues showed only small variations in their MIC values and membrane binding. In contrast, for citropin 1.1, the analogue replacing the aspartate with a lysine showed the lowest MIC against Gram-positive bacteria and best membrane binding to* E. coli *lipid extracts, coinciding with an increased hydrophobic moment of the peptide. These data give further insight into these antimicrobial natural products, toward the development and evaluation of these and other analogues as potential antibiotics. © 2010 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 96: 147–157, 2011.*

Keywords: antimicrobial peptides; peptide antibiotic; electron microscopy; phospholipids; membrane selectivity; surface plasmon resonance

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

Current address: The Experimental Research Center, China Academy of Chinese Medical Sciences, Beijing 100700, P.R. China.
Correspondence to: Matthew A. Cooper; e-mail: m.cooper@uq.edu.au
Contract grant sponsors: BBSRC, ARC

INTRODUCTION

To increase their chances of survival in the wild, many organisms use bioactive chemicals to defend themselves against predators and pathogenic micro-organisms.[1–3] Amphibian skin is a well-known source of such chemicals[4–7] and most were found to be peptides. A search in

the antimicrobial peptide database[8] (APS) reveals around 580 peptides that have been isolated from frogs or toads. Around 100 of these were isolated from the *Litoria* genus, an Australian tree frog species.[9] These peptides, which vary between 10 and 25 residues, are obtained from skin glands found on the dorsal surface of the amphibian, and include peptides such as aurein, caeridin, citropin, dahlein, gastrin, and maculatin, most of which were found to possess broad-spectrum antibiotic activity. Furthermore, most were found to have low-haemolytic activity. These compounds are hence potential candidates for the development of novel antibiotics,[10] and have therefore attracted the interest of several research groups.[7,11–15]

The antibacterial peptides from the Australian frog species *Litoria* belong to the class of linear helical peptides, which are cationic and adopt amphipathic α-helical structure in membrane and membrane-like environments.[14,16] They bind or aggregate with the membrane in an unspecific manner, and disrupt the bacterial membrane integrity.[17] Several models have been proposed to describe how these helical peptides disrupt bacterial membrane; two pore-forming mechanisms (a) barrel-stave and (b) toroidal models; and a surface-interaction mechanism (c) the carpet model.[18] In the barrel-stave model, the peptide forms transmembrane aggregates that form a pore lined with either the peptide or with a mixture of peptide and lipid. In the toroidal model, the peptide disrupts the lipid–lipid interaction, leading to a thinning of the membrane by mediating a curvature of the inner and outer surface, and eventually the formation of a pore. The pores can then act as nonselective channels, allowing the free flow of ions, solutes and metabolites, destabilizing and killing the bacteria. For both models, it is suggested that the peptide is able to span the lipid membrane (>20 residues). In the carpet model, shorter (<20 residues) peptides bind parallel to the membrane surface to form clusters, exerting disruptive forces to the membrane, without explicit pores. Independently of the chosen membrane perturbation model, an implicit concentration threshold is always required for disruption.[19]

Some members of the α-helical, antibacterial peptides are reported to have antitumor activity,[20–22] i.e., aurein and magainin. Both are suggested to act in a similar mode of action as with bacteria, by disrupting the membrane. In the better studied case of magainin, the peptide is suggested to penetrate the cell membrane and disrupt the mitochondrial membrane, thereby triggering apoptosis.[21]

Citropin 1.1 is a 16-residue antimicrobial peptide, produced by both the dorsal and submental skin glands of the Australian green tree frog *Litoria citropa*.[15,23] The peptide shows antibacterial activity mainly against Gram-positive bacteria,[13] but it also shows inhibition of nitric oxide synthases (NOS).[24] No antitumor activity has been reported for citropin 1.1 or its derivatives so far. The structure of citropin 1.1 is reported to form a random coil in aqueous environment but forms a defined α-helical structure in hydrophobic, membrane-like environments, such as 50% trifluoroethanol (TFE)[23] or in the presence of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC).[25] A more recent study indicated that citropin 1.1 in the presence of SDS micelles actually forms two α-helical fragments (residue 4–7 and 10–16) with a βIV turn in between residues 8 and 9.[26]

Caerin 1.1 is a 25-residue peptide from various Australian green tree frog (*Litoria* species), showing similar activity profile as citropin, i.e., acting mainly on Gram-positive bacteria. Caerin was also reported to have inhibitory effect on mucosal HIV transmission.[27] Solution nuclear magnetic resonance (NMR) structure of caerin 1.1[28] in membrane-like environments revealed a predominately α-helical structure, but with a distinct bend due to the two prolines. Both prolines are important for the antimicrobial activity.[29] Peptide–lipid interaction studies suggest that caerin 1.1 spans the membrane by forming pores in a barrel-stave like model.[30]

Maculatin is a 21-residue peptide, similar to caerin in its primary sequence. The peptide, which was isolated from *Litoria genimaculata*, shows also similar antibacterial activity, i.e., mainly against Gram-positive bacteria, and similar inhibitory effect on mucosal HIV transmission.[27] Solution NMR structure of maculatin 1.1 in membrane-like environments also shows a predominately α-helical structure with a bend due to the single proline.[31] As is the case with caerin, the proline is important for antimicrobial activity. One study of the peptide-lipid interaction using a quartz crystal microbalance with DMPC/1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) membranes showed some differences compared to maculatin, suggesting a more toroidal like model for caerin rather than a pore forming model.[30]

In this article, we studied minimum inhibitory concentrations (MICs) and haemolytic activity of citropin 1.1, maculatin 1.1, caerin 1.1, and analogues, and investigated their effects on membrane disruption. Most peptide-lipid interaction studies reported to date have been carried out using synthetic DMPC, DMPG, or 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE). However, native membranes are comprised of a variety of saturated, unsaturated, and derivatized lipids, which result in membranes with different physicochemical properties to those made using a single synthetic phospholipid. In this article, we study the peptide-lipid interaction of some of the α-helical antimicrobial peptides with total lipid extracts of *E. coli* and of bovine brain cells. For citropin 1.1, three analogues were designed to change the electrostatic properties of the peptide. For maculatin 1.1, a single analogue was designed to change the α-helical conformation

and thereby potentially disrupt the amphipathic moment of the peptide. For caerin 1.1, we selected two variants which change the hydrophobic property of the peptide but retain the range of antimicrobial activity.[32] In addition, we determined what effect these changes had on membrane specificity with regard to lipid composition of model membranes and lipid extracts, using a surface plasmon resonance (SPR) biosensor.[33] We have also used transmission electron microscopy and a membrane-potential fluorescence leakage probe to gain a better insight into the mechanism of bactericidal action of these peptide antibiotics on Gram-positive bacteria.

