

Action of antimicrobial peptides and their prodrugs on model and biological membranes.

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Full title: Action of Antimicrobial Peptides and their prodrugs on model and biological membranes

Short title: Enhanced membrane targeting potential of AMP prodrugs

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Abstract

Antimicrobial peptides (AMPs) are promising broad-spectrum antibiotic candidates in the wake of multi-resistant pathogens. Their clinical use still requires a solution based on lead optimisation and/or formulation to overcome certain limitations, such as unwanted cytotoxicity. A prodrug approach could overcome this safety barrier and can be achieved through reversible reduction or neutralisation of the AMPs' net cationic charge. By prodrug activation through pathogen associated enzymes, this approach could increase the therapeutic index of membrane active peptides. P18, a cecropin/magainin hybrid, and WMR, a myxinidin analogue from hagfish, were used as templates for the design strategy. The membrane permeabilizing activities of these AMPs and their prodrugs are reported here for liposomes of either *Escherichia coli* polar lipid extract or a human model lipid system of phosphatidylcholine and cholesterol. These results are compared with their antibacterial and haemolytic activities. Overall, correlation between liposome permeabilization and the corresponding bioactivity is observed and indicate that the broad-spectrum antibacterial effect exerted by these peptides is associated with membrane disruption. Furthermore, the prodrug modification had a general negative influence on membrane disruption and bioactivity, notably as much on bacterial as on human membranes. This prodrug strategy is particularly successful when complete neutralisation of the AMP's net charge occurs. Thus, on-target selectivity between bacterial and human membranes can be improved, which may be used to prevent the unnecessary exposure of host cells and commensal bacteria to active AMPs.

Keywords

Antimicrobial Peptides; Prodrugs; Liposomes; Membrane Permeabilization; Therapeutic Index.

Abbreviations

AMP: Antimicrobial Peptide; CF: Cystic Fibrosis; CL: cardiolipin; EC₅₀: half maximal effective concentration; IC₅₀: half maximal inhibitory concentration; MBC: Minimal Bactericidal Concentration; MIC: Minimum Inhibitory Concentration; NE: Neutrophil Elastase; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine; WMR: W and R modified Myxinidin peptide.

Introduction

The history of antibiotic discovery is marked by a golden era, starting in 1929 with the discovery of penicillin and lasting approximately 30 years, and a recent ‘discovery void’ of nearly 30 years.¹ The latter period might augur a post-antibiotic era, if the need for novel-mechanism antibiotics is not met. This global public health threat is further compounded by the challenge to outpace the mutation rates in antibiotic resistance with novel antibacterial agents. It takes an average of twelve years to develop a new drug, against a dozen days for a resistance mechanism to evolve.^{2,3} Therefore, novel-mechanism antimicrobials associated with high fitness costs of resistance are critically required to constrain the evolution of bacteria under antibiotic selective pressure.⁴

Antimicrobial Peptides (AMPs) are among the handful of antibiotic candidates currently investigated that potentially fulfil this requirement.⁵ These molecular mediators of innate immunity in multicellular organisms, also known as Host Defence Peptides, have a complex pharmacology that contributes to innate as well as adaptive immunity.⁶ Under appropriate conditions, AMPs can display a broad spectrum of antimicrobial activity, exerting bactericidal effects in minutes at low micromolar concentrations⁷ and contending with bacteria in both planktonic and biofilm growth modes.⁶ Notably, these host defence components, ubiquitous throughout the animal and plant kingdoms, have remained effective over millions of years of co-evolution with bacteria.^{8,9} Although AMPs are not immune to resistance mechanisms and selection pressures, the evolution of resistance to synthetic AMPs and cross-resistance to natural AMPs appears to be incomplete and to progress slowly *in vitro*,¹⁰ and possibly even more so *in vivo* owing to combination effects.¹¹

