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Fluorescent Ion Efflux Screening Assay for Determining Membrane-Active Peptides

Neil M. O'Brien-Simpson^{A,B,C,F}, Wenyi Li^{D,E,F}, Namfon Pantarat^{A,B,C}, Mohammed Akhter Hossain^{D,E}, Frances Separovic^D, John D. Wade^{D,E,G} and Eric C. Reynolds^{A,B,C,G}

^AMelbourne Dental School, ^BOral Health CRC, ^CBio21 Institute, ^DSchool of Chemistry, ^EThe Florey Institute of Neuroscience and Mental Health, University of Melbourne, Victoria 3010, Australia.

^FThese authors contributed equally to this article.

^GCorresponding authors: Eric C. Reynolds, Email: e.reynolds@unimelb.edu.au and John D. Wade, Email: john.wade@florey.edu.au

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Abstract (250 words)

A major global health threat is the emergence of antibiotic-resistant microbes. Coupled with a lack of development of modified antibiotics, there is a need to develop new antimicrobial molecules and screening assays for them. In this study we provide proof of concept that a large unilamellar vesicle (LUV) method used to study chloride ion efflux facilitated by ionophores and surfactant-like molecules that disrupt membrane integrity can be adapted to identify membrane interactive antimicrobial peptides (AMPs) and to screen relative activity of AMPs. Lucigenin was encapsulated in LUVs in the presence of Cl^- ion (NaCl) which quenches fluorescence and then incubated with AMPs in 100 mM NaNO_3 buffer. With AMP membrane interaction/disruption, the Cl^- ion is exchanged with the NO_3^- ion and the resultant lucigenin fluorescence is indicative of relative AMP activity. Seven AMPs were synthesised by solid phase peptide chemistry and incubated with LUVs of different phospholipid compositions. Each AMP resulted in lucigenin fluorescence which was dose dependent and relative fluorescence correlated with the MIC and MBC values for the corresponding peptide. Furthermore, using mammalian model phospholipid LUVs, lucigenin-induced fluorescence also correlated with the AMP cytotoxicity IC_{50} values. The proline rich AMP, Chex1-Arg20, which is non-lytic but does interact with the bacterial membrane resulted in lucigenin fluorescence of bacterial-membrane model LUVs but not mammalian-membrane model LUVs. The fluorescent ion efflux assay developed here should have applicability for the majority of AMPs and can be tailored to target particular bacterial species membrane composition which may lead to the identification of novel membrane-interactive AMPs. The rapid high-throughput method also allows for screening of relative AMP activity and toxicity prior to biological testing.

Introduction

Infections with antibiotic resistant microbes are recognised as a developing worldwide health problem. This has been highlighted in a recent United Kingdom Government report estimating that without new antimicrobial compounds the deaths from antibiotic resistant microbes may reach 10 million per year by 2050, a mortality rate in excess of those projected for cancer-related deaths.^[1] A World Health Organisation report on antibiotic resistance has also stressed the need for “fostering innovation and research and development of new tools” to advance progress in antimicrobial discoveries.^[2] Despite these reported concerns, the pharmaceutical pipeline for the development of new antibiotics has significantly diminished. This has been largely attributed to the revenue return being low for antibiotics as a result of the rapid resistance development to traditional antibiotic mimics.^[3-4] Thus there is a clear need for the development of new, effective antimicrobials which do not induce resistance like the traditional antibiotics; however, to assist with this development there is a requirement for relevant, high throughput screening assays.

Over the past two decades, hundreds of peptides, readily water soluble, with potent antimicrobial activity have been isolated from single celled organisms, invertebrates and vertebrates.^[5] These antimicrobial peptides (AMPs) are being heralded as a new class of therapeutics for several reasons.^[6] Their mechanism of microbial killing is distinct from currently used antibiotics and they do not readily induce resistance.^[7] Furthermore, modified natural AMPs can have enhanced microbial membrane targeting ability and can be effective at nanomolar (nM) concentrations.^[8] Typically, AMPs are 7-50 amino acids long making them accessible to chemical synthesis and modification. This has led to a large number of studies systematically investigating the importance of the size, sequence, net charge,

conformation, structure, hydrophobicity and amphipathicity on the activity of AMPs.^[9-10] These studies have shown that each of these peptide characteristics is important for AMP activity and that modifying these characteristics can have significant effects on AMP activity.^[5]

A prerequisite for the action of any AMP is its interaction with the cell membrane of a microbe. By studying these interactions and binding kinetics with membrane and cytosolic molecules the mode of action of AMPs has been described as targeting either the inner/cytoplasmic membrane or intracellular molecules such as a protein or DNA/RNA.^[5] Thus a microbe is killed via either membrane barrier disruption (channel/pore formation resulting in membrane depolarization and/or lysis) or inhibiting specific metabolic pathways, gene regulation or inducing aggregation/inactivation of intracellular macromolecular structures. These different mechanisms of killing are thought to be AMP sequence dependent. However, the stratification of AMPs based on their specific mechanism of activity may not be that straightforward as some AMPs have recently been shown to have multimodal mechanisms of action. AMPs once thought to only target intracellular molecules and processes can also interact with the inner/cytoplasmic membrane of bacteria disrupting integrity.^[11-13]

