Upsala Journal of Medical Sciences

ISSN: 0300-9734 (Print) 2000-1967 (Online) Journal homepage: informahealthcare.com/journals/iups20

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To cite this article: Martin Malmsten (2014) Antimicrobial peptides, Upsala Journal of Medical Sciences, 119:2, 199-204, DOI: 10.3109/03009734.2014.899278

To link to this article: https://doi.org/10.3109/03009734.2014.899278

Upsala Journal of Medical Sciences. 2014; 119: 199–204

informa
healthcare

REVIEW ARTICLE

Antimicrobial peptides

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Abstract
With increasing antibiotics resistance, there is an urgent need for novel infection therapeutics. Since antimicrobial peptides provide opportunities for this, identification and optimization of such peptides have attracted much interest during recent years. Here, a brief overview of antimicrobial peptides is provided, with focus placed on how selected hydrophobic modifications of antimicrobial peptides can be employed to combat also more demanding pathogens, including multi-resistant strains, without conferring unacceptable toxicity.

Key words: *AMP, antimicrobial peptide, bacteria, liposome, membrane*

Introduction

Due to increasing antibiotic resistance, there is much current interest in novel therapeutic approaches, including antimicrobial peptides (AMPs) (1-3). AMPs are key components of the innate immune system, ≈10–40 amino-acids long, generally net positively charged, and containing a substantial fraction of hydrophobic residues. Although AMPs induce multiple damages in bacteria, including inhibition of DNA, RNA, and protein synthesis, their main mode of action is disruption of bacterial membranes (1-3). Several mechanisms for this have been suggested, including detergency-like packing disruption and formation of barrel-stave or toroidal pores (Figure 1) (1-5). Chemical potential gradients may also cause transient defects due to peptide translocation through the membrane (6). Furthermore, lateral membrane expansion due to peptide binding allows relaxation of the alkyl chains and causes membrane thinning, further facilitating membrane destabilization (7). Also peptide-induced lipid segregation and/or phase transition may contribute to peptide-induced membrane rupture (8).

Critical for the therapeutic application of AMP is that it is selective, efficiently disrupting bacteria and other pathogens, but causing limited damage to human cell membranes. By careful selection of peptide properties, however, such selectivity is possible through composition differences between bacterial and human membranes. Thus, human cell membranes are rich in cholesterol (20–50 mol%) (9), while fungal membranes instead contain ergosterol, and bacteria no sterol at all. There are also differences in phospholipid composition. For example, the outer leaflet of erythrocyte membranes is dominated by zwitterionic lipids, including sphingomyelin and phosphatidylcholine, making it essentially uncharged (9), while bacteria membranes are rich in anionic lipids (10,11). Such composition differences contribute to differences in peptide binding and membrane destabilization (1-4).

Effects of peptide properties on membrane disruption

Peptide length

Due to the increased entropy penalty, per amino-acid, on adsorption, membrane binding is expected to decrease with decreasing peptide length. For similar reasons, there is also a decreased tendency to form

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(Received 2 January 2014; accepted 25 February 2014)