However, the approval pathway of AMPs as antibiotics is fraught with challenges. Limited therapeutic windows, lack of bioavailability and (bio)stability, as well as elevated costs of production are the main issues impeding their clinical use, in particular as systemic therapies.¹² Diverse strategies to improve the pharmaco-kinetic and pharmaco-dynamic properties of peptide therapeutics have been applied to AMPs. They include peptidomimetic conversion, including the inverso modification and polymer-based analogues; optimisation of their therapeutic window through Structure-Activity Relationship studies; conjugation to polymers and application of formulation technologies.¹²⁻¹⁴ Additionally, the design of immunomodulatory peptides devoid of direct antimicrobial activities¹⁵ and the development of an AMP candidate with a good safety profile, metabolically stable and amenable to large scale

production by a fermentation technique, have been reported,¹⁶ while a notable reduction in the cost of synthetic production of peptides on the whole has been achieved.¹⁷

The major challenge in the development of AMP-based systemic therapies is their narrow therapeutic window. This can be addressed by a prodrug approach where a biologically inactive drug precursor employs a (patho)physiological process to release the active agent *in vivo*.¹⁸ A prodrug candidate is usually generated by modifying a parent drug with a promoiety, a functional group or molecular entity which transiently modifies the biological and/or physicochemical properties of this therapeutic candidate.¹⁹ In the case of AMPs, this approach could overcome the safety barrier in their clinical use, but also possibly delay the potential evolution of cross-resistance to innate immunity peptides,²⁰ by limiting the exposure of commensal bacteria to exogenous, active, AMPs.

The main characteristic and activity determinant of AMPs is their amphiphilic and cationic nature. It is determined by their amino acid content, nearly equally shared between hydrophobic and basic residues, for these sequences generally shorter than 50 amino acids.^{8,18} Here the prodrug concept can permit the cationic feature to be transiently reduced or annihilated by reversible conjugation of an anionic promoiety. The bioreversibility can then be imparted by a peptidic linker, introduced between the active AMP sequence and the promoiety, if it constitutes a substrate for a pathology associated enzyme. Since AMPs typically do not exert their direct antibiotic activity by specific structural affinities, dynamic metabolic stability can be gained by assembling the prodrug sequence from heterochiral residues. Thus the AMP itself is assembled from D-amino acids, whereas the promoiety and linker are made of L-amino acids.²¹ For example, fusion sequences encompassing an oligo-glutamate, a Neutrophil Elastase (NE)-sensitive tetrapeptide motif (AAAG) and an all-D AMP could target *Pseudomonas aeruginosa* in chronically infected Cystic Fibrosis (CF) patients. These prodrug candidates can confine the activity of linear AMPs to the endobronchial space and reduce the toxicity of their parent peptides *in vivo*.²² As these AMP prodrugs are activated by an extracellular protease, ultimately their behaviour is expected to conform to the mechanism(s) of action of the original peptides. On the other hand, the neutralisation of their net charge is likely to reduce their propensity to affect biological membranes and to translocate into cells.²³ In theory, this could prevent the unnecessary exposure of host cells and commensal bacteria to active AMPs. However, there is no evidence to date that this benefit is achieved with AMP prodrugs. Accordingly, the action of two AMPs and their prodrug candidates with model membranes of bacterial and mammalian cells are described here, using a membrane permeabilization assay, and compared to their experimental haemolytic and antimicrobial activities.

Materials and Methods

Materials

The Fmoc-protected amino acids and the Rink Amide MBHA resin were obtained from Novabiochem (Merck Millipore, UK). HATU and NMP were purchased from ChemPep Inc

(USA) and BioSciences, respectively. The phospholipids were sourced from Avanti Polar Lipids (Alabaster, USA). All other reagents and solvents were supplied by Sigma-Aldrich Ireland.

The reference strains of *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC15692, PAO1) were obtained from the American Type Culture Collection (Manassas, VA, USA).

The WMR and pro-WMR peptides used for susceptibility testing were from a scaled-up synthesis batch manufactured by Almac (Craigavon, UK). Other peptides were synthesised at RCSI in the Department of Pharmaceutical and Medicinal Chemistry as described below.