Methods currently used to identify peptides as having antimicrobial activity are based on bacterial growth assays to determine Minimum Inhibitory Concentration (MIC) and/or Minimum Bactericidal Concentration (MBC) against *Escherichia coli* (Gram negative) and *Staphylococcus aureus* (Gram positive) as the typical test bacteria. These assays have evolved into 96 well plate formats which has allowed some increase in throughput but they are still limited as they are still dependent on growth of the bacterial species which may require a

level of biological containment and expertise, particularly for fastidious, pathogenic multidrug resistant species, that are often not readily available to a peptide/organic chemist. This has led to the development of *in silico* systems^[14-15] and the use of dye release large unilamellar vesicles (LUV) assays.^[16-17] The *in silico* methods have had successes^[14] but are limited to using natural amino acids to alter the AMP sequence and to model activity. The lipid vesicle assays use a combination of phospholipids palmitoylcholine (POPC), palmitoylcholine (POPG), palmitoylcholine (POPE), cardiolipin (CL) and cholesterol to mimic Gram negative (POPE/POPG; 7:3, w/w) or Gram positive (POPG/CL; 6:4, w/w) bacterial cytoplasmic membranes or those of mammalian cells (POPC without or with CL or cholesterol).^[17-18] Fluorescent dyes such as calcein, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), *p*-xylenebispyridinium bromide (DPX) and fluorescently labeled dextrans are encapsulated into the LUVs and the release of these dyes or dextrans after incubation with an AMP indicates membrane pore formation and pore size.^[17, 19-20] The LUV dye release assays have the advantage that they are highly accessible, rapid and can be used for screening, but are limited to AMPs that disrupt or form pores in the membrane. Further these assays are dependent on the Stoke's radius of the dye; the small dyes (calcein, ANTS and DPX) with a Stoke's radius of 0.4-0.6 nm are not able to detect the pore forming peptide alamethicin which can form a pore with an internal diameter of 0.25 nm.^[13]

In this proof-of-concept study we show that a LUV method used to study chloride ion efflux facilitated by ionophores and surfactant-like molecules that disrupt membrane integrity.^[21-22] can be adapted to identify membrane interactive AMPs and screen relative activity of AMPs (Scheme 1).

Results and Discussion

The chloride-ion LUV fluorescent assay is based on encapsulating the fluorophore lucigenin in phosphate buffer containing 100 mM NaCl. Lucigenin is fluorescently quenched in the presence of Cl^- ion. The quenching of the fluorophore can be reduced by exchanging the chloride ions for nitrate ions. . Thus a membrane interactive molecule (in phosphate buffer containing 100 mM NaNO_3) able to disrupt the membrane integrity to form an ion channel or pore will allow efflux of the intravesicular Cl^- ion in exchange for the extravesicular NO_3^- ion which will activate lucigenin fluorescence (Scheme 1). As the Cl^- and NO_3^- ions have similar ionic radii, 0.18 nm and 0.19 nm ,respectively,^[23] this assay has been used to monitor Cl^- ion movement across membranes. We hypothesised that this lucigenin-based Cl^- ion efflux assay could be a sensitive assay for identifying membrane interactive AMPs. The assay could also be used to compare the relative activity of different AMPs and be a pre-biological screening assay to help identify lead AMPs and analogues for biological testing.

The AMPs used in this study were synthesized by solid phase peptide chemistry using the Fmoc/tBu protocol on Rink AMSURE resin or Fmoc Rink amide polystyrene resin as previously described.^[12-13] The purified (>95%) AMPs and their observed and calculated masses are listed in Table 1. As lucigenin fluorescence is affected by pH,^[22] our initial studies using two well defined pore forming peptides, maculatin 1.1 and ovispirin, were completed over a pH range from 5.5 to 8.0. Lucigenin/NaCl/phosphate buffer LUVs (lucigenin-LUVs) were incubated with maculatin 1.1 or ovispirin in NaNO_3 /phosphate buffer at different lipid to peptide ratios (50:1-6400:1) at 37 °C for 30 minutes and fluorescence (ext 440 nm, em 530 nm) measured and the normalized fluorescence intensity determined against the positive control (full disruption of the LUVs with Triton X100). For the aforementioned lipid