MATERIALS AND METHODS

Materials

Peptides were purchased from Chiron Mimotopes (Victoria, Australia) and were used without further purification. The lipids DMPC and DMPG were purchased from Aldrich (UK), and bovine brain and *E. coli* total lipid extracts were purchased from Avanti Polar Lipids (USA). Bacteria assayed were laboratory strains or were obtained from the American Type Culture Collection (http://www.atcc.org/).

Peptide Properties

The properties of the peptides, such as isoelectric point, charge at physiological pH, hydrophobic moment, and hydrophobicity were calculated using the EMBOSS[34] software suite and the tools at the ExPASy Proteomics Server (http://www.expasy.org/tools/). For the hydrophobic moment, the maximum value is reported, using 8-sequence window and α-helical structure. The hydrophobicity[35] is calculated as average value over all amino acids in the corresponding peptide.

Broth Microdilution Antimicrobial Assays

Enterococcus faecium STR 207 (VanA phenotype), *Enterococcus faecium* STR 211 (a vancomycin susceptible derivative of STR 207 lacking the plasmid containing Tn1546), *Enterococcus faecalis* V 583 (VanB phenotype), *Bacillus subtilis* (ATCC 6633), and *Staphylococcus aureus* H (a laboratory strain) were cultured in brain heart/0.5% yeast (BHY) extract broth and incubated at 37°C. A sample of each culture was then diluted 40-fold in fresh BHY broth and incubated at 37°C for 1 h. The resultant mid-log phase cultures were diluted to a final concentration of 5×10^5 colony forming unit (CFU)/ml, and then added to wells of 96-well nonbinding surface plates (NBS, Corning). All compounds were serially diluted twofold across the culture-containing wells from 128 μg/ml to 0.063 μg/ml. Vancomycin was included as a reference. The 96-well plates were covered and incubated at 37°C for 24 h. MICs were the lowest concentration showing no visible growth. Bactericidal activity (minimal bactericidal concentration, MBC) was determined by subculturing 10 μl from wells at the MIC value, and adjacent to it, onto BHY agar plates. The plates were then incubated at 37°C for 24 h and examined for bacterial cultures.

Time of Killing Assays

Time of killing testing was performed in BHY medium using a single colony to inoculate the broth followed by overnight incubation. Dilutions were performed to provide an approximate inoculum of 10^6 CFU/ml in 3 ml of broth. Compounds were tested at 0.5, 1, 2, and 4 times the antibiotic MIC values for *S. aureus* H (a laboratory strain) and for *B. subtilis* (ATCC 6633). Samples (0.1 ml) were removed from each tube at 0, 1, 4, 10, 20, and 30 min and diluted appropriately in cold 0.9% sodium chloride. Samples were then plated in triplicate on BHY agar plates. Plates were incubated at 37°C for 20 h, and colony counts ($\log_{10}$[cfu]/ml) were determined.

Haemolytic Assay of hRBCs

The peptides were tested for haemolytic activity using human red blood cells (hRBC). Freshly prepared hRBC with 1 m*M* ethylenediaminetetraacetic acid (EDTA) were rinsed 3 times with phosphate buffer saline (PBS) (600 μl, 35 m*M*, pH 7.4) by centrifugation for 10 min at 800*g* and resuspension in PBS. The appropriate peptide was dissolved in PBS to make up a concentration of 1024 μg/ml. Hundred microliter of this solution was then added to a solution of the stock hRBC in PBS (100 μl) to give a final volume of 200 μl (final erythrocyte concentration, 5% v/v). The resulting suspension was incubated with gentle agitation for 1 h at 37°C. The samples were then centrifuged at 800*g* for 10 min. Release of haemoglobin was monitored by measuring the absorbance of the supernatant at 510 nm. Controls for zero haemolysis (blank) and 100% haemolysis consisted of hRBC suspended in PBS and in pure water, respectively.

Electron Microscopy

Samples containing either *E. coli* B or *S. aureus* H (1 ml, 10^6 CFU/ml) in BHY medium were incubated with the various peptides at their MIC and at twice the MIC for 30 min. After centrifugation, the pellets were resuspended in a solution of 2% glutaraldehyde (1 ml) and 33% hydrogen peroxide (100 μl). The samples were stained with osmium tetroxide and lead-uranium salt, and then 50 nm sections were mounted on a copper grid (3 mm) and examined using a Philips CM100 transmission electron microscope.

Cytoplasmic Membrane Permeability

Bacteria were grown at 37°C with shaking to mid-logarithmic phase (OD_{600} = 0.5–0.6). Cells were collected by centrifugation, washed once with buffer (5 m*M* HEPES, pH 7.2, 5 m*M* glucose), and resuspended in the same buffer to an OD_{600} of 0.05. The cell suspension was incubated with 0.4 μ*M* 3,3′-dipropylthiadicarbocyanine iodide-(5) [DiSC3(5)] until dye uptake was maximal (as indicated by a stable reduction in fluorescence due to fluorescence quenching as the dye became concentrated in the cell by the membrane potential). KCl (100 m*M*) was then added to equilibrate the cytoplasmic and external K^+ concentrations. A 1 ml cell suspension was placed in a 1 cm cuvette, and the desired concentration of peptide was added. The fluorescence reading was monitored by using an Aminco Bowman Series 2 luminescence spectrophotometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The maximal fluorescence increase due to the disruption of the cytoplasmic membrane was then subtracted from a blank cuvette containing only bacteria and the dye to correct for background signal drift.

Table I Minimum Inhibitory Concentrations (MICs, μg/ml) and Haemolytic Activity (μg/ml) of the Peptide and Their Synthetic Analogues

Organism/Cell	*E. faecalis* Van-sens	*E. faecalis* VanA	*E. faecalis* VanB	*B. subtilis*	*S. aureus* H	*E. coli* B	*E. coli* DC2	hRBC (IH 50 μg/ml)
Vancomycin	4	>512	64	0.125	4	>512	64	ND
Citropin 1.1	4	4	8	4	8	128	64	256
Citropin 1.1 M14	8	8	16	8	8	128	n/a	256
Citropin 1.1 M15	8	8	16	8	8	128	n/a	128
Citropin 1.1 M20	2	2	4	2	4	128	n/a	256
Caerin 1.1	32	32	32	32	8	>512	256	128
Caerin 1.6	8	16	16	16	16	>512	256	64
Caerin 1.8	8	16	16	16	16	>512	256	64
Maculatin 1.1	32	32	32	2	8	>512	256	128
Maculatin 1.1 P15A	>512	>512	>512	>512	>521	>512	>512	>512

These organisms were only assayed with vancomycin and Citropin 1.1. in the presence of 0.1 M KCl (the conditions used in the membrane permeability assay).

