Cell. Mol. Life Sci. 65 (2008) 2450 – 2460
1420-682X/08/162450-11
DOI 10.1007/s00018-008-8188-x

Cellular and Molecular Life Sciences

Visions & Reflections (Minireview)

Antimicrobial peptides: natural templates for synthetic membrane-active compounds

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Received 08 April 2008; received after revision 20 June 2008; accepted 25 June 2008
Online First 26 July 2008

Abstract. The innate immunity of multicellular organisms relies in large part on the action of antimicrobial peptides (AMPs) to resist microbial invasion. Crafted by evolution into an extremely diversified array of sequences and folds, AMPs do share a common amphiphilic 3-D arrangement. This feature is directly linked with a common mechanism of action that predominantly (although not exclusively) develops upon interaction of peptides with cell membranes of target cells. This minireview reports on current understanding of the modes of interaction of AMPs with biological and model membranes, especially focusing on recent insights into the folding and oligomerization requirements of peptides to bind and insert into lipid membranes and exert their antibiotic effects. Given the potential of AMPs to be developed into a new class of anti-infective agents, emphasis is placed on how the information on peptide-membrane interactions could direct the design and selection of improved biomimetic synthetic peptides with antibiotic properties.

Keywords. Antimicrobial peptides, lipid membranes, folding, oligomerization, synthetic analogues, peptidomimetics.

Antimicrobial peptides everywhere!

Pathogenic microbes pervade the biosphere, deploying a potentially lethal threat to any life form. It is therefore no surprise that virtually all multicellular organisms have evolved some type of defense system, either based on molecular and/or cellular components. The innate immune system provides such protection. A broad-ranging but otherwise non-specific shield, this ancient host defense mechanism ultimately proved well suited for its task, since it has only been recently (in evolutionary terms) that vertebrates flanked (but did not substitute) it with the more sophisticated adaptive immunity, endowed with antigenic specificity and immunologic memory. Following immune recognition, inborn immune responses commonly include the release of antimicrobial peptides (AMPs), a vast group of molecules variously active against bacteria, enveloped viruses, protozoa and fungi. Gene-encoded and ribosome-synthesized, AMPs do share some common features, such as usually being cationic and amphipathic, but are otherwise highly diversified from the structural point

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of view. Following their initial discovery in insects and amphibians, hundreds of AMPs have subsequently been identified and isolated from both prokaryotes and eukaryotes, either invertebrates and vertebrates, including humans [1–4], and dedicated web based repositories such as AMSDb (http://www.bbcm.univ.-trieste.it/~tossi/pag1.htm) and ANTIMIC (http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC/), strive to keep up the pace of discovery of new peptides from many natural sources [5, 6].

With microbial resistance to conventional antibiotics rising at an alarming rate, and with the not-so-remote possibility of remaining practically unarmed against a growing number of noxious infectious agents, AMPs have received an ever-expanding dose of attention in the last two decades or so as potential constituents of a novel class of anti-infective therapeutic agents [7]. The main fact supporting this interest stems from the appreciation of AMPs as phylogenetically successful effectors of innate immunity, crafted by evolution to withstand multiple challenges posed by continuous changes in the target microbial biota. Trying to tap into this potential, researchers from a wide range of disciplines that include microbiology, organic chemistry, biochemistry, and biophysics are teaming up to disclose the details of AMPs' mechanism(s) of action and to turn them into much-needed antimicrobial drugs. AMPs are generally considered to kill their microbial targets through insertion and damage/permeabilization of the cytoplasmic membranes of target cells [8]. However, recent observations suggest that a number of defense peptides may also interact with intracellular targets such as DNA and RNA, presumably interfering with their metabolic functions and thus leading to cell death [9, 10]. They can alter cytoplasmic membrane septum formation (e.g., PR-39, indolicidin, and microcin 25); inhibit cell wall synthesis (e.g., mersacidin); inhibit nucleic acid and protein synthesis (e.g., pleurocidin, dermaseptin, PR-39, HNP-1, HNP-2, and indolicidin); or inhibit enzymatic activity (e.g., histatins, pyrrhocoricin, drosocin, and apidaecin) [9]. Additionally, as experimental evidence has accumulated it has also became evident that some AMPs not only act directly as microbe killers, but may also play a distinct role as signalling molecules at the so far poorly defined interface between innate and adaptive immunity. In mammals, indeed, peptides such as defensins and cathelicidins, among others, have been shown to possess different immunomodulatory and immunostimulatory functions aimed to reinforce secondary host protection from infections, including chemotactic activities on dendritic and T-cells and induction of proinflammatory cytokines or chemokines [11, 12]. To account for this 'extension of duties', the term 'host defense peptides' has been increasingly used by many authors.