Peptide synthesis

Peptide sequences were assembled by standard Solid Phase Peptide Synthesis (SPPS) according to the Fmoc-*t*Bu strategy with HATU/DIEA coupling chemistry, from a Rink Amide MBHA resin. NMP was used as the solvent for coupling reactions and washes; a solution of 20% piperidine in NMP (automated synthesis) or DMF (manual synthesis) was used for the Fmoc-deprotection reactions. Single coupling cycles, using a total 10-fold excess of Fmoc-amino acid derivatives to resin-bound peptide were used, except for leucine following (N-terminal side) the proline residue in the P18 sequence, for which a double coupling cycle was used (10-fold excess each). The side-chain protecting groups were Boc for lysine and tryptophan, *t*Bu for glutamic acid and Trt for histidine. Assembly of the parent P18 and WMR sequences from D-amino acids and their elongation with the AAG motif, using L-alanine, was carried out on a 100 μ mol scale, on an automated peptide synthesizer (433 Applied Biosystems, Warrington, UK). For the prodrug candidates, elongation of the previous sequences with the P1 residue of the NE-sensitive linker (L-alanine) and the tetra-glutamate motif (L-glutamic acid), N-terminally acetylated, was carried out by manual SPPS, using a fritted reaction vessel placed on an orbital shaker. Qualitative monitoring of the deprotection and coupling reactions was performed by the Kaiser test.

Following assembly, peptides were deprotected and cleaved from the resin, by treatment with a solution consisting of 80% trifluoroacetic acid, 5% water, 5% thioanisole, 5% triisopropylsilane and 5% 1, 2-ethanedithiol, at RT for 2 h. They were then precipitated with diethyl ether from the cleavage cocktail, subsequently washed three times with diethyl ether, air dried, dissolved in distilled water and lyophilized.

Chromatographic analysis and purification were performed on a BioCAD SPRINT Perfusion Chromatography Workstation (PerSeptive Biosystems) using Gemini columns (Phenomenex, 110 Å, 5 μ m, C18, 4.6 mmd / 250 mmL and 100 mmd / 250 mmL, for the analytic and semi-preparative columns, respectively). Buffers used were mobile phase A (0.1% TFA in water), mobile phase B (0.1% TFA in acetonitrile), with a gradient of 5 to 65 % B in 18 column volumes (analytical) or 5 column volumes (semi-preparative) with a flow rate of 1 ml/min (analytical) or 5 ml/min (semi-preparative) and dual wavelength detection at 214 and 280 nm. Purified peptides were finally characterised by MALDI-TOF MS, using either a Bruker Reflex Mass Spectrometer (Conway Institute, University College Dublin) or a Waters MALDI Q-ToF Premier Mass Spectrometer (School of Chemistry, Trinity College Dublin), using α -cyano-4-hydroxy-cinnamic acid as the matrix.

Membrane permeabilization assay

Liposomes were manufactured and permeabilization assayed as described previously,²⁴ with minor alterations. Briefly, dry lipid films of *E. coli* polar lipid extract or of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine with cholesterol (POPC:cho in 3:2 molar ratio), were formed on round-bottom flask walls. This was achieved by dissolving lipids in chloroform and evaporating the solvent under agitation and nitrogen gas flow, followed by incubation in vacuum chamber. Lipid films were re-suspended by either 30 min stirring (*E. coli*), or repeated freeze-thawing using liquid nitrogen (POPC:cho), at 55°C in an aqueous solution of 100 mM 5(6)-carboxyfluorescein in 10 mM Tris (set to pH 7.4 at 37°C). Suspensions were subjected to repeated extrusion through a 100 nm pore size polycarbonate membrane in order to reduce multilamellar structures and polydispersity. Un-trapped carboxyfluorescein was removed by gel filtration. Membrane permeability was measured by monitoring carboxyfluorescein efflux from the liposomes to the external low concentration environment, resulting in loss of self-quenching and an increased fluorescence signal. The 96-well plates were prepared with a 2-fold serial dilution of the peptides in Tris buffer, as well as controls without peptides (background) and 0.16% Triton X-100 (maximum leakage). The plates were pre-heated to incubation temperature (37°C) and administered a liposome suspension, to a final lipid concentration of 10 µM in 200 µl. The effects of each peptide concentration on the liposome systems were monitored for 45 min, at which point the initial leakage had largely subsided. Results shown represent the mean from four experiments with standard deviations and are expressed as percent of total leakage generated with Triton X-100 and subtraction of the baseline value. The EC₅₀-values, fixed at 50% total leakage, are calculated from a sigmoidal dose-response curve fitting with variable slope to the leakage percentage as a function of the peptide concentration (log₁₀).

