Optimisation of antimicrobial peptide design: insights from action mechanisms

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Abstract

Antimicrobial peptides (AMPs) have emerged as one of several viable options for treatment of infections caused by multidrug-resistant microorganisms, which continue to pose the single greatest public health challenge to humankind. Unlike conventional antibiotics, AMPs affects several key extracellular and intracellular targets in bacteria simultaneously, drastically limiting chances of drug-resistance development. Over the years it has been possible to manipulate their chemical structures to design novel anti-infectives for the treatment of systemic and topical infections based on detailed information generated from structural-activity studies. These novel synthetic AMPs exhibit enhanced antimicrobial activity, less cytotoxicity to mammalian cells and enhanced stability to proteases. This review discusses some current developments that have expanded our understanding of their diverse killing mechanisms and shows how this has been employed in the design of AMPs with predictable activity.

Introduction

AMPs are considered as natural antibiotics that play key functions in the innate immune system of all living organisms. Unlike conventional antibiotics that inhibit a limited number of targets in microorganisms, AMPs attack a broad range of extra- and intracellular targets simultaneously, drastically limiting chances of resistance development [1,2]. Furthermore, several AMPs including defensins and cathelicidns, display both immunomodulatory and antimicrobial activities[3,4]. Currently, more than 5,500 natural and synthetic AMPs with antimicrobial, antiviral, antiparasitic and anticancer activities have been deposited in several peptide databases, including http://aps.unmc.edu/AP/main.php/) and Dragon Antimicrobial Peptide Database (DAMPD, http://apps.sanbi.ac.za/dampd). Most of the novel synthetic AMPs deposited in these databases were designed using natural AMPs as templates[5].

Detailed studies have outlined the series of molecular events by which AMPs induce cell death. Common themes identified are binding to receptors on the cell surface, translocation across the cell membrane through pore formation and accumulation of peptide, intracellularly followed by inhibition of critical biosynthetic pathways, protein synthesis and DNA replication. Attempts will be made in the next section to discuss details of these mechanisms in the light of structural-activity relationships and how these have contributed to the design of more potent AMPs.

The molecular dynamics of AMP-bacterial membrane interaction: the role of arginine, lysine and lipid composition

In spite of the large variability in their primary structure, all cationic and anionic AMPs have the ability to associate with anionic bacteria membranes through long-range electrostatic interactions with the lipopolysaccharides or teichoic acids in the outer membrane of Gram-

negative and Gram-positive bacteria, respectively. This interaction is facilitated by weak non-covalent bonds between the cationic or anionic groups and phosphate groups in lipids, nucleic acids and proteins, and hydroxyl or hydrogen of water. For anionic AMPs such as dermicidin and maximinH5 this interaction with the capsular polysaccharides is mediated by Zn (2+) salt brides [6]. The presence of a short site-specific H-bond distance of (~ 4 Å) between the guanidinium-bearing carbon Cξ of Arg and ammonium-bearing carbon Cε of Lys and the phosphate of lipid head groups has been confirmed by solid state NMR, indicating the critical role of cationic amino acids in the antimicrobial action of AMPs [7]. The function of the H-bond however, is not limited to the initial electrostatic attraction. Its formation enables the peptide bond and charged residues to overcome the Born repulsion and facilitates their insertion into the lipid bilayer [8,9]. This explains why abolishment or reduction of the hydrogen-bonding ability in the guanidinium group either by dimethylation or charge reduction drastically reduces antimicrobial activity in some AMPs, such as PG-1[10]. Thus, the antimicrobial action of AMPs is highly correlated with the Arg or Lys content, as confirmed by several studies using histone-derived peptides – buforin II, DesHDAP1, Arg-rich and Lys-rich histones[11-13]. However, significant differences in the stabilities of H-bond of Lys and Arg influence the depth of insertion into the bilipid layer and consequently their contribution to antimicrobial activity [14]. This is because Lys –ammonium is monodentate and adopts a relatively mobile β-turn state in its interaction with the phosphate head groups, resulting in the formation of a weak and transient H-bond. In contrast, Arg-guanidinium is bidentate and adopts a rigid β-strand conformation which stabilises the H-bond and favours a deeper insertion into bacterial cell membrane. The difference in stability of H-bonds explains the several observations of changes in antimicrobial activity in Lys- for- Arg substitution[15,16]. It must be stated emphatically that depth of insertion into membranes is multifaceted and not dependent only on electrostatic interaction between Arg and Lys residues and LPS of Gram-negative bacteria or teichoic acid of Gram-positive bacteria, because other amino acids such as Pro have been found to modulate depth of insertion [17]. It has been demonstrated that differences in the molecular densities and constitution of the inner and outer leaflet of the membrane expressed as spontaneous lipid curvature (SC) drastically influence the depth and orientation of insertion (SC)[18, 19]. The SC influences the folding and insertion of α- helical and β-barrel AMPs into the membranes. For example, α- helical AMPs adopt a parallel orientation in membranes with negative SC such as 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), but assume a tilted orientation or become completely buried in positive SC like dimyristoyl phosphatidylcholine (DMPC). For beta-barrel AMPs, a positive SC greatly facilitates membrane insertion, but insertion is severely limited in negative SC.