composition, the ratio of POPE:POPG (7:3) was chosen to mimic the negatively charged liposome to optimize the pH effects of maculatin 1.1 or ovispirin. The results in **Figure 1** show that both maculatin 1.1 and ovispirin induced chloride ion efflux and lucigenin fluorescence in a dose dependent manner (**Figure 1A**). By plotting the lucigenin fluorescence induced at a lipid to peptide ratio of 100:1 across the pH range tested, it can be seen that a pH of 7.0 resulted in the maximal fluorescence for both peptides (**Figure 1 B and C**). Our data showing maximal lucigenin fluorescence at pH 7.0 is in contrast to Graefe et al.^[22] who showed that lucigenin in solution or incorporated into polyacrylamide nanoparticles had greater fluorescence at pH values less than 7.0. This discrepancy may be explained as lucigenin fluorescence is reported to be stabilized and enhanced in water/organic solvent mixtures and particularly with aliphatic compounds.^[24] Thus a liposome environment may mimic this water/organic solvent effect on lucigenin fluorescence. Based on these results all further lucigenin-LUV assays were conducted at pH 7.0.

As AMPs are able to induce chloride ion efflux and lucigenin fluorescence in the lucigenin-LUV assay, we wanted to then investigate whether relative activity of AMPs could be differentiated and if LUV lipid composition would have an effect on this relative activity. Two sets of LUVs (negatively charged liposomes and overall neutral liposomes), were produced to mimic the membrane of Gram negative bacteria (POPE:POPG, 7:3 w/w) and mammalian cells (POPC) and incubated with four known, well-defined and characterised AMPs (caerin 1.1, pardaxin IV, dermaseptin B2 and magainin II). **Figure 2A** shows that there is a clear distinction in the fluorescence signals induced by each of the AMPs in the POPE:POPG lucigenin-LUV assay, with relative activity at 0.5 F.U. being pardaxin IV > caerin 1.1 = dermaseptic B2 > magainin II. Each of these peptides were tested for antimicrobial activity against the Gram negative bacterium *Escherichia coli* to determine MIC

and MBC, using standard AMP protocols (**Table 2**). The relative order of activity of each AMP was similar for MIC and MBC which was in close agreement with the relative order of activity found in the lucigenin-LUV assay. It is noteworthy that the relative order of activity shown in the lucigenin-LUV assay more closely resembles the MBC order of AMP activity indicating that the lucigenin-LUV assay may provide a surrogate measure of cell death (MBC) rather than growth inhibition (MIC). Using POPC lucigenin-LUVs to mimic mammalian cell membranes the induced fluorescence by each AMP at 0.5 F.U. was pardaxin IV > caerin 1.1 > dermaseptic B2 > magainin II (**Figure 2B**). This order of activity was found to correlate with the IC₅₀ values of the AMPs obtained in epithelial cytotoxicity studies (**Table 2**) suggesting that the POPC lucigenin-LUV assay can be used to determine the relative toxicity of different AMPs. Surprisingly, the relative fluorescence for each AMP across each of the lipid to peptide ratios for the POPC LUVs was higher than for the POPE:POPG LUVs suggesting that the AMPs may be more cytotoxic than antimicrobial. However, the MIC/MBC and IC₅₀ values in **Table 2** demonstrate that this is not the case. This phenomenon where AMPs interact more with an overall neutral lipid (POPC) LUV than a negatively charged lipid (POPE:POPG) LUV has been reported previously by Sani et al.^[25] using maculatin 1.1 and aurein 1.2 AMPs. In the Sani et al. study,^[25] it was found that although the AMPs were more potent against neutral vesicles in the dye release assay, upon mixing the neutral lipid vesicles with anionic lipid vesicles the AMPs migrated to the anionic lipids indicating more stable binding to these molecules. This highlights the importance of closely replicating the lipid composition of the bacterial and host cell membranes in the liposomes produced for the assay. This is further supported as the lipid composition of LUVs is well known to alter the binding of AMPs and membrane interactive compounds.^[18, 26] Here we have used published model lipid compositions to represent “typical” Gram negative bacteria and mammalian cells as a proof of concept for the assay. This is a limitation in our

current study and one that is prominent in LUV research. The cytoplasmic/inner membrane phospholipid compositions of the most common bacteria studied in AMP research is listed in **Table 3** and shows the considerable variation in the concentration and type of lipid in the different species. To further the research from this study, formulating LUVs to represent the major cytoplasmic/inner membrane lipids of different bacterial species as described in **Table 3** would enhance specificity and give greater insight into targeting particular species. Further, by using the co-mixing strategy described in the Sani et al. study^[25] this would give insight into AMP specificity toward particular bacteria and/or mammalian cells. These are part of an ongoing investigations in our laboratory.