Haemolysis assay

Human blood was collected from consenting healthy volunteers and centrifuged at 1000 g for 5 min. The supernatant was removed and the erythrocyte-rich pellet re-suspended and washed twice in sterile PBS before re-suspending in PBS at twice the original volume. Serial doubling dilutions of peptides were prepared (3.9µM-1mM) in PBS and 50µl of each dilution added to 50 µl of the erythrocyte suspension in 96-well plates, resulting in a final peptide concentration range of 1.95-500 µM. The assay was performed in triplicate. Positive controls contained 0.1% Triton X-100 and negative controls contained no peptide. After 24 h incubation at 37°C, absorbance at 570 nm was recorded and used to calculate percent haemolysis relative to controls.

Bacterial susceptibility testing

Minimum Inhibitory Concentrations (MICs) were determined using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI),²⁵ with modifications for cationic peptides as described by M. Wu and R. Hancock.²⁶ Briefly, serial doubling dilutions of peptides were prepared at 10 times the final assay concentration (10µg/ml-2.56mg/ml for P18-based peptides and 5µg/ml-1.28mg/ml for WMR-based peptides)

in a sterile solution containing 0.2% w/v bovine serum albumin (BSA) and 0.01% v/v acetic acid. Assays were prepared in 96-wells plates where triplicate wells contained 10 μ l of each 10X peptide dilution and approximately 1.4×10^4 CFU/well of bacterial inoculum in 100 μ l Mueller-Hinton (MH) broth (non-cation adjusted, Oxoid, UK) resulting in final peptide concentrations of 1-256 μ g/ml and 0.5-128 μ g/ml for P-18 and WMR-based peptides respectively). The MIC was recorded as the lowest peptide concentration showing no visible growth after approximately 18 h incubation with reference to positive growth control (no peptide) and negative controls (no inoculum).

Results

The two AMPs selected in these studies are linear sequences, which can adopt α -helical structures in α -helix-promoting solvents mimicking the environment of cell membranes. The first one, P18, is a hybrid sequence of two innate immunity peptides, cecropin A (residues 1-8) from the moth *Hyalophora cecropia* and magainin 2 (residues 1-12) from the frog *Xenopus laevis*.²⁷ The second one, WMR, is an innate immunity peptide from the hagfish *Myxine glutinosa* with an optimised spectrum of antibacterial activity.²⁸ These peptides were assembled from D-amino acids for proteolytic stability and with a homologous substitution of isoleucine with leucine in the P18 sequence (on the N-terminal side of the proline residue) for the purpose of reducing production costs of this peptide. Moreover, its antimicrobial activity benefits from these combined modifications.²⁹ Both peptides were modified at their C-termini by amidation and at their N-termini with residual amino acids (AAG) from the NE-sensitive linker (Table 1), to match the sequences released from the prodrugs.²⁹ Accordingly, the sequences of the active peptides were AAGkwkllfkkllpkflhlakkf, for P18 (named AAG-P18) and AAGwglrrllkygkrs, for WMR (named AAG-WMR). The prodrugs, pro-P18 and pro-WMR, were produced by elongating (C \rightarrow N) these sequences with the last alanine of the NE-sensitive linker and the acetylated tetraglutamate promoiety (Table 1).

Interaction with model membranes

The interactions of two cationic AMPs, and their prodrugs of reduced net charges, with model membranes were studied with a leakage assay. This was accomplished by measuring the efflux of carboxyfluorescein from liposomes of either *Escherichia coli* polar lipid extract or the human model lipid system POPC:cholesterol (3:2). The cytolytic AMP melittin from bee venom was used for comparison.

In this assay, AAG-P18 induced significant leakage from the *E. coli* liposomes, with an EC₅₀ lower than melittin (0.37 vs. 0.49 μ M, respectively), as presented in Figure 1A and Table 2. Note that melittin is considered as one of the most potent AMPs in this regard. AAG-WMR was slightly less potent than melittin (0.59 vs. 0.49 μ M, respectively). Prodrug modification of these peptides reduced their EC₅₀ against this lipid system, modestly for P18 (0.58 μ M, 1.6 fold reduction), but more substantially for WMR (11 μ M, 19 fold reduction).