These results highlight the interdependence of AMP insertion into membranes on peptide backbone rigidity or flexibility, amphipathicity and lipid composition of the bacterial membrane. It is not possible to manipulate one factor without affecting the others because they are not mutually exclusive. This explains why the role of primary structure in antimicrobial activity must be considered on a one-on-one basis rather than a generalisation.

AMPs that enter cells via membrane-bound surface receptors

The antimicrobial activity of several AMPs appears to be dependent on interaction with membrane receptors as illustrated in Figure 1[20]. These include lantibiotics such as copsin from *Coprinopsis cinerea*[21,22], the fungal defensin, eurocin from *Eurotium amstelodami* [23], human AMPs such as HNP-1 and hBD-3[24] and teixobactin from *Eleftheria terrae* [25], which bind to lipid II on the bacterial surface. Lipid is required in shuttling of building blocks for peptidoglycan synthesis, and disruption of its activity severely impairs cell wall synthesis. Overall, it appears that blocking of bacterial cell wall synthesis via lipid II binding is a

common but very potent antimicrobial strategy among all eukaryotes and prokaryotes, possibly due to its highly conserved domain[25], and this represents an attractive target for drug development.

Recently, several receptors other than lipid II have been described in bacteria. These include UppP (undecapreny pyrophophosphate phosphatase), the enzyme involved in the biosynthesis of lipopolysaccharide and peptidoglycan, which acts as a receptor for lactococcin G and enterocin 1071 (two-chain bacteriocins)[26]. Garvicin ML (GarML) and the leaderless class II bacteriocin LsbB, produced by *Lactococcus lactis* subsp. lactis BGMN1-5,interact with receptors such as maltose ABC transporter and the *yvjB* gene, respectively [27,28]. The maltose ABC transporter plays a critical role in starch and maltose utilisation in the bacterial cell. Mutants that lack the *malEFG* gene cluster for this transporter are resistant to GarML. The mannose phosphotransferase system (man-PTS) has also been identified as a receptor for some class II nonlantibiotics, including pediocin-like bacteriocins, the lactococcal bacteriocins lactococcin A and B and microcin E492 from *Klebsiella*[29]. This system plays a key role in sugar uptake among Firmicutes and Gamaproteobacteria.

Various antifungal defensins are known to act through sphingolipid and phospholipid receptors in the plasma membrane and cell wall of yeast and fungal cells. The major receptors that mediate antifungal activity are the sphingolipids receptor glucosylceramide (GlcCer), and the phospholipids receptors inositolphosphorylceramide (IPC), mannosyl-IPC (MIPC) and mannosyldi-IPC (M(IP)2C). It has been demonstrated by Aerts *et al.*[30] that the plant defensin RsAFP2 from *Raphanus sativus* binds to GlcCer and induces apoptosis and activation of caspases, leading to the accumulation of ROS-susceptible fungal and yeast cells such as *Candida albicans*. The absence of the glucosylceramide synthase gene (GCS) or GlcCer receptors with modified ceramide moiety confers resistance to the antifungal. Similarly Dm AMP1 from *Dahlia merckii* has been found to exhibit its fungicidal activity in *S. cerevisiae* using M(IP)2C as a receptor. This is premised on the observation that *S. cerevisiae* strains with a non-functional IPT1 allele, the gene that codes for M(IP)2C receptors, and thus lacking M(IP)2C in their membranes, were found to be highly resistant towards DmAMP1 as compared to the wild type (WT)[31]. Some antifungal proteins such as *Penicillium chrysogenum* antifungal protein (PAF) have been reported to act through a G-protein-coupled signal transduction pathway, triggering cell death by apoptosis or inducing imbalance in Ca(+2) homeostasis in filamentous fungi, including *Aspergillus nidulans* and *Aspergillus fumigatus* [32].