Recently we have shown that the proline-rich, cationic AMP Chex1-Arg20, which is thought to kill bacteria via a mechanism mediated by binding to ~~the~~ intracellular targets, heat-shock protein DnaK also strongly interacts with the cytoplasmic/inner membrane.^[12] Furthermore, Chex1-Arg20 interacts with negatively charged liposomes and sequentially induces their aggregation without calcein leakage.^[27] It has been reported that for a few cationic AMPs, initial membrane interaction to form a surface lipid raft can result in a hyperpolarized membrane due to the additional positive charge of the AMP at the surface. However, with time and an increased concentration of the AMP it can insert into the membrane to span the membrane and form an ion channel. In the presence of high concentrations of extracellular Cl⁻, the anion can diffuse down a concentration gradient into the cell which increases the membrane potential even further thereby enhancing the membrane hyperpolarization.^[28-29] Using Chex1-Arg20 in the lucigenin-LUV assay, it can be clearly seen that for the POPE:POPG LUVs there was a strong chloride ion efflux and lucigenin fluorescence and that this was dose dependent (**Figure 3A**). However, unlike the pore-forming peptides above, Chex1-Arg20 did not induce lucigenin fluorescence in the POPC LUVs (**Figure 3A**). Using

the ion sensitive fluorophore 3,3'-diethyloxacarbocyanine iodide (DiOC2) that fluoresces red in a hyperpolarized membrane and fluoresces green in a depolarized membrane state, we determined the membrane potential changes of *E. coli* incubated with Chex1-Arg20 at 0.5 x MIC and 1.0 x MIC by flow cytometry (**Figure 3B and 3C**). The flow cytometry dot plots (**Figure 3B**) show a significant shift in the red fluorescence of the *E. coli* population at both concentrations indicating a hyperpolarized membrane state. This is significantly different from incubating *E. coli* with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) which collapses the proton motive force and so produces a depolarized membrane state (increased green fluorescence) which is similar to that induced by pore forming and gross membrane disrupting peptides (**Figure 3B and 3C**).^[12] The transmembrane Cl⁻ ion movement seen in the lucigenin-(POPE:POPG) LUV assay with Chex1-Arg20 suggests that the hyperpolarization of *E. coli* by Chex1-Arg20 is the result of chloride ion influx as described above. Further it may suggest that Chex1-Arg20 has a multi-modal action of killing: an intracellular mechanism and another mechanism involving membrane hyperpolarization. The inability of Chex1-Arg20 to induce chloride ion efflux in the POPC LUVs is consistent with its very low/no cytotoxicity to mammalian cells (**Table 2**) and its reported low overall toxicity.^[12, 30] It is noteworthy that our assay result may provide, for the first time, a mechanism which explains that the lack of toxicity of Chex-Arg20 may be attributable to the peptide not interacting in a stable way with the mammalian membrane.

Conclusions

There is a need for the development of new antimicrobial molecules to combat the rise in antibiotic resistant bacteria. To assist in this development there is also a requirement for peptide and medicinal chemists to have a chemical based assay to pre-screen and identify lead AMPs prior to biological testing. Here we provide proof of concept that a chloride ion efflux

assay based on lucigenin loaded LUVs is an effective tool for the identification of lead AMPs as increased LUV fluorescence correlated with MIC and MBC of the peptides. Further the assay was able to provide data which showed that a proline-rich, cationic AMP induced ion flux in bacterial model membranes but not in mammalian model membranes which is consistent with the biological activity of the AMP. Finally, we showed that our LUV assay produced data consistent with toxicity data for both the membrane pore forming peptides and the internal acting Chex1-Arg20. Thus the fluorescent ion efflux assay developed here should have applicability for the majority of AMPs that interact with cytoplasmic/inner membranes. The information on membrane phospholipid composition will allow, the ability to tailor an AMP assay specific for the target microorganism. A high-throughput screen using this assay should lead to the identification of novel membrane interactive AMPs with high antimicrobial activity and low cell toxicity. This would provide a shortlist of lead candidates prior to biological testing saving considerable time and money.

Experimental

Synthesis and Purification of Antimicrobial Peptides

O-Benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIPEA), *N,N*-dimethylformamide (DMF), piperidine, trifluoroacetic acid (TFA) and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from Auspep Pty Ltd (Melbourne, Australia). Triisopropylsilane (TIPS), cyclohexane-carboxylic acid, and dimethyl sulfoxide (DMSO) were obtained from Aldrich (New South Wales). Diethyl ether and dichloromethane (DCM) were obtained from BDH (Poole, UK). Unless otherwise stated chemicals were of peptide synthesis grade or its equivalent.