In terms of peptide induced permeability on the human model membrane system (POPC:cholesterol liposomes), both P18 and WMR derivatives were less active than melittin (0.064 and 0.55 μM , vs. 0.0093 μM , respectively). Accordingly, similar to the results on bacterial membrane system, AAG-P18 is more potent than AAG-WMR (Figure 1B). The prodrug modification of these peptides also increased their EC_{50} against this lipid system, with the greater effect again being observed with WMR (10 μM , 19 fold reduction), compared to P18 (0.23 μM , 3.6 fold reduction). The peptide-to-pro-peptide window of activity for P18 was more than twice as broad with the human model as with the bacterial counterpart, while no significant difference was observed with WMR and its prodrug.

Haemolytic activity

The haemolytic activities of the AAG-modified AMPs were compared to those of their prodrugs. Triton X-100 (0.1%) was used as a positive control here. Both P18-based peptides caused dose-dependent lysis of erythrocytes. AAG-P18 induced 75% haemolysis at 250 μM , similar therefore to the level of activity reported with unmodified P18.³⁰ However, the pro-peptide was substantially less lytic, with roughly 8 times higher concentrations required to reach 10% haemolysis, and a similar ratio in lysis levels at lower peptide concentrations (Figure 2A). Neither of the two WMR-based peptides exhibited any dose-dependent haemolysis within the concentration range (Figure 2B). In this regard, there were no significant differences observed between these AAG-modified all-D peptides and the unmodified parent all-L peptide.³¹

Antibacterial activity

In bacterial susceptibility testing, AAG-P18 was more effective than AAG-WMR at inhibiting the growth of *E. coli* (0.8 vs. 8.7 μM , respectively) as presented in Table 3. The same MICs were obtained for these peptides with another Gram-negative organism, *P. aeruginosa*. Activity differentials up to 128 for P18, and at least of 8, for WMR, were obtained against *E. coli* by prodrug modification of these peptides (MICs of 256 and >128 μM , respectively). The parent sequences of the peptides investigated here have reported MICs against *E. coli* of 3.125 to 6.25 μM for P18 and 2 μM for WMR.^{27,31,32} It appears therefore that the addition of AAG residues at the N-termini of the parent peptides had no marked effect on the antimicrobial activity of P18, but reduced the potency of WMR against *E. coli*. However, there are limitations to this comparison, as performed between heterochiral peptides (D-AAG-modified peptides vs. L-parent peptides) and using different bacterial strains (ATCC 33694 or KCTC 1682 for P18 and ATCC 11219 for WMR, for reported MICs).

Discussion

Influence of the pro-peptide sequence on membrane permeabilization

The relative levels of membrane leakage within each individual lipid system follows the order expected from the antimicrobial and haemolytic activities of these peptides, as well as the

anticipated effect of the net charge reduction for their prodrugs. However, the relative EC_{50s} between these systems, for each peptide, may appear inconsistent with the selectivity of cationic AMPs for anionic lipid systems over zwitterionic ones. Instead these cationic sequences are as potent (AAG-WMR), or even more potent (AAG-P18), at permeabilizing the membranes of POPC:cholesterol liposomes than those of *E. coli* phospholipids. The primary reason for this is that the homogenous alkyl groups of synthetic POPC-based liposomes renders them far more susceptible to peptide generated leakage.²⁴ The underlying theory refers to the inability of homogenous lipid compositions to accommodate membrane curvature stress by demixing. Inherently, when discussing the membrane activity of these peptides and the influence of the pro-peptide modification, it is important to not compare results from the two liposome systems in absolute terms, due to the different nature of the two models, but instead to focus on the relative results within each system. Notable therefore is that the activity ratio for Pro-P18 compared to AAG-P18 was more than twice as high on POPC:cholesterol than on *E. coli* liposomes. For the WMR-based peptides, the promoiety conferred a 19 fold decrease in activity in both membrane types.