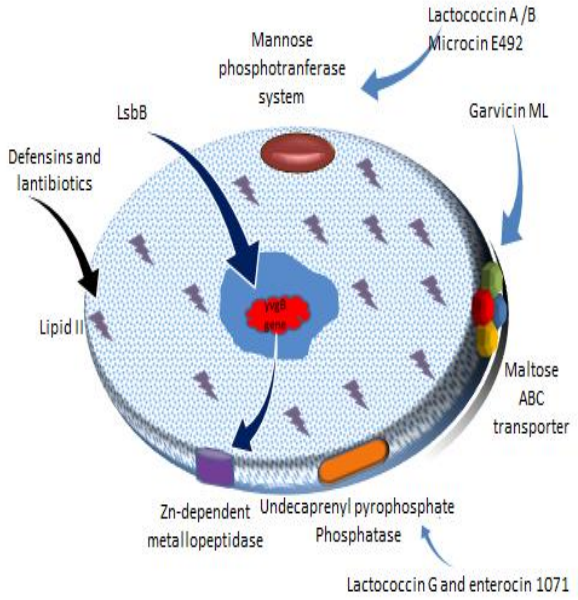

Figure 1: Overview of receptors in bacterial cells and the AMPs that bind to them. These receptors include membrane-bound receptors like Lipid II, undecaprenyl pyrophosphate phosphatase (UppP), the maltose ABC transporter and the mannose phosphotransferase system. The maltose ABC transporter is a tetramer of MalE, MalF, MalG and MalK, each of which is represented by a colour. The *yvjB* gene serves as a receptor for the class II leaderless bacterion LsbB, produced by *Lactococcus lactis* subsp. lactis BGMN1-5 and

codes for the Zn-dependent metallopeptidase enzyme is shown in red within the bacterial cell.

Molecular insight into membrane translocation models and membrane disruption mechanisms

The membrane-bound proteins in bacterial cells form a dynamic complex with the lipids and polysaccharides that are involved in key processes such as cell-cell communication or signalling, membrane transport and cellular energetics. Disruption of these critical functions was considered as the main mechanism of action of AMPs. Several studies on the interaction of AMPs with bacterial cells indicate leakage of cell content and delocalisation of peripheral membrane proteins in proportion to the ratio of AMP concentration to phospholipid content[33-35]. Given the wide variation in membrane ultrastructure, it is not difficult to predict that a given AMP may act by different mechanisms in different membrane environments due to the formation of a repertoire of varied peptide-peptide and peptide-lipid complexes. The formation of these complex multimeric structures upon interaction and insertion into the membrane results in the formation of transmembrane channels or pores, which have long been associated with the antimicrobial activity of AMPs[6,10].