Surface Plasmon Resonance Assays

The SPR assay was done with a BIACORE 2000 (Biacore, UK) using a Biacore L1 chip. The Biocore L1 chip consists of a gold slide coated with carboxymethylated dextran derivatized with hydrophobic anchors. Each chip consists of four flow-cells (2.4 mm × 0.5 mm × 0.05 mm) with a probing spot for SPR signal of 0.26 mm^2 for each flow cell. Degassed and filtered PBS (100 m*M*, pH 7.4) was used as the running buffer for all experiments.

Loading of the Sensor Chip With Lipid

Small unilamellar vesicles (SUVs) at 2 m*M* total lipid concentration in PBS with 0.05% EDTA were formed as described previously.[36] They were immediately passed across the surface of a Biacore L1 sensor chip[37] for 20 min at a low flow rate (60 μl, 10 m*M*, 2 μl/min), following a 2-min injection of 3-cholamidopropyl-dimethylammonio-1-propane sulphonate (CHAPS, 20 m*M*) solution at high flow rate (20 μl/min). The captured vesicles could be completely removed from the sensor chip after a binding assay by a 2 min injection of 20 m*M* CHAPS. The degree of coverage of the surface was determined from the extent of nonspecific binding of bovine serum albumin (BSA) (0.1 mg/ml in PBS, 5 min injection).

Peptide Binding Assay

The antimicrobial peptides were diluted serially in PBS to 4 μ*M* and were passed sequentially over the different phospholipid-containing flow cells. The amount of peptide bound at equilibrium just before the end of the injection was corrected by subtraction of the bulk-refractive index difference observed at the beginning and the end of each injection. The surface was then cleaned with two 5 min injections of 20 m*M* CHAPS and the lipids reloaded as described above. The binding assay was performed at 25°C in random order in triplicate and the binding response values obtained were then normalized by dividing the average observed response (RU_{Bound}) by the different peptide molecular weights (MW) of the corresponding peptides, and the amount of lipid captured on the surface of the chip (RU_{Lipid})), i.e. $RU_{Bound}/(MW \times RU_{Lipid})$. Normalization against the molecular weight is used to account for the fact that the response unit (RU) is mass dependent. Normalization against the amount of lipid captured was used as the capacity of the membrane to bind peptide was correlated with the total amount of lipid loaded, which varied typically by 10%.

RESULTS

Minimal Inhibition Concentrations of Peptides

MIC assays showed that all peptides, with the exception of the P15A mutant of Maculatin 1.1, possess wide-spectrum antibacterial activities against Gram-positive bacteria (Table I). In particular, the active compounds were active against vancomycin-resistant strains of *E. faecium* (MIC 2-16 μg/ml). None of the peptides tested were significantly active against the Gram-negative *E. coli* B with MIC ≥512 μg/ml for most peptides. Only Citropin and its analogues show some weak activity (MIC 128 μg/ml) against *E. coli* B. In general, the MBC was observed at, or within a factor of two of, the MIC (data not shown).

Haemolytic Assay of Peptides

Haemolytic assays revealed that the peptides were haemolytic towards hRBC only at concentrations much higher than the MIC values for Gram-positive bacteria (see Table I). Although all peptides showed selectivity for the bacteria compared to the red blood cells (see Figure 2c), citropins showed the best selectivity as a group, compared to the caerin and maculatin group. Within the citropin group, citropin 1.1 M20 exhibits the best selectivity for Gram-positive bacteria, with the other variations, M14 and M15 exhibiting a small selectivity compared to the native citropin 1.1. The differen-

ces in the selectivity is thereby mainly due to the difference in the MIC values, rather than difference in the rRBC.

Electron Microscopy Study on Bacterial Lysis

The effect of citropin 1.1 and of maculatin 1.1 on the morphology of *S. aureus* cell membranes was visualized using transmission electron microscopy (Figure 1a). At peptide concentrations equal to the MIC several darker areas, possibly due to interactions of peptide with intracellular organelles, were observed on the bacterial membrane (Figure 1b) for both peptides. At twice the MIC, both peptides caused total lysis of the bacteria (Figure 1c). No effects on bacterial morphology were observed when *E. coli* B was used in place of *S. aureus* H (data not shown).

SPR Spectrometry

The amount of lipid loaded on the sensor chip varied considerably, depending on the lipid type, from 5000 (DMPC and DMPG) to 14,000 RU (bovine brain and *E. coli* lipid extract). Because the correlation between response units and absorbed mass is known,[36,38] the values for DMPC and DMPG correspond to surface densities equivalent to $\sim$5 ng/mm^2. This was roughly the amount of material expected for formation of a planar lipid bilayer ($\sim$4.4 ng/mm^2).[36] The response levels after the injection of the vesicles drifted at a rate of approximately $\sim$2 RU/min with a buffer flow of 20 μl/min. Following injection of 10 m*M* sodium hydroxide, a stable baseline resulted with a drift $<$0.3 RU/min.

Citropin 1.1 and citropin 1.1 M20, which possessed the lowest MICs against Gram-positive organisms (Table I), showed the greatest specificity for negatively charged membranes (DMPG and *E. coli* lipids) over more neutral membranes (DMPC and bovine lipids) (see Figure 2). However, the very low activity against Gram-negative *E. coli* establishes that antibiotic activity is not determined alone by the higher affinity for negatively charged membranes. (Table I).

Cytoplasmic Membrane Permeability

Cytoplasmic membrane permeabilization was determined by using the membrane potential-sensitive carbocyanine dye DiSC3-(5)[39] together with *B. subtilis* ATCC 6633. Following the addition of DiSC3-(5) to bacterial suspensions, equilibration between extra-cellular and intra-cellular dye (as determined by a stable fluorescence signal) was complete within 30 s for *E. coli* DC2 and 30 min for *B. subtilis* ATCC 6633. To balance the intra- and extra-cellular chemical potential of the cells, 0.1*M* KCl was then added to the buffer. Hence, the MICs of the peptides against *E. coli* DC2 and *B. subtilis* ATCC 6633 were also determined in the presence of 0.1*M* KCl (Table I) for this part of the study. A blank with only cells and the dye was used to subtract the background.