Both AAG-P18 and AAG-WMR were potent membrane permeabilizing agents on the bacterial model liposomes. These were reconstituted from an *E. coli* polar lipid extract and are, in terms of phospholipid composition, representative of the outer membrane phospholipid composition of a broad range of Gram-negative bacteria. *P. aeruginosa*, for example, has a composition of 65% phosphatidylethanolamine (PE), 23% phosphatidylglycerol (PG) and 12% cardiolipin (CL) (area%),³³ which is comparable to the *E. coli* extracts consisting of 67% PE, 23% PG and 10% CL (mass%). This is in agreement with the comparable MICs displayed by the active P18- and WMR-based peptides against these two bacteria (Table 3). In a recent study that showed WMR to be more active on model membranes of DOPE/DOPG/CL (65/23/12 mol%) than of DOPE/DOPG (80/20 mol%), the cardiolipin (CL) was proposed to play a key role.³⁴ This was in turn suggested to be the reason for WMR having more activity on *P. aeruginosa* than *E. coli*. However, as cardiolipins are in principle two covalently adjoined two-tailed phospholipids, this results in both mass% and area% per molecule (on which the original lipid reference for *P. aeruginosa* rely)³³ having to be roughly halved when translated into mol% (*i.e.* 5-6 mol%). This number becomes equivalent to typical *E. coli* membranes, such as the lipid extract used in this study. Another unknown factor is how CL affects artificial alkyl chain compositions as compared to natural compositions. Other antimicrobial peptides that are more active on *P. aeruginosa* than *E. coli*, such as cyclotides,³⁵ exhibit less membrane disruption on the natural *E. coli* phospholipid liposomes containing CL than artificial model systems devoid of CL. The reason for higher susceptibility for *P. aeruginosa*, compared to *E. coli*, regarding effects of membrane disrupting peptides, is still unknown and might be linked to membrane target areas displayed during various stages of the cell cycle rather than general membrane attributes.

Influence of membrane permeabilization on antimicrobial activity

It is important to note that, while AAG-P18 and AAG-WMR exhibited membrane permeabilization activity on bacterial liposomes in the same range as melittin, the activity of these peptides on the human liposomal model were on the other hand 7 and 59 fold lower, respectively, as compared to melittin. Since melittin is at least as antibacterial as it is cytotoxic

and is regarded as one of the most potent AMPs in this regard, with activity at sub-micromolar concentrations in microdilution assays,³⁵ these results can be extrapolated to indicate that there is a substantial amount of selectivity in bacterial membrane targeting achieved by P18 and WMR. This is even more pronounced for the pro-peptides that are 25 and 1100 times less membrane lytic (POPC:cholesterol liposomes) compared to melittin, for Pro-P18 and Pro-WMR, respectively.

Noteworthy is that the promoiety did not convey the same magnitude of reduced activity for the P18-based peptide on the *E. coli* liposomes as it did against bacteria themselves. In contrast, the correlation between bacterial model and biological membranes observed with WMR/Pro-WMR was more to scale. This was relevant also considering the activity on human model membrane and haemolysis. In this case both assays largely followed the relative activity for each peptide, although not reaching haemolytic activity within the concentration interval used for the WMR-based peptides. The effects of the prodrug modification were more variable with P18 than WMR, as Pro-P18 can still display a noticeable antimicrobial activity against *P. aeruginosa*, as well as cytotoxicity against human bronchial epithelial cells,²⁹ while Pro-WMR is essentially inactive. Of note also, is that the prodrug modification (including the inclusion of the NE-sensitive linker) increased the retention time of WMR in RP-HPLC by 6%, whereas it only increased by 2% in the case of P18 (Supplementary Figures S1, S5, S7 and S11). The ratio of these percentages is more than twice higher than the one related to the sequence length increase. On the other hand, Pro-P18 differed from Pro-WMR by its residual, relatively high, cationic net charge. Therefore, the main impact of the prodrug modification appears to occur through the reduction of the AMP's net charge, rather than the modulation of its hydrophobicity, according to RP-HPLC data, or even of its helix propensity, as already shown with a P18 prodrug candidate.³⁰ In this case, it is important to consider that the *E. coli* liposomes have a high anionic charge with a zeta potential of -41 mV comparable with their bacterial counterpart,³⁶ whereas the typical PC/cholesterol human model liposomes has a zeta potential of -9 mV.³⁷ For these anionic membranes the adsorption is to a large extent driven by electrostatic attraction and all peptides with positive net charge will subsequently partition to these membranes at a higher rate. In the present study, a (residual) net charge greater than three seems to be conveying distinct advantage in this regard. However, the significant impact of the promoiety on the susceptibility of *E. coli* and *P. aeruginosa* against P18 indicate that effects other than electrostatics also operate. In this regard, it is significant that adding a fifth glutamic acid in the promoiety of P18, and therefore reducing its net charge to +2.5, did not further reduce the susceptibility of *P. aeruginosa* against the prodrugs of P18 (Supplementary Table S1). Further research, such as conformational and aggregation studies, membrane depolarization and cell penetration measures, will be required to identify these additional contributions. While cationic properties appear important for bacterial membrane permeabilization, both P18 and Pro-P18 were relatively more active on the human model than WMR and its pro-peptide. This is likely due to the higher number of bulky hydrophobic residues in P18, notably phenylalanines, which are particularly important for adsorption on these zwitterionic membrane systems.