The antimicrobial peptide (AMPs) (**Table 1**) were chemically synthesized by solid phase peptide synthesis (SPPS) on a CEM Liberty microwave peptide synthesizer (Ai Scientific, Victoria) as previously described.^[12-13] Briefly, the peptide-resins were assembled from Fmoc-Rink-AM SURETM Resin (Merck Millipore Pty Ltd, NSW) or Fmoc Rink amide polystyrene resin (Rapp Polymer, Tübingen, Germany) to produce the C-terminal carboxamide peptides in the Fmoc/tBu mode of synthesis. For a 0.1 mmol reaction scale, Fmoc-deprotection was performed in two stages by initial treatment with 20% piperidine/0.1 M HOBt/DMF (v/v, 7 mL) under microwave radiation for 30 s (40 W, 40 °C), followed by filtration and a second addition of the above solution (45 W, 75 °C; 3 min). The peptide-resins were then rinsed with DMF (4 × 7 mL). Acylation, where required, was achieved by the addition of a solution containing amino acid (5 eq, relative to reaction scale), HBTU (5 eq) and DIEA (10 eq) in DMF/NMP (7:1, v/v; 4 mL) to the $N\alpha$ -deprotected peptide-resin and the mixture agitated under microwave radiation for 10 min (30 W, 75 °C, vessel under external chilled air flow). Dichloromethane (DCM) (5 × 2 min) was used to rinse the peptide-

resins prior to the cleavage step. For the synthesis of Chex1-Arg20, the cyclohexane-carboxylic acid (Chex) was coupled as the last residue in the sequence using the acylation conditions above. The peptide was cleaved from the resin support by the addition of TFA/TIPS/phenol/water (95:3:1:1, % v/v/v/v; 10 mL) for 2.5 h, after which the cleavage filtrates were evaporated under nitrogen flow to 0.5-1.0 mL and the crude product was isolated by precipitation in cold ether (4×30 mL).

The crude peptides were dissolved initially in Buffer-Eluant B (0.1% v/v TFA in 90% v/v aqueous acetonitrile) and then Buffer-Eluent A (0.1% v/v TFA in Milli-Q water) was added dropwise to give a final peptide solution of 10% v/v Buffer-Eluent B in Buffer-Eluent A. The dissolved peptide was purified using an Agilent 1200 series liquid chromatograph instrument (Agilent, NSW) equipped with a UV detector (model G1316A) and a Zorbax 300 SB-C18 reversed phase column (9.4 mm $\times$ 25 cm). Crude peptide analysis/purification was achieved using a linear acetonitrile gradient in 0.1% TFA at a flow rate of 2 mL/min (linear gradient of 10 to 50% CH₃CN over 15 min, followed by a 50 to 100% CH₃CN over 50 min). Analytical RP-HPLC of the purified peptide was achieved using a Zorbax 300 SB-C18 reversed phase column (4.6 mm $\times$ 15 cm) using a linear acetonitrile gradient in 0.1% TFA at a flow rate of 2 mL/min (linear gradient of 10 to 100% CH₃CN over 30 min). Analysis of the purified peptide was performed using an Bruker Ultraflex III MALDI TOF/TOF. One microliter of HPLC eluted purified peptide was deposited on to a prespotted HCCA (α -cyano-4-hydroxycinnamic acid) Anchor Chip II (PAC II) and MS spectra acquisition performed manually was carried in reflectron mode measuring from 700 to 4000 Da, using an accelerating voltage of 25 kV. All MS spectra were produced from five sets of 100 laser shots and calibration of the instrument was performed externally with ions of pre-spotted internal standards. Each of the purified peptides gave the expected masses (**Table 1**).

Bacterial Strains and Growth Conditions

Escherichia coli ATCC 25922 lyophilised or glycerol stocks were obtained from the culture collection of the Oral Health Cooperative Research Centre, The Melbourne Dental School, University of Melbourne, Victoria. This strain was selected for this study as it is widely used in AMP studies and is a Gram negative bacterium and growth conditions were as previously described.^[13] Briefly, bacteria were grown aerobically and maintained by passage at ambient temperature on horse blood agar (10% v/v defibrinated horse blood, 4.4% w/v Oxoid Blood Agar Base No. 2). For the antimicrobial peptide assays a 20 mL starter culture was produced by taking single colonies from blood agar plates, to inoculate either: Luria Broth (LB; 1% w/v Bacto™ Tryptone, 1% w/v NaCl, 0.5% w/v Oxoid Yeast Extract, pH 7.5, Thermo Scientific Pty Ltd, Sydney, NSW) which were grown aerobically at 37 °C. After overnight incubation, 0.5-2.0 mL of the starter culture was used to inoculate the appropriate fresh LB broth (200 mL) and growth monitored at 650 nm using a spectrophotometer (model 275E; Perkin-Elmer, Sydney, NSW) with culture purity checked by microscopic examination and culture. Bacteria were harvested during late exponential growth phase and viability and bacteria/mL determined using a BacLight viability kit (Invitrogen, Sydney, NSW) and a Quanta SC-MPL flow cytometer (Beckman Coulter Pty Ltd, Sydney, NSW).