Citropin 1.1 behaved differently to vancomycin with respect to cytoplasmic membrane permeabilization (depolarization of the membrane potential gradient). Fluorescence leakage from *B. subtilis* ATCC 6633 and *E. coli* DC2 was observed immediately upon addition of the citropin at the MIC concentration. There was no leakage observed when vancomycin (which is known to inhibit the transglycosylation and transpeptidation steps of peptidoglycan biosynthesis in Gram-positive bacteria and not directly compromise membrane integrity[40]) was used, even at 10 times its MIC (Figure 3a).

Fluorescence leakage from *E. coli* DC2 was observed after addition of citropin at concentrations as low as 5% of the MIC value in the presence of 0.1*M* KCl, and reached a maximum at 50% of the MIC value in the presence of 0.1*M* KCl (data not shown). The exposure of the dye-saturated *B. subtilis* ATCC 6633 at various concentrations (0.5–10 times the MIC in the presence of 0.1*M* KCl), lead to immediate fluorescence leakage. Additionally, a slow and minor change in membrane potential was observed when citropin was added at concentrations at 10% of the MIC value (in the presence of 0.1*M* KCl) (Figure 3c).

Time of Killing Assay

The killing ability of the citropin against *S. aureus* was assayed over 30 min (see Table III). Citropin 1.1 reduced the amount of viable bacteria with respect to the initial inoculum significantly within one minute at two and at four times the MIC. At four times the MIC, citropin 1.1 completely killed the starting inoculum after 10 min as no regrowth was observed. At two times MIC citropin 1.1 effected in >98% killing within 20 h (data not shown). Thus, citropin 1.1 was clearly bacteriocidal, rather than simply bacteriostatic. This conclusion is in accord with the evidence for the occurrence of cellular fragments in Figure 1c.

DISCUSSION

All peptides showed good activity against Gram-positive bacteria and no or very poor activity against the Gram-negative *E. coli* B. Citropin 1.1 and its analogues showed good activity (MIC 4-16 μg/ml) against all tested Gram-positive bacteria, in particular against vancomycin-resistant strains of *E. faecium*. The single residue variation D4K in citropin 1.1 M20 reduced the MIC fourfold. Caerin 1.1 and its analogues showed only medium activity (MIC 8–32 μg/ml) against the Gram-positive bacteria, with a slightly better activity among

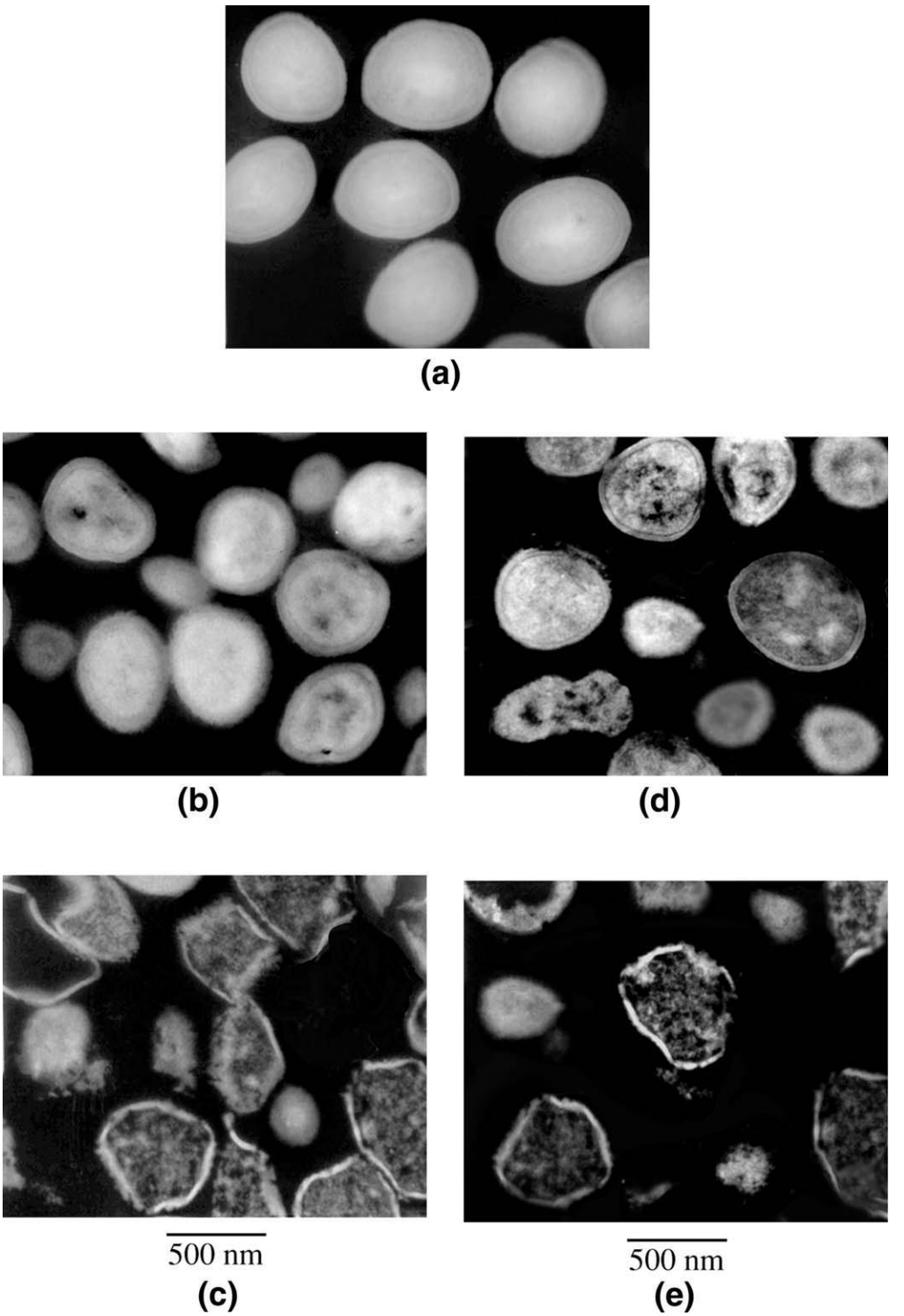
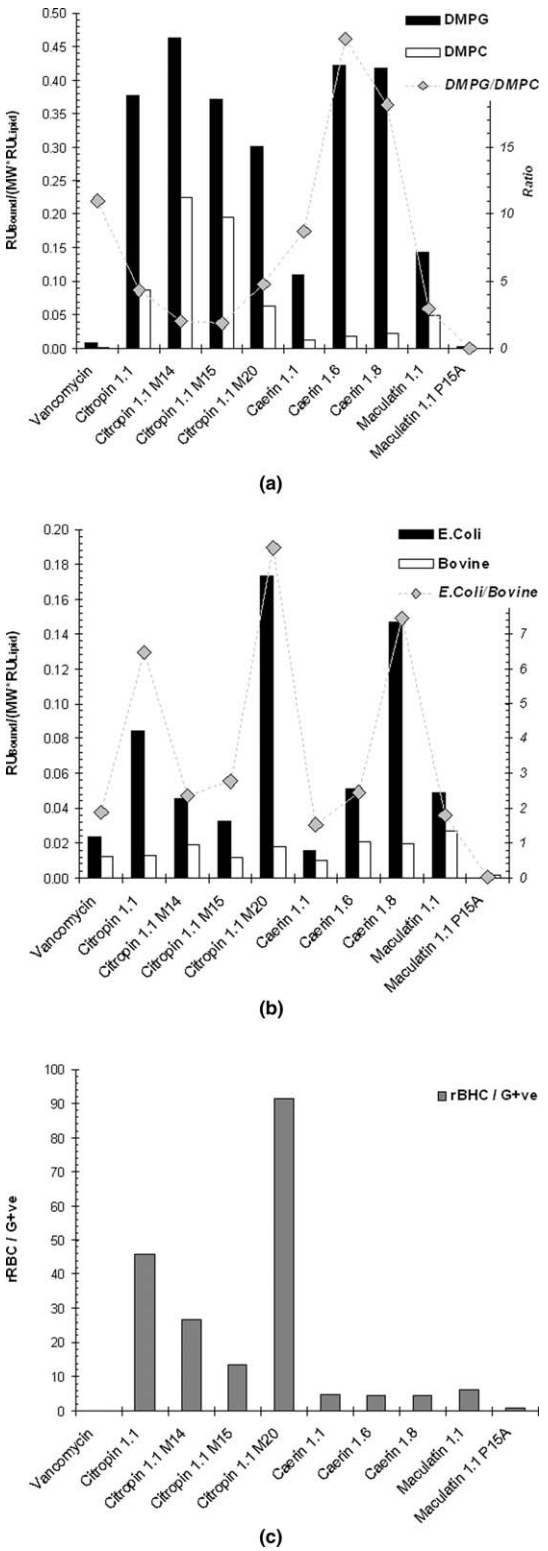