Some AMPs such as alamethicin are able to insert perpendicularly into the cell membrane and disperse the lipids to form pores stabilised by the wall formed by the peptides[36]. This barrel-stave model of peptide translocation has the hydrophobic domain of the peptide interacting with the fatty acyl group of the membrane lipids, whilst the hydrophilic groups line the pore. Pore diameter widens as peptide concentration increases, resulting in rapid leakage of cellular content and eventually cell death[37]. Alternatively, other AMPs orient parallel to the surface and induce weak transient toroidal or worm-hole pores in the membrane, as a result of displacement of phospholipid head groups. The displacement of lipids strains and thins the membrane, and closure of the pores allows the peptide to translocate the membrane. This has been characterised for α-helical peptides such as megainins, protegrins and melittin[6,36]. However, at very low peptide concentrations, a spontaneous disordered pore is observed in the interaction of these AMPs with membranes, but with only one or two peptides lining the pore[38]. Alternatively, the AMPs can attach to various locations on the surface of the cell membrane, induce a localised displacement of lipids to form numerous small pores and disintegrate the membrane by micellisation or in a detergent-like manner at a threshold concentration. Cecropins derived from haemolymph of *Hyalophora cecropia* destroy *E.coli* membranes using this carpet model of membrane lysis[39]. Doherty *et al*. [40] describe an in-plane diffusion or partial insertion model for short peptides like tachyplesin-1 that are unable to induce transmembrane pores. Their fast diffusion across the membrane forms transient pores that compromise membrane integrity. Epand *et al*. [41] describe an induction of anionic lipid clustering away from zwitterionic ones in Gram-negative bacteria as a possible mechanism of membrane perturbation. This clustering was attributed to their higher charge density, since this is absent in peptides with lower charge densities such as magainin. Mattila *et al*. [42] described a novel mechanism of membrane targeting in which temporin B and L, indolicidin bind to oxidised phospholipids through the formation of Schiff base between the amino group of peptides and the functional group of oxidised lipid aldehydes. Indolicidin is also able to compromise the osmotic balance of cells by forming complexes with anionic organic compounds, and it translocates them across the cell membrane without forming pores[43]. Other peptides such as NK-lysin have been found to induce permeability by electroporation[44]. Overall, the insertion of AMPs into membranes induces collapse of the cell wall or cell shape, resulting in decreased adhesiveness, leakage of water and intracellular materials causing loss of turgidity, impairment of osmoregulation and reorientation in time and

space of membrane-bound protein complexes that are crucial in respiration and cell-wall biosynthesis[45-47].

Although membrane perturbation does not always lead to cell death it remains a very simple but complex and unspecific mechanism with a debilitating effect on cell viability [48].

Inhibition of cell wall biosynthesis and other extracellular processes

As stated previously, some AMPs such as Bac7, thrombin-induced platelet microbicidal protein 1 (tPMP-1), gramicidin D, and protamine are microbicidal without membrane perturbations, indicating the existence of other molecular mechanisms and targets[49]. These molecular targets, as revealed by several transcriptomic studies, are numerous, form a continuum and principally involve the inhibition of various components of cell wall synthesis, such as lipid II and UppP or the uncontrolled release of cell wall-degrading enzymes, including autolysins or phospholipases by pep5, and some defensins[10, 24] .

Genome-wide transcriptional analysis of molecular events triggered by the insertion of AMPs into the membrane reveals a complicated cellular response pattern aimed at counteracting the myriad of defects caused by the AMP, mainly by reinforcing cell wall synthesis. The presence of an AMP in the membrane triggers an entire set of genes or regulons involved in aerobic energy production, cell wall biosynthesis and lipid metabolism extra-cytoplasmic-function sigma factors, post-translational modification, protein turnover and chaperones[50-52]. Examples of these genes include *msrA,* which encodes methionine sulfoxide reductase, an enzyme that protects against oxidative damage and the *recU* gene, which is involved in DNA replication, recombination and repair . There is a general up-regulation of the stress molecular chaperones/ proteases HtrA and Hsp33 and genes related to carbohydrate metabolism and energy production, such as NADH dehydrogenase I (nuoA), succinate dehydrogenase (*sdhD*), fumarate reductase (*frdC*), and nitrate reductase (*nirD*) genes.

Inhibition of intracellular targets

Most AMPs rich in cationic amino acids or bearing a sequence similarity to histones, including buforin II, are able to accumulate rapidly within cells without significant membrane perturbation and bind to DNA and RNA[53]. Similar results have been reported for tryptophan-proline-rich AMP indolicidin[54] and the proline-rich AMPs (Pr-AMP) like apaedicins and oncocins that bind to the 70S of ribosomes, as well as molecular chaperons inhibiting protein biosynthesis and proper folding of nascent proteins[55] . Kriszan *et al*.[56] have shown that capistruin inhibits the growth of *Burkholderia* and closely related *Pseudomonas* strains, by interaction with DNA-dependent RNA polymerase, in just the same way as its structural analogue micrococin (MccJ25) produced by *E. coli*. The molecular basis of preference for nucleic acids appears to be related to the cationic and hydrophobic residues, because apaedicins and oncocins bind to DNA and RNA via the active site residues (Arg17 and Leu18) and (Lys3, Tyr6, Leu7, and Arg11), respectively.