Antimicrobial Peptide Assays

Antibacterial assays were undertaken to determinate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for each of the AMPs using assays previously described.^[13] Briefly, *E. coli* stock solution (2.5×10^6 cells/mL) in the LB media was made and an aliquot incubated with AMPs within 15 minutes from viable cell count and stock preparation. All AMPs were dissolved in DMSO and a 500 µM stock solution prepared

by adding the respective media and serial dilutions (250-0.244 μ M) of the AMP in media (100 μ L/well) made just prior addition of bacteria. The final assay concentration of DMSO was $\leq 2.5\%$ v/v, which does not affect bacteria viability or susceptibility to AMPs. One hundred microliter aliquots of the bacterial stock solution (2.5×10^5 cells/well) were added to the AMP serial dilutions and incubated at 37 °C for 90 min. Bacteria were also incubated in the absence of AMP to serve as a growth control for the assay. After the 90 minute incubation period the antimicrobial activity (MIC and MBC) was determined as follows: For MIC, the CLSI broth microdilution assay^[31] was followed and, for determination of MBC, the CLSI protocol^[31] was also followed as we have previously described.^[13] A minimum of 3 biological replicates were conducted for each AMP.

Mammalian Cell Cytotoxicity Assay

To determine the cytotoxicity of the AMPs, 100 μ L of HEK-293 (ATCC CRL-1573TM) cells (5×10^3) in media (Eagle's Minimum Essential Medium (EMEM) supplemented with 10% v/v foetal bovine serum) or OKF6/TERT-2 (TERT-2), an immortalized oral epithelial cell line were provided by J. Rheinwald (Harvard Medical School, Cambridge, USA)^[32] in keratinocyte serum-free medium supplemented with 0.2 ng/mL human EGF, 25 mg/mL bovine pituitary extract, 0.4 mM CaCl_2 , 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM GlutaMAX-1 were seeded into 96-well plates containing 100 μ L serial dilutions (250-0.244 μ M) of the AMP in media and cultured at 37°C, 5% CO_2 incubator. After 28 h, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay kit, Promega) were added to each well and the plates were incubated for a further 1-2 h at 37°C, 5% CO_2 incubator. Cell cytotoxicity was determined as previously described.^[13] A minimum of 3 biological replicates were conducted for each AMP.

Large Unilamellar Vesicle (LUV) Encapsulated Lucigenin Preparation

Lucigenin, Triton X-100, and general chemicals were purchased from Sigma-Aldrich (Sydney, NSW). Phospholipids palmitoylcholine (POPC), palmitoylcholine (POPG) and palmitoylcholine (POPE) were purchased from Avanti Polar Lipids (Alabaster, USA) and were used without further purification. Milli-Q water was used in all experiments.

Large unilamellar vesicles of POPC or POPE:POPG (7:3) to mimic mammalian or Gram negative bacterium membranes, respectively were produced as we have previously described.^[17] Briefly, phospholipids were co-solubilized in chloroform-methanol (3:1 [vol/vol]) and the solvents removed by rotatory evaporation. Phospholipids were hydrated in Milli-Q water and lyophilized overnight. The resultant lipid powders were resuspended in 5 mM lucigenin (100 mM NaCl, 20 mM phosphate) and subjected to 20 freeze-thaw cycles. The homogeneous dispersions were then extruded 10 times through an Avanti-Mini-Extruder (Alabaster, USA) using 0.1- μ m-pore-size polycarbonate filters to produce large unilamellar vesicles (LUV) 100 nm in diameter. The LUVs were suspended in 100 mM NaCl (with 20 mM phosphate buffer). Then the LUVs encapsulated lucigenin were dialysis *via* SnakeSkin Pleated Dialysis Tubing (7000 MWCO, 22 mm $\times$ 35 feet, Thermo Scientific, VIC) to remove un-encapsulated lucigenin with 100 mM NaNO₃ (20 mM phosphate buffer) for 6 hours.

Fluorescent Ion Efflux Assay

For fluorescent ion efflux assay AMPs were dissolved in 100 mM NaNO₃ (20 mM phosphate buffer) and 250- μ L reaction samples were made of 250 μ M LUV and appropriate amount of peptide to obtain an initial Lipid/Peptide (L/P) molar ratio of 50:1, where the initial peptide

concentration for each peptide was 10 μ M. Analogous samples were made up with 100 mM NaNO₃ (20 mM phosphate buffer) instead of peptide stock solution to produce negative controls. Complete LUV disruption was achieved by treating LUV with 10 μ L of 1.0% Triton X-100 (Tx). After the addition of peptide, buffer or Tx, the samples were incubated at 37 °C for 30 min and the lucigenin fluorescence was determined using a FLUOstar Optima plate reader (BMG Labtech, USA). All assays were conducted in 96 well plates. The excitation wavelength was 440 nm and the bottom-read emission was recorded at 530 nm with 3 cycles at 37 °C. The fluorescence intensities were averaged and calculated to percentage lucigenin fluorescence *via* the following equation.

$$\text{Fluorescence \%} = \frac{(I - I_n)}{I_p - I_n} \times 100\%$$

in which *I* represents the fluorescence of LUVs with peptides, *I_n* represents the fluorescence of the LUVs only as negative control and *I_p* represents the fluorescence of the LUVs with 1% Triton X-100 as positive control. Then, the normalised intensities were plotted against the lipid to peptide molar ratio (L/P). A minimum of 3 biological replicates were conducted for each AMP.