ISSN 0300-9734 print/ISSN 2000-1967 online
DOI: 10.3109/03009734.2014.899278

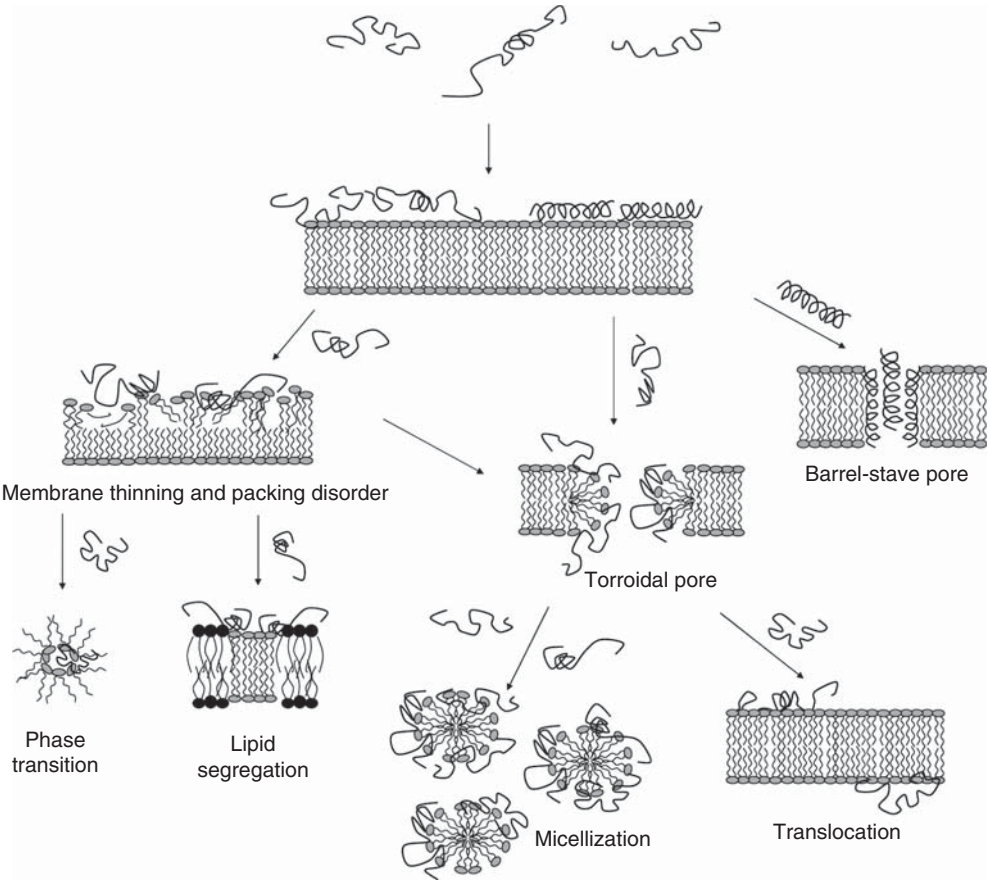

Figure 1. Schematic illustration of AMP interaction with lipid membranes. In barrel-stave pores, peptide oligomers organize in a transmembrane structure, while toroidal pores are disorganized membrane defects caused by curvature strain. Higher peptide densities may subsequently cause complete membrane disintegration (micellization). Furthermore, peptide binding to the polar headgroup region allows relaxation of the alkyl chains and causes membrane thinning. In addition, chemical potential gradients may result in peptide translocation across the membrane. Finally, peptide-induced lipid segregation or phase separation may contribute to AMP-induced membrane rupture.

(amphiphilic) secondary structures with decreasing peptide length. Together, these effects are expected to result in decreased membrane lysis and antimicrobial effect with decreasing peptide length, as indeed generally found (12,13). Having said that, there may be room for *selective* truncations of longer AMPs without losing too much activity (depending on composition), in some cases even reaching improved performance of the truncated peptide (14). Ultimately, however, selective membrane-rupturing capacity is lost when AMPs become sufficiently short.

Peptide charge

Since bacterial membranes are anionic, peptide-induced lysis of bacterial membranes generally increases with peptide positive charge. For example, Ringstad et al. found membrane rupture by the C3a-derived peptide CNY21 to increase with peptide positive charge, but to be completely lost after removal of all positive charges in the peptide (15). Through titrating histidine groups, AMPs may furthermore display pronounced pH-dependent charge, resulting in membrane lysis and antibacterial activity at acidic conditions (i.e. pH < pKa), but abrogated antimicrobial activities above the pKa of histidine (≈6.0 for an isolated H) (12). Also the charge distribution and nature of the charged group have been found to affect membrane interactions of AMPs (2).

Peptide secondary structure

Formation of amphiphilic ordered structures, particularly α-helices, has been found to correlate to peptide-induced membrane disruption (1-4). Illustrating this, GKE21 is an internal peptide sequence that maintains the bactericidal potency of LL-37, but displays lower toxicity (14). GKE21 forms a nearly perfectly amphiphilic helix, with polar/charged and hydrophobic/non-polar residues localized on opposite sides. Consequently, helix induction provides a