Both AAG-modified peptides induced similar and potent permeabilization of the bacterial model liposomes, whereas AAG-P18 was 9-fold more lytic than AAG-WMR with the human model liposomes. The trend seen in these results with the active peptides, and the overall correlation between liposome permeabilization and the corresponding bioactivity, indicate that the broad-spectrum antibacterial effect exerted by these peptides is likely to be associated with membrane disruption. The pro-peptide sequence exerted a (desired) general negative influence on antimicrobial and haemolytic activities, which, according to the results with the model membranes, also appears to be related to membrane permeabilization. It substantially improved on-target selectivity between bacterial and human membranes, reducing toxicity against the latter for both candidate peptides. This strategy appeared to be particularly successful when (near) complete neutralisation of the AMP's net charge occurs, as in the case of the WMR peptide. These data suggest that balancing of charge and hydrophobicity between AMP and pro-sequence and their resulting structures can enhance target selectivity of the prodrug AMP concept. This may therefore be an approach applicable to many promising AMP drugs, to further increase their therapeutic concentration range.

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Table 1

Active and prodrug peptides.

Name	Sequence ^a	Residues	Net charge ^b
AAG-P18	AAGkwklfkklpkflhlakkf-NH ₂	18	+ 8.5
Pro-P18	Ac-EEEEAAAGkwklfkklpkflhlakkf-NH ₂	26	+ 3.5
AAG-WMR	AAGwglrrllkygkrs-NH ₂	13	+ 6
Pro-WMR	Ac-EEEEAAAGwglrrllkygkrs-NH ₂	21	+ 1
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH ₂	26	+ 6

^a Lowercase letters denote D-amino acids; uppercase letters denote L-amino acids. Ac-: N-terminal acetylation; -NH₂: C-terminal amidation.

^b At pH = 7

Table 2

Peptide concentrations required to reach 50% liposome leakage within 45 minutes.

Lipids	EC ₅₀ (μM)				Melittin
	AAG-P18	Pro-P18	AAG-WMR	Pro-WMR	
<i>E. coli</i> (extract)	0.37	0.58	0.59	11	0.49
POPC:cholesterol (3:2)	0.064	0.23	0.55	10	0.0093

Table 3

Peptide concentrations required to inhibit bacterial growth.

Organism	MIC in μg/ml [μM]			
	AAG-P18	Pro-P18	AAG-WMR	Pro-WMR
<i>E. coli</i>	2 [0.8]	256 [82]	16 [8.7]	>128 ^a [>52]
<i>P. aeruginosa</i>	2 [0.8]	64 [20]	16 [8.7]	64 [26]