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Figure Legends

Scheme 1. Fluorescent ion efflux assay. LUV encapsulated lucigenin fluorescence is quenched in the presence of chloride ions (NaCl). With the addition of a membrane interactive peptide such as an AMP in NaNO₃ the LUV membrane integrity is disrupted by the AMP to allow efflux of the chloride ions in exchange for the nitrate ions so that lucigenin fluoresces.

Figure 1. The relative activity of maculatin 1.1 and ovispirin in the lucigenin-LUV assay at different pHs. For all assays the intravesicular conditions were 100 mM NaCl, 10 mM phosphate buffer, 2 mM lucigenin; extravesicular conditions were AMP in 100 mM NaNO₃, 10 mM phosphate buffer at varying pH. **A.** Effect of ovispirin and maculatin 1.1 on the fluorescence of lucigenin at different lipid to peptide ratios of POPE:POPG (7:3) at pH 7.0. **B and C.** The effect of pH on AMP induced fluorescence of lucigenin-LUVs at a 100:1 lipid to peptide ratio of POPE:POPG (7:3). Data are expressed as normalised fluorescence intensity and each point is representative of 3 replicate experiments.

Figure 2. The relative activity of different AMPs in the lucigenin-LUV assay using different phospholipid LUVs. For all assays the intravesicular conditions were 100 mM NaCl, 10 mM phosphate buffer, pH 7.0, 2 mM lucigenin; extravesicular conditions were AMP in 100 mM NaNO₃, 10 mM phosphate buffer pH 7.0. **A.** Effect of AMPs on the fluorescence of lucigenin at different lipid to peptide ratios using the Gram negative bacteria membrane model LUVs containing POPE:POPG at a ratio of 7:3 w/w. **B.** Effect of AMPs on the fluorescence of lucigenin at different lipid to peptide ratios using the mammalian membrane model LUVs

containing POPC. Data are expressed as normalised fluorescence intensity and each point is representative of 3 replicate experiments.

Figure 3. The relative activity of Chex1-Arg20 in the lucigenin-LUV assay using different phospholipid LUVs. For all assays the intravesicular conditions were 100 mM NaCl, 10 mM phosphate buffer, pH 7.0, 2 mM lucigenin; extravesicular conditions were AMP in 100 mM NaNO₃, 10 mM phosphate buffer pH 7.0. **A.** Effect of Chex1-Arg20 on the fluorescence of lucigenin at different lipid to peptide ratios using the Gram negative bacteria membrane model LUVs containing POPE:POPG at a ratio of 7:3 w/w. and on the mammalian membrane model LUVs containing POPC. Data is expressed as normalised fluorescence intensity and is representative of 3 replicate experiments. **B.** Flow cytometry dot plots of the membrane potential of *E. coli* incubated without or with the depolarizing compound CCCP and with 0.5 x and 1.0 x MIC concentration of Chex1-Arg20. Data is expressed as red or green fluorescence of DiOC2. **C.** Membrane potential of *E. coli* expressed as Red/green ratios were calculated using mean fluorescence intensities of populations incubated with 30 mM DiOC2 for 30 min in the presence or absence of CCCP and with 0.5 x and 1.0 x MIC concentration of Chex1-Arg20. All data are expressed as mean $\pm$ standard deviation as indicated by the error bars, based on values obtained from at least two biological replicates.

Table 1. Antimicrobial peptide sequences.

Peptide	Sequence ^a	Mass – Obs (Calc) (Da)
Maculatin 1.1	GLFGVLAKVAAHVVPVPAIAEHF-amide	2144.3 (2144.2)
Caerin 1.1	GLLSVLGSAKHVLPVVPVIAEHL-amide	2582.9 (2582.6)
Ovispirin	KNLRRIIRKIIHIIKKYG-acid	2261.8 (2261.5)
Magainin II	GIGKFLHSAKKFGKAFVGEIMNS-amide	2464.5 (2464.4)
Dermaseptin B2	GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAV-amide	3178.8 (3178.8)
Pardaxin IV	GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE-amide	3320.8 (3320.8)
Chex1-Arg20	Chex-RPDKPRPYLPRPRPPRPV-amide ^b	2479.8 (2479.8)

a single letter amino acid code

b Chex = 1-amino-cyclohexane-carboxylic acid

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Table 2. Antimicrobial and cytotoxic activity of peptides against *E. coli* and epithelial cells.