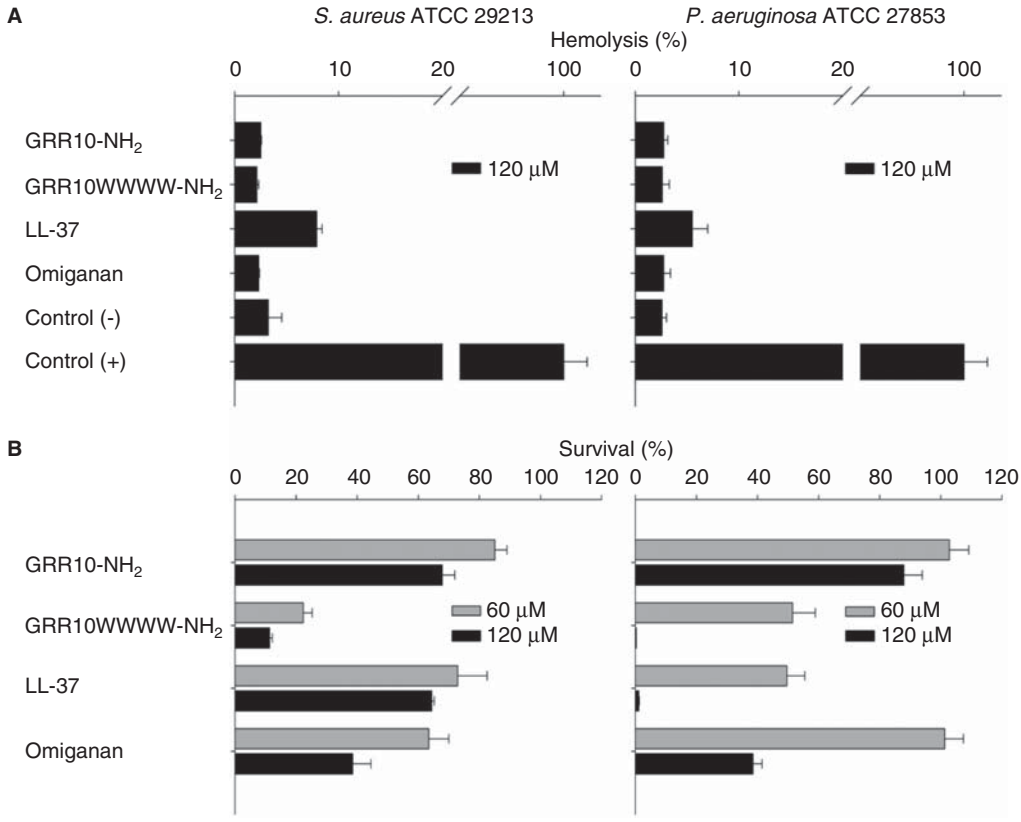

Figure 2. Combined hemolysis (A) and viable count (VCA), (B) assay for *S. aureus* and *P. aeruginosa* (both 2×10^8 cfu/mL) added to 50% citrate blood at peptide concentrations of 60 and 120 μM (23).

driving force for membrane binding. Thus, reducing the helix-related amphiphilicity through selected D-amino-acid substitutions results in reduced membrane disruption and bacterial killing (16). In parallel, helix destabilization generally reduces cytotoxicity of AMPs, although such toxicity reduction may ultimately result in reduction or elimination of antimicrobial effects.

Peptide hydrophobicity

Due to electrostatic screening, highly charged and hydrophilic peptides lose much of their membrane-disrupting effect at high ionic strength (e.g. at physiological conditions). By increasing AMP hydrophobicity, such inactivation can be avoided (1-3,17). For very hydrophobic peptides, however, binding occurs irrespectively of membrane composition. This, in turn, results in lysis of both bacteria and human cells. For example, hydrophobic K/L peptides are even more hemolytic than the bee venom melittin (18). Thus, AMP hydrophobicity can only be moderately increased to boost peptide potency without losing selectivity and inducing toxicity.

End-tagging with hydrophobic amino-acids

In analogy to hydrophobic point mutations, hydrophobic end-modifications promote membrane binding and antimicrobial activity of AMPs, increasing with the length of the hydrophobic moiety (19,20). Due to their long acyl chains, commonly investigated lipopeptides (including therapeutically used ones) are exceedingly efficient in their ability to insert into lipid membranes, essentially independent of the composition of the latter (21). Such lipopeptides therefore display substantial toxicity, which has restricted their use to local applications, and to severe indications for which other antibiotics are ineffective, e.g. multi-resistant *Pseudomonas aeruginosa* infections in cystic fibrosis (19). Alternatively, however, end-tagging with hydrophobic amino-acid stretches (notably W and F) offers an approach to achieve high, but *selective*, AMP activity (22). Demonstrating this, Figure 2 shows antimicrobial and cell toxicity data for the peptide GRR10W5N, displaying potent broad-spectrum antimicrobial effects, also at physiological ionic strength (23,24). At the same time, however, toxicity to human cells is limited. This pronounced selectivity can be understood as follows: For a peptide to be able to