FIGURE 1 Electron micrographs of (a) *Staphylococcus aureus* control, (b) *S. aureus* treated with citropin 1.1 at the MIC (8 μg/ml), (c) *S. aureus* treated with citropin 1.1 at twice the MIC (16 μg/ml), (d) *S. aureus* treated with maculatin 1.1 at the MIC (8 μg/ml), and (e) *S. aureus* treated with maculatin 1.1 at twice the MIC (16 μg/ml).

the analogues compared to Caerin 1.1. Maculatin 1.1 also showed only medium activity (MIC 8–32 μg/ml) against the Gram-positive bacteria, with the exception of *B. subtilis* (MIC 2 μg/ml). The P15A variation showed no activity at all. All peptides show no haemolytic activity against human red blood cells, suggesting potentially high selectivity of the peptide for Gram-positive bacteria over Gram-negative bacteria and eukaryotic cells. The antibacterial activities reported here are broadly in line with other reported values for similar or identical peptide sequences.[7,13]



To gain insight into the cell-specificity of amphibian antimicrobial peptides and its role in selective killing activity, we used a vesicle capture sensor chip together with a SPR biosensor to determine the propensity of the peptides to bind to different membrane types.[13,41,42] Zwitterionic phospholipid (such as PC) containing membranes are regarded as mimics of mammalian cells while anionic phosphlipid (such as PG) containing membranes are used as mimics for bacterial cells. We used both synthetic lipid extracts of *E. coli* and bovine brain to mimic a bacterial and a mammalian cell membrane, respectively. The total *E. coli* lipid extract (using MeOH/CCl_4) better represents a Gram-negative cell membrane and is comprised of ~67% PE, ~23% PG, ~10% cardiolipin, and a mixture of fatty acids.[43] Notably, the Gram-positive bacterial lipid extract also contains teichoic acid (a polymer of glycerol phosphate linked to the peptidoglycan), which serves to increase the anionic character of the membrane,[44] making the membrane more attractive to cationic peptides. Gram-positive bacteria have however developed several antibiotic defense mechanisms via D-alanylating the teichoic acids or incorporating lysyl-phosphatidylglycerol, both of which serve to annul the positive charged groups thereby decreasing the efficacy of cationic peptides.[45,46]

All peptides, expect for maculatin P15A, show a relatively weak binding of the peptides to DMPC and stronger binding to DMPG (Figure 2a), confirming that electrostatic interactions play an important role in the binding of the peptides to the membrane. All peptides are cationic and have an amphipathic property when adopting a α-helical structure,[28] but neither the number of amines nor the charge at physiological pH or the hydrophobic moment (Table II) would explain the

FIGURE 2 Graphs showing the relative amount of different peptides bound to (a) DMPC (white), DMPG (black) and their ratio (grey diamonds), and (b) bovine lipid extract (white), *E. coli* lipid extract (black) and their ratio (grey diamonds). The response (RU_{Bound}) has been normalized for the peptide molecular weight (MW) and the amount of lipid captured on the chip surface (RU_{Lipid}) as discussed in *Methods*. Also shown is the cytotoxic selectivity of the peptides between prokaryotic and eukaryotic cells, given as (c) the ratio between the haemolytic IC_{50} against human red blood cells (hRBC) and the average MIC value against Gram-positive bacteria (G+ve), both given as μg/ml. High values indicate a low haemolytic activity (high IC_{50} value) and high antimicrobial activity (low MIC value).