The aim of this minireview is focused on and limited to a summary of recent advances in understanding the modes of interaction of AMPs with biological and model membranes. In particular, we intend to highlight the results obtained with molecular dynamics (MD) and computational methods (together with other approaches), showing the structural (folding and/or oligomerization) requirements for a peptide to interact in a dynamic way with cell-membranes and eventually exert membrane perturbation-related antibiotic effects on target microbial cells. The information provided by these observations is indeed of significant importance in the selection and design of biomimetic synthetic peptides with antibiotic properties as drug candidates, a viable – and perhaps preferable – alternative to the use of naturally-occurring molecules and a research avenue which we will also discuss in some detail. Many other general features of AMPs and specific groups of them are regularly reviewed, and the interested reader is directed to the current literature. Although essentially focused on AMPs, our discussion will also hint at other examples of naturally-occurring antimicrobial agents that do not properly belong to this class, such as the non-ribosomally biosynthesized lipopeptides.

Membranes as the primary target

An impressive amount of experimental data has accumulated in the last decade or so unequivocally indicating that AMPs act predominantly by disrupting the integrity of cell membranes through interaction with the phospholipid component [8, 13–16]. It is generally agreed that AMPs are essentially unstructured in the aqueous phase and fold upon contact with the membrane, adopting an amphiphilic fold. This favours absorption of peptides onto the lipid bilayer and their subsequent integration into the membrane with expansion of the outer leaflet, which in turn leads to membrane thinning. The latter effect is not uniformly distributed over the entire bilayer area, but rather is concentrated in distinct domains [e.g., 17]. Over a certain concentration threshold, peptides perturb membranes by forming transient pores via one of the various models proposed to account for this step, i.e. barrel-stave, carpet-like, toroidal (or 'wormhole') pore formation, detergent-type micellization, and induction of non-lamellar phases, leading to membrane permeabilization and either leakage of cell content and osmotic instability, and/or peptide diffusion to intracellular targets (Fig. 1). Looking for a unifying model that could help to rationalize, at the

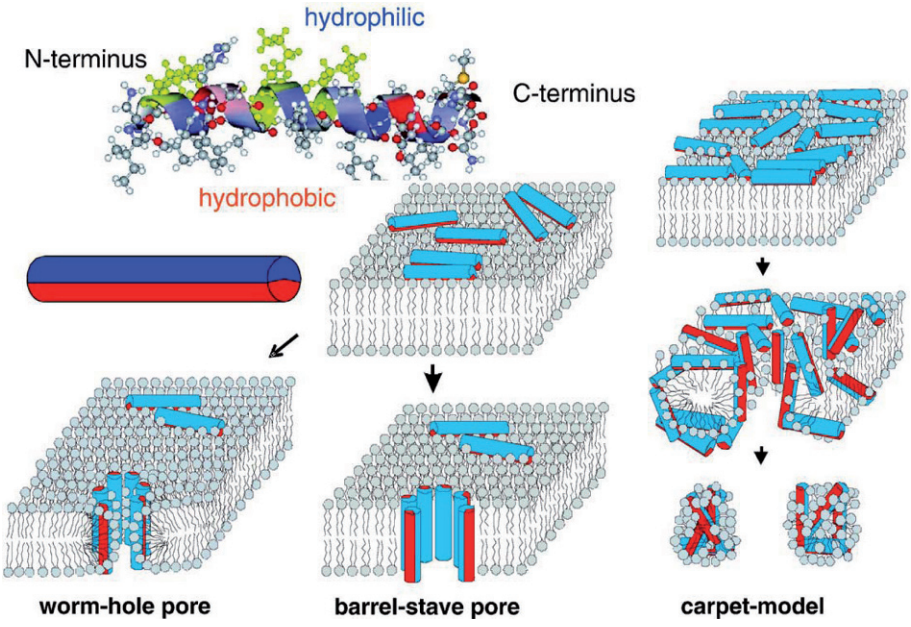

Figure 1. Sketch of different models describing the functional mechanisms of linear antimicrobial peptides interacting with lipid bilayers. (Top, left) Many AMPs have the potential to fold into amphipathic α-helices with hydrophilic and hydrophobic sides. This conformation is here schematically represented as an amphiphilic cylinder, with hydrophobic (red) and hydrophilic (blue) halves. (Center) Antimicrobial peptides bind to the membrane surface with the hydrophobic side groups anchored in the hydrophobic lipidic core of the bilayer, leading to different outcomes. (Bottom, left) The wormhole (or toroidal) pore model as proposed for magainin (Bottom, center) Barrel-stave pore model, as proposed for alamethicin, a peptide antibiotic produced by the soil fungus *Trichoderma viridis*. (Bottom, right) Carpet model: antimicrobial peptides crowd on the membrane's surface and lead subsequently to micellation. From ref. [16], reproduced with permission. See main text for further details.

molecular level, the membrane activity of most AMPs, Michael Zasloff of Georgetown University (Washington, D.C., USA) has proposed the so-called Shai-Matsuzaki-Huang (SMH) model, which envisages the entrance of the peptide inside the microbial cell and can also accommodate the action of short peptides that cannot span the membrane and thus presumably cannot form a classic multimeric transmembrane pore [2]. Briefly, the SMH model proposes that a peptide would initially carpet the outer