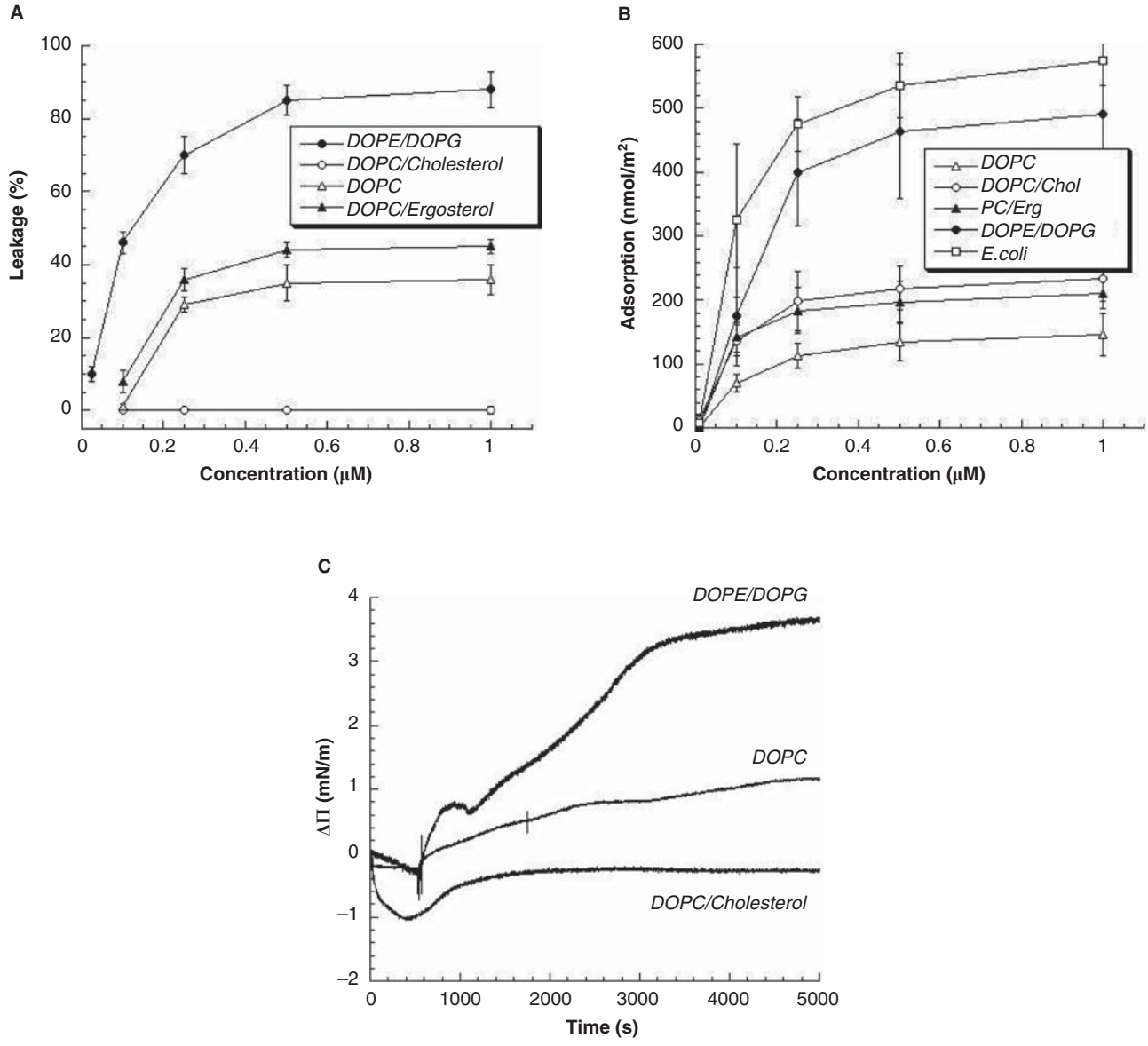

Figure 3. (A) Peptide-induced liposome leakage for GRR10W4N at 10 mM Tris, pH 7.4. Shown in (B) and (C) is the adsorption of the same peptide to supported lipid bilayers, as well as the change in surface pressure (ΔΠ) due to insertion of GRR10W4N to zwitterionic DOPC and DOPC/cholesterol, and anionic DOPE/DOPG monolayers from Tris buffer, pH 7.4 (23).

insert, it must overcome the cohesive energy of the membrane. Particularly for bulky groups such as W and F, which require substantial area expansion, insertion into membranes containing cholesterol (known to condense lipid bilayers (25)) is precluded. This difference in membrane interactions in the presence and absence of cholesterol, and that due to the charge difference between anionic and zwitterionic membranes, contributes to the pronounced selectivity between bacteria and human cells (Figure 3) (23).