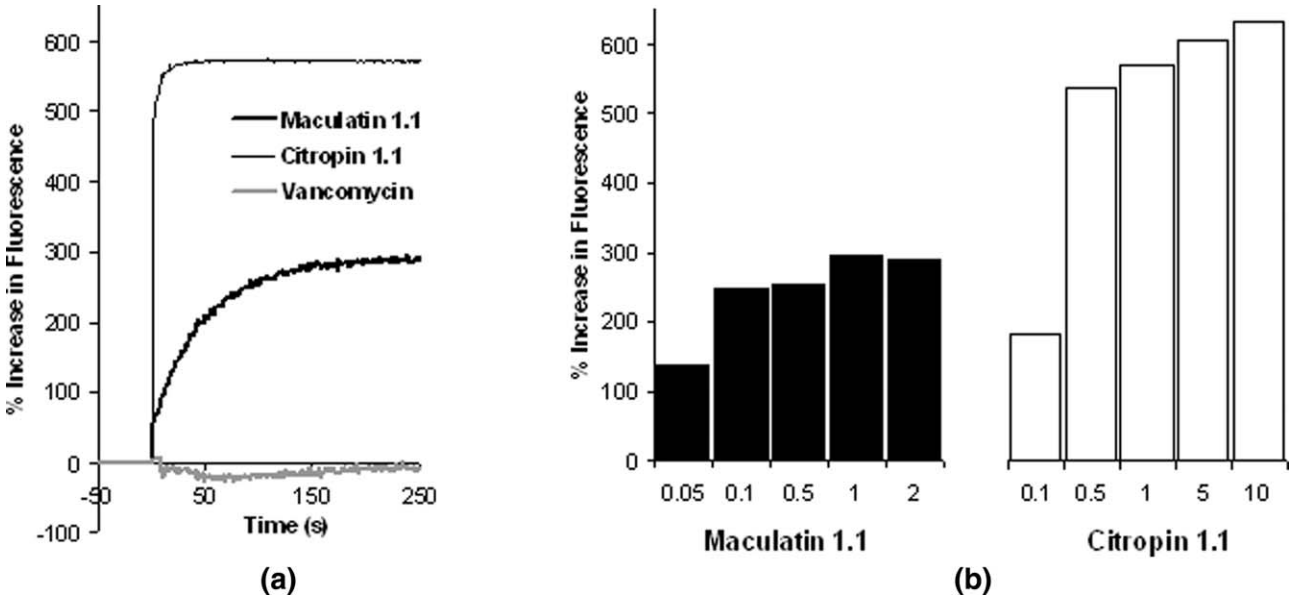


FIGURE 3 Effects of maculatin 1.1, citropin 1.1, and vancomycin (control) on the permeabilization of the cytoplasmic membrane of the Gram-positive *B. subtilis*. (a) Change of fluorescence for each compound at their MIC concentration, (b) change of fluorescence at the equilibrium phase (average between 400 and 500 sec) for different concentration, given as times of their MIC concentrations (MIC for Maculatin 1.1 = 2 μg/ml, Citropin 1.1 = 4 μg/ml, and vancomycin = 0.125 μg/ml).

trends in the binding to DMPG or the selectivity in binding between DMPG an DMPC. Similarly, no correlation is observed between the absolute magnitude of peptide-DMPG binding and bactericidal activity (Table I).

Binding of the peptides to the native lipid extracts showed a similar trend as with the synthetic phospholipids, weak binding to the bovine brain lipid extracts and stronger binding to the *E. coli* lipid extract (Figure 2b), confirming the selectivity of the peptides for prokaryotic cell membranes. While there is no correlation between the magnitude of peptide-lipid binding and any of the peptide properties, there is a correlation between the peptide-*E.coli* lipid binding (Figure 2b) and the selectivity of the peptide for prokaryotic over eukaryotic cells, expressed as the ratio between the IC_{50} of hRBC and the MIC of the Gram-positive bacteria (Figure 2c). Comparison of the figures indicated that both show similar trends, with citropin 1.1 M20 as a strong selective peptide, citropin 1.1 as medium selective and the others with low selectivity. Exception to this trend is Caerin 1.8, which despite showing a strong selectivity for *E. coli* lipid extract, shows only a low selectivity in its toxic activity (MIC_{G+ve} : 16 μg/ml compared to hRBC IC_{50} of 64 μg/ml)

Detailed NMR structure analysis of citropin 1.1 and derivatives[26] has revealed that the full length (1–16) citropin adopts a structure with two α-helical fragments with a βIV turn in between. On the other hand, a C-terminal truncated version (1–12) citropin showed a single α-helical, without a turn at the residues 8 and 9, and eliminating or at least drastically reducing the activity against both Gram-positive and negative bacteria. Several detailed peptide–lipid interaction studies between citropin 1.1 and model membranes[47] indicated that citropin can adopt an amphipatic α-helical structure, able to bind to the membrane, but too short to span the membrane, suggesting a carpet model for the disruption of the membrane.[30] The analogues of citropin 1.1 show similar antibacterial activity against Gram-positive bacteria, with the single Asp4Lys variation showing an overall better activity. A similar trend can be observed with the membrane binding to the *E. coli* lipid extract (Figure 2b), in which the same Citropin variation exhibits greater binding. These results are in contrast to earlier reported results, obtained by measuring inhibition zones on an agarose plate,[24] where the citropin 1.1 M14 analogue is reported with slightly better antibacterial activity. Common to both results, though, is that citropin 1.1 and analogues show some low activity against Gram-negative bacteria. All modifications of citropin used in this study are not regarded to change the α-helical structure of the Citropin.[24] The trend within this small set of peptides suggested that the hydrophobic moment (Table II) of the α-helical pep-

Table II Amino Acid Sequences of Citropin 1.1 and Its Synthetic Analogues

Peptide	Amino Acid Sequence	MW	Charge at pH 7.4	Isoelectric Point	Number of Amine Groups	Max Hm[a]	⟨H⟩[b]
Citropin 1.1	GLFDV IKKVA SVI-- ------ --GGL-NH_2	1615.0	+2	9.55	3	0.796	1.28
Citropin 1.1 M14	GLFDV I**A**KVA SVI-- ------ --**KK**L-NH_2	1700.1	+3	10.51	4	0.708	1.20
Citropin 1.1 M15	GLF**A**V IKKVA SVI-- ------ --**KK**L-NH_2	1713.2	+5	11.28	5	0.726	1.17
Citropin 1.1 M20	GLF**K**V IKKVA SVI-- ------ --GGL-NH_2	1628.1	+4	11.10	4	0.870	1.26
Caerin 1.1	GLLSV LGSVA KHVLP HVVPV IAEHL-NH_2	2584.1	+1.7	7.98	2	0.579	1.19
Caerin 1.6	GL**F**SV LG**A**VA KHVLP HVVPV IAE**KL**-NH_2	2593.2	+2.5	9.56	3	0.505	1.22
Caerin 1.8	GL**FK**V LGSVA KH**L**LP HVVPV IAE**KL**-NH_2	2664.3	+3.5	10.51	4	0.650	0.98
Maculatin 1.1	GLFGV LAKVA AH--- -VVPA IAEHF-NH_2	2145.4	+1.5	7.93	2	0.528	1.30
Maculatin 1.1 P15A	GLFGV LAKVA AH--- -VV**A**A IAEHF-NH_2	2119.5	+1.5	7.93	2	0.528	1.46

[a] Maximum hydrophobic moment (Hm), using a 8 amino acid window and assuming α-helical structure.
[b] Average hydrophobicity/residue using Kyte and Doolittle protein scale.

tides correlated with better activity and better membrane binding to *E. coli*.