^a Highest concentration tested

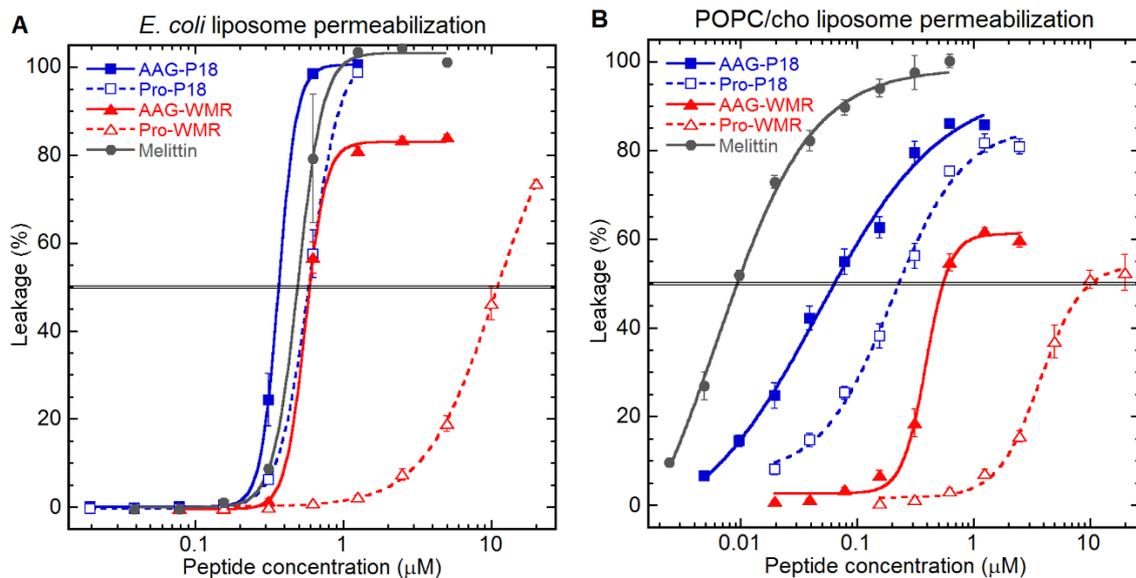


Fig. 1. Membrane leakage levels as a function of peptide concentration. The levels of carboxyfluorescein efflux after 45 min of incubation for liposomes composed of either *E. coli* polar lipid extract (A) or POPC:cholesterol (3:2) (B). Each marker represents the mean leakage at 37°C in 10 mM Tris buffer (pH 7.4) with standard deviation from four experiments done at individual peptide concentrations, *i.e.* no cumulative additions. The sigmoidal dose-response curves for the pro-peptides are dashed and the EC₅₀ level is highlighted with a double line.

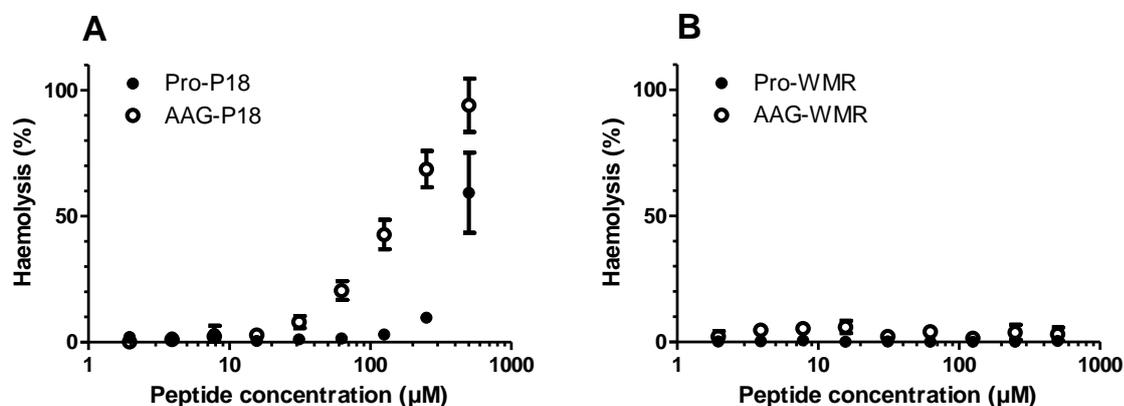


Fig. 2. Lysis of human erythrocytes after 24 h incubation at 37°C in 100 μl of a P18 (A) and WMR (B) peptide solution in PBS. Each column represents the mean percentage of haemolysis normalised to 0.1% Triton X-100, from three independent experiments.