Rational AMP design

The overall objective of rational design of AMPs is not limited to enhancing activity but also encompasses minimising production costs by limiting the length/structure to the smallest amino acid domain that is responsible for the antimicrobial activity. Again, the modified peptide must be able to retain its original activity or have a narrower spectrum of activity against a select microbe under physiological saline conditions and in complex biological fluids, whilst resisting degradation by host enzymes [57].

It is clear from the various studies that a given AMP employs a myriad of different mechanisms in a panel of microbes, whilst a given microbe displays different levels of sensitivity to different classes of AMP. Details of the molecular basis for these differences have not been explained from

the huge data collected so far, making the design and prediction of synthetic AMPs a complicated task. However, results from several structure-activity studies in different membrane environments have identified the size, the sequence, the degree of structuring (for example, helical content), the net charge, the overall hydrophobicity, the amphipathicity and the respective widths of the hydrophobic and hydrophilic faces of the helix as key determinants of antimicrobial activity[20]. Although these parameters are interrelated, the general strategy of increasing the cationicity and keeping moderate hydrophobicity of the template AMP has been found to be very effective in improving antimicrobial or antitumor activity, whilst supressing haemolytic activity [58]. This is probably because the active regions of AMPs are generally amphipathic[59]. Yang et al.[58] reported enhanced anticancer activity in six analogues of temporin-1CEa, a naturally occurring α- helical and amphipathic AMP derived from skin secretions of the Chinese brown frog *Rana chensinensis,* by either increasing cationicity or increasing/decreasing hydrophobicity. This is generally done by replacing neutral and acidic amino acids with lysine or leucine residues on the polar face and non-polar face of the α-helix. This strategy has recently been employed to improve the antimicrobial and anticancer activity of antimicrobial peptides present in the venom of scorpions [60,61].

Several AMP modification techniques involving changing the cationic and hydrophilic character have been described and reviewed. The most common involve designing molecules imitating AMP structure and function, such as AMP mimetics and hybrid AMPs. Mimetics are synthetic, non-peptidic molecules constructed with peptoids, β-peptides, arylamides, oligomers or phenylene ethynylenes that mimic the properties and activities of naturally occurring AMPs. Examples include the anti-Mycobacterium tetrameric, alkylated, cationic peptoid (1-C134mer) and antimicrobial and antitumour mimetics based on enhanced cationicity such as Oligoacyl- lysine (OAK)[62,63].

The hybrids differ from the mimetics in that they are conjugates of the active regions of two to three naturally occurring peptides. Common examples include the family of cecropin A–melittin (CEME) and CEMA family of peptides. CEME is composed of amino acid residues 1–8 of cecropin A and amino acid residues 1–18 of melittin, whilst CEMA is a congener of CEME with a modified and enhanced cationic C-terminus. The formation of hybrids with extra cationicity improves their ability to bind lipopolysaccharide (LPS) and lipoteichoic acid (LTA) and increase membrane permeabilising ability whilst reducing haemolytic activity[64]. Some recent techniques include the incorporation of transition metal complexes with protein binding motifs to form metallo drugs, as demonstrated by Libardo *et al*.[65].

Conclusion

Modification of natural AMP templates to produce novel anti-infective drugs holds great potential in the fight against multi-drug resistant microorganisms. The singular advantage of reducing the emergence of drug-resistant strains still remains a major motivation in this pursuit. Although many templates with enhanced biological activity have been designed using various concepts, the challenge of treatment failures still remains a threat to public health. It is very possible to overcome this challenge, but more information from structural-activity studies is needed to guide drug design concepts. For now, the preferred strategy in all these developments remains modifying the amphipathicity by reducing minimising hydrophobicity and increasing the cationicity to improve membrane disruption, or attaching AMPs to transition metals to form metallodrugs.

Acknowledgements

This work was supported by CAPES, CNPq, FUNDECT and FAPDF and the University of Cape Coast, Ghana.

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