Maculatin 1.1 loses all its antibacterial activity, when proline at position 15 is replaced with alanine. Similar loss of antibacterial activity has been reported for the same modification previously,[31] including a reduction in membrane leakage experiment,[48] even though others report similar antibacterial activity with the unmodified maculatin.[49] The mode of action for maculatin 1.1 is currently viewed as between a pore forming[50] and a torodial like model[30] A P15A mutation of the peptide is shown to remove the bend between the two α-helical structure, forming a single α-helix.[31] Our data clearly shows that P15A loses all membrane binding capability, with no binding observed to either the synthetic or native lipid extracts. This is, in a sense a surprising result, as it is commonly suggested that small structural changes do not affect the rather unspecific membrane binding ability of a peptide compared to the more specific membrane disruption. Our data suggests that in the case of Maculatin 1.1 a small structural change can dramatically alter its membrane binding capability.

NMR experiments with Caerin 1.1 revealed a predominately α-helical structure, but with a distinct bend due to the two prolines.[29] Modification of those two prolines, as in maculatin 1.1 P15A, resulted in a loss of antimicrobial activity, and a straightening of its structure to a single α-helix.[29] The caerin 1.1 analogues included in this study were designed in a similar manner as with the Citropin analogues; by changing the hydrophobic and electrostatic properties of the peptide, without altering its overall structure. The antimicrobial activity of caerin 1.1 and its analogues reported herein are in agreement with earlier results,[32] with all peptides within one dilution factor. Notably in the case of the caerins one modification (caerin 1.8) showed increased membrane binding to *E. coli* lipid extract, not reflected in an increase in antimicrobial ac-

Table III Time-of-Killing of Citropin 1.1 at Concentrations of 4, 2, 1, 0.5, and 0 Times the MIC Against an Initial Innoculum of 100×10^4 CFU/ml of *S. aureus* H

		S. aureus H (CFU/ml) $\times 10^4$	
	× MIC (8 μg/ml)	5 min	60 min
No antibiotic	–	152	267
Citropin 1.1	0.5	92	150
	1	78	88
	2	56	26
	4	9	0

Values given for two time points, 5 and 60 min.

tivity. No correlation of that increased affinity can be found to any property of the peptide, apart from the fact that caerin 1.8 has the highest charge within that set of peptides.

Both citropin 1.1 and maculatin 1.1 show similar effects on the morphology of *S. aureus* cells, using transmission electron microscopy (see Figure 1). Both show a few areas of lysis at MIC level and total lysis of the bacteria at twice their MIC level. Although the effect on the morphology looks similar, their effect on the membrane permeabilization of *B. subtilis* using a DiSC3(5) dye looks slightly different. Maculatin 1.1 displays only half the increase in fluorescence of Citropin 1.1, but is able to trigger the leakage at a 0.1 times MIC level (Figure 3). Citropin 1.1 on the other hand triggers a higher and faster leakage, but only at a higher concentration (>0.5 times the MIC level). Comparable reports[48] using a carboxyfluorescein leakage of POPC large unilamellar vesicles (LUV's) report similar effect with maculatin 1.1 showing a leakage at a significant lower lipid/peptide ratio (higher peptide concentration) than citropin 1.1.

This work suggests that citropin 1.1 and citropin 1.1 M20, both with low haemolytic and broad spectrum antibiotic activities, are potential candidates for further investigation and development. For example, future studies could involve substitution of glycine residues for lysine, which has been used to improve the bactericidal activity of other antimicrobial peptides.[51,52]

The authors thank Dr. Jeremy Skepper (Atomic Imaging Centre, University of Cambridge) for the electron microscopy work and Jennifer White (Adprotech Ltd.) for hRBC assays.