For reasons of cost and stability, reducing peptide length is of major importance in the development of AMPs towards novel therapeutics. Here, W/F-tagging offers opportunities for efficient and selective ultrashort AMPs, allowing potency and salt resistance to be maintained down to 4–7 amino-acids in the hydrophilic template peptide (26). Short W-tagged peptides furthermore offer opportunities to combat difficult pathogens. Demonstrating this, the peptide RRPRPRPRPWWWW-NH_2 was found to be effective against a range of 'superbugs', including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, and multi-drug resistant *P. aeruginosa*, yet displaying very limited toxicity (24).

Resistance issues of AMPs

Particularly in early literature, microbes were speculated to be unable to develop resistance towards AMPs. More recently, however, a number of resistance mechanisms have been reported, including upregulation of proteolytic enzymes able to degrade AMPs, membrane modifications resulting in decreased negative potential of bacterial membranes, and release of glucose aminoglycans (GAGs), polysaccharides, and other polyanionic species able to scavenge AMPs (27). For example, *S. aureus* can resist various AMPs by reducing its net membrane charge through introduction of cationic moieties (28). Similarly, *Serratia* sp. and *Morganella* sp. have an outer membrane lacking acidic lipids (29), while *Shigella* sp. releases plasmid DNA, inactivating AMPs by scavenging. In an alternative strategy, *Streptococcus pyogenes*, *Pseudomonas* spp., *S. aureus*, *Staphylococcus epidermidis*, and *Porphyromonas gingivalis* produce AMP-degrading proteases. In addition, *Pseudomonas* spp., *Enterococcus faecalis*, and *S. pyogenes* degrade host macromolecules, including GAGs and collagen, so that that the degradation products can scavenge AMPs (2). More relevant than the occurrence of mutations as such, however, are consequences thereof on bacteria viability and fitness. For example, mutations in the putative transport protein SbmA have been shown to be responsible for the resistance of *Salmonella enterica* against PR-39. Importantly, *sbmA* mutants were found to be as fit as the wild type regarding growth and survivability (30), while investigations with protamine demonstrated the corresponding mutants to display drastically reduced fitness and growth (31). Reduced fitness was observed also for mutants against LL-37, CNY100HL, and wheat germ histones (32). Important from a 'resistance spread' perspective, the PR-39 mutants display similar susceptibility as the wild type to different types of antibiotics and AMPs (30), while protamine mutants were less susceptible than the parental strain to several other antimicrobials, including colistin, gentamacin, lactoferrin, and human defensin HNP-1 (31). Further work on these important aspects is, however, needed. Furthermore, despite having been convincingly demonstrated *in vitro*, resistance development to AMPs *in vivo* needs to be further clarified, since conditions experienced by bacteria in a laboratory setting are likely to differ from those *in vivo*. In the latter case, the microbes are exposed to a cocktail of AMPs, which may reduce or alter the selection pressure underlying resistance development. Furthermore, microbes are not continuously exposed to the same specific stress, as they encounter different AMPs within the host tissue and species. It remains of major importance, however, to clarify whether, and under what circumstances, resistance to antimicrobial peptides can develop to provide cross-resistance to AMPs, and provide AMP-resistant mutants with a competitive advantage also *in vivo*.

Summary

AMPs affect bacteria in numerous ways, but their main mode of action is the disruption of bacterial membranes. Peptide length, charge, secondary structure, and hydrophobicity all influence AMP–membrane interactions. Contrary to hydrophobic point mutations and acyl modifications, end-tagging with W and F stretches allows AMP potency to be boosted without causing unacceptable toxicity effects. Such W/F-tagged peptides are efficient against a wide spectrum of micro-organisms, including various 'superbugs'. Particularly for AMPs not sensitive to infection-related proteolysis, this also opens up applications characterized by high proteolytic activity, such as infected wounds, eye infections, and cystic fibrosis.

Acknowledgements

This work was financed by the Swedish Research Council.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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