	MIC (μM) ^{a, b}	MBC (μM) ^{a, b}	IC ₅₀ (μM) ^c
Pardaxin IV	7.5±1.0	7.5±0.8	6.6±1.1 ^d
Caerin 1.1	8.5±0.2	15.6±0.8	8.1±2.4 ^d
Dermaseptin B2	18.3±3.8	14.2±1.2	21.6±4.6 ^d
Magainin II	29.5±3.7	48.1±12.4	80.4±2.9 ^d
Chex1-Arg20	28.6±0.4	38.9±0.9	>100 ^e

a Antimicrobial peptide activity expressed as μM is the average of 3 biological replicates ± standard deviation.

b MIC and MBC determined following incubation of *E. coli* ATCC 25922 with peptide for 90 mins using standard protocols.^[13]

c Cytotoxic activity expressed as IC₅₀ is the peptide concentration (μM) that results in death in 50% of the cell population and is the average 3 biological replicates ± standard deviation.

d Cytotoxicity assay data for human oral epithelial cell line OKF6.

e Cytotoxicity assay data for human epithelial cell line HEK-293.

Table 3. Major phospholipid composition of bacterial cytoplasmic/inner membranes and of mammalian cells^a.

	Total membrane phospholipid ^b (%)									
	PE + lysoPE ^c	PG + lysylPG ^d	CL	PC	Chol	PS	PI	NL	SM	Other
Gram negative bacteria										
Model Gram negative membrane	70	30	-	-	-	-	-	-	-	-
<i>E. coli</i>	80	15	5	-	-	-	-	-	-	-
<i>Enterobacter cloacae</i>	74	21	3	-	-	-	-	-	-	2
<i>Yersinia kristensenii</i>	60	20	20	-	-	-	-	-	-	-
<i>Proteus mirabilis</i>	80	10	5	-	-	-	-	-	-	5
<i>Klebsiella pneumoniae</i>	82	5	6	-	-	-	-	-	-	7
<i>Pseudomonas aeruginosa</i>	60	21	11	-	-	-	-	-	-	8
<i>Neisseria gonorrhoeae</i>	75	23	2	-	-	-	-	-	-	-
<i>Bordetella pertussis</i>	71	11	18	-	-	-	-	-	-	-
<i>Vibrio cholerae</i>	84	11	5	-	-	-	-	-	-	-
<i>Salmonella typhimurium</i>	83	11	4	-	-	-	-	-	-	2
<i>Legionella pneumophila</i>	40	18	15	27	-	-	-	-	-	-
<i>Helicobacter pylori</i> (pH 7.4 grown)	69(2)	4	10	4	9	-	-	-	2	-
<i>Helicobacter pylori</i> (pH 5.0 grown)	14(33)	4	14	8	19	4	-	-	4	-
Gram positive bacteria										
Model Gram positive membrane	-	60	40	-	-	-	-	-	-	-
<i>Bacillus polymyxa</i>	60	3	8	-	-	-	-	-	-	29
<i>Bacillus cereus</i>	43	40	17	-	-	-	-	-	-	-
<i>Bacillus anthracis</i>	43	40	17	-	-	-	-	-	-	-
<i>Bacillus subtilis</i>	12	70	4	-	-	-	-	-	-	14
<i>Staphylococcus aureus</i>	-	58	42	-	-	-	-	-	-	-
<i>Streptococcus pneumonia</i>	-	50	50	-	-	-	-	-	-	-
<i>Streptococcus sanguis</i>	-	82	18	-	-	-	-	-	-	-
<i>Listeria monocytogenes</i>	9	29(23)	22	-	-	-	-	-	-	17
<i>Clostridium difficile</i>	-	100	-	-	-	-	-	-	-	-
<i>Enterococcus faecium</i>	-	34(14)	39	-	-	-	-	-	-	13
<i>Streptococcus</i> (Group A)	-	50	50	-	-	-	-	-	-	-
<i>Mycobacterium tuberculosis</i> ^e	5	-	6	-	-	-	13	54	-	22
Model eukaryote membrane	-	-	-	100	-	-	-	-	-	-
Mammalian plasma membrane	16	18	0	55	5	3	-	-	3	-
Erythrocyte (human)	16	0	0	20	23	5	-	-	20	-

a known phospholipid compositions of bacteria compiled from^[26, 33-35] and references therein.

b PE = phosphatidylethanolamine, PG = phosphatidylglycerol, CL = cardiolipin, PC = phosphatidylcholine, Chol = cholesterol, PS = phosphatidylserine, PI = phosphatidylinositol, NL = neutral lipids, SM = sphingomyelin, Others = mainly glycolipids.

c the total membrane percent of PE is given in no brackets and the total membrane percent of lysoPE (lysophosphatidylethanolamine) is given in brackets.

d the total membrane percent of PG is given in no brackets and the total membrane percent of lysyl-PG (lysyl-phosphatidylglycerol) is given in brackets.

d *Mycobacterium tuberculosis* is considered as Gram neutral as it has Gram positive and negative staining.

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Figure 1

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Figure 2