REFERENCES

1. Boman, H. G. Cell 1991, 65, 205–207.
2. Epand, R. M.; Vogel, H. J. Biochim Biophys Acta 1999, 1422, 11–28.
3. Vogel, H.; Jahnig, F. Biophys J 1986, 50, 573–582.
4. Erspamer, V. In Amphibian Biology. Vol 1: The Integument; Heatwole, H.; Barthalamus, G. T., Eds; Chipping Norton, Surrey Beatty & Sons, Australia, 1994; pp 178–350.
5. Bevins, C. L.; Zasloff, M. Annu Rev Biochem 1990, 59, 395–414.
6. Zasloff, M. Proc Natl Acad Sci USA 1987, 84, 5449–5453.
7. Apponyi, M. A.; Pukala, T. L.; Brinkworth, C. S.; Maselli, V. M.; Bowie, J. H.; Tyler, M. J.; Booker, G. W.; Wallace, J. C.; Carver, J. A.; Separovic, F.; Doyle, J.; Llewellyn, L. E. Peptides (New York, NY) 2004, 25, 1035–1054.
8. Wang, Z.; Wang, G. Nucleic Acids Res 2004, 32, D590–D592.
9. Bowie, J. H.; Wegener, K. L.; Wabnitz, P. A.; Chia, B. C. S.; Carver, J. A.; Wallace, J. C.; Tyler, M. J. Protein Pept Lett 1999, 6, 259–269.
10. Hancock, R. E. W.; Sahl, H.-G. Nat Biotechnol 2006, 24, 1551–1557.
11. Simmaco, M.; Mignogna, G.; Barra, D. Biopolymers 1998, 47, 435–450.
12. Boland, M. P.; Separovic, F. Biochim Biophys Acta Biomemb 2006, 1758, 1178–1183.
13. Fernandez, D. I.; Gehman, J. D.; Separovic, F. Biochim Biophys Acta Biomemb 2009, 1788, 1630–1638.
14. Gehman, J. D.; Luc, F.; Hall, K.; Lee, T.-H.; Boland, M. P.; Pukala, T. L.; Bowie, J. H.; Aguilar, M.-I.; Separovic, F. Biochemistry 2008, 47, 8557–8565.
15. Seto, G. W. J.; Marwaha, S.; Kobewka, D. M.; Lewis, R. N. A. H.; Separovic, F.; McElhaney, R. N. Biochim Biophys Acta Biomemb 2007, 1768, 2787–2800.
16. Jenssen, H.; Hamill, P.; Hancock, R. E. W. Clin Microbiol Rev 2006, 19, 491–511.
17. Shai, Y. Biochim Biophys Acta 1999, 1462, 55–70.
18. Sanderson, J. M. Org Biomol Chem 2005, 3, 201–212.
19. Melo, M. N.; Ferre, R.; Castanho, M. A. Nat Rev Microbiol 2009, 7, 245–250.
20. Rozek, T.; Wegener, K. L.; Bowie, J. H.; Olver, I. N.; Carver, J. A.; Wallace, J. C.; Tyler, M. J. Eur J Biochem 2000, 267, 5330–5341.
21. Mader, J. S.; Hoskin, D. W. Expert Opin Invest Drugs 2006, 15, 933–946.
22. Lu, C.-X.; Nan, K.-J.; Lei, Y. Anticancer Drugs 2008, 19, 931–939.
23. Wegener, K. L.; Wabnitz, P. A.; Bowie, J. H.; Carver, J. A.; Wallace, J. C.; Tyler, M. J. Eur J Biochem 1999, 265, 627–635.
24. Doyle, J.; Brinkworth, C. S.; Wegener, K. L.; Carver, J. A.; Llewellyn, L. E.; Olver, I. N.; Bowie, J. H.; Wabnitz, P. A.; Tyler, M. J. Eur J Biochem 2003, 270, 1141–1153.
25. Marcotte, I.; Wegener, K. L.; Lam, Y. H.; Chia, B. C.; de Planque, M. R.; Bowie, J. H.; Auger, M.; Separovic, F. Chem Phys Lipids 2003, 122, 107–120.
26. Sikorska, E.; Greber, K.; Rodziewicz-Motowidlo, S.; Szultka, L.; Lukasiak, J.; Kamysz, W. J Struct Biol 2009, 168, 250–258.
27. VanCompernolle, S. E.; Taylor, R. J.; Oswald-Richter, K.; Jiang, J.; Youree, B. E.; Bowie, J. H.; Tyler, M. J.; Conlon, J. M.; Wade, D.; Aiken, C.; Dermody, T. S.; KewalRamani, V. N.; Rollins-Smith, L. A.; Unutmaz, D. J Virol 2005, 79, 11598–11606.
28. Haney, E. F.; Hunter, H. N.; Matsuzaki, K.; Vogel, H. J. Biochim Biophys Acta 2009, 1788, 1639–1655.
29. Pukala, T. L.; Brinkworth, C. S.; Carver, J. A.; Bowie, J. H. Biochemistry 2004, 43, 937–944.
30. Mechler, A.; Praporski, S.; Atmuri, K.; Boland, M.; Separovic, F.; Martin, L. L. Biophys J 2007, 93, 3907–3916.
31. Chia, B. C. S.; Carver, J. A.; Mulhern, T. D.; Bowie, J. H. Eur J Biochem 2000, 267, 1894–1908.
32. Steinborner, S. T.; Currie, G. J.; Bowie, J. H.; Wallace, J. C.; Tyler, M. J. J Pept Res 1998, 51, 121–126.
33. Cooper, M. A. Curr Opin Pharmacol 2003, 3, 557–562.
34. Rice, P.; Longden, I.; Bleasby, A. Trends Genet 2000, 16, 276–277.
35. Kyte, J.; Doolittle, R. F. J Mol Biol 1982, 157, 105–132.
36. Cooper, M. A.; Try, A. C.; Carroll, J.; Ellar, D. J.; Williams, D. H. Biochim Biophys Acta 1998, 1373, 101–111.
37. Cooper, M. A.; Hansson, A.; Löfås, S.; Williams, D. H. Anal Biochem 2000, 277, 196–205.
38. Stenberg, E.; Persson, B.; Roos, H.; Urbaniczky, C. J Colloid Interface Sci 1991, 143, 513–526.
39. Sims, P. J.; Waggoner, A. S.; Wang, C. H.; Hoffman, J. F. Biochemistry 1974, 13, 3315–3329.
40. Walsh, C. T.; Fisher, S. L.; Park, I.-S.; Prahalad, M.; Wu, Z. Chem Biol 1996, 3, 21–28.
41. Papo, N.; Shai, Y. Biochemistry 2003, 42, 9346–9354.
42. Hall, K.; Aguilar, M. I. Biopolymers 2009, 92, 554–564.

43. Andrushchenko, V. V.; Aarabi, M. H.; Nguyen, L. T.; Prenner, E. J.; Vogel, H. J. Biochim Biophys Acta 2008, 1778, 1004–1014.
44. Xia, G.; Kohler, T.; Peschel, A. Int J Med Microbiol 2010, 300, 148–154.
45. Li, M.; Cha, D. J.; Lai, Y.; Villaruz, A. E.; Sturdevant, D. E.; Otto, M. Mol Microbiol 2007, 66, 1136–1147.
46. Otto, M. Contrib Microbiol 2009, 16, 136–149.
47. Balla, M. S.; Bowie, J. H.; Separovic, F. Eur Biophys J 2004, 33, 109–116.
48. Ambroggio, E. E.; Separovic, F.; Bowie, J. H.; Fidelio, G. D.; Bagatolli, L. A. Biophys J 2005, 89, 1874–1881.
49. Niidome, T.; Kobayashi, K.; Arakawa, H.; Hatakeyama, T.; Aoyagi, H. J Pept Sci 2004, 10, 414–422.
50. Chia, C. S.; Torres, J.; Cooper, M. A.; Arkin, I. T.; Bowie, J. H. FEBS Lett 2002, 512, 47–51.
51. Bessalle, R.; Haas, H.; Goria, A.; Shalit, I.; Fridkin, M. Chemother Antimicrob Agents 1992, 36, 313–317.
52. Piers, K. L.; Brown, M. H.; Hancock, R. E. W. Antimicrob Agents Chemother 1994, 38, 2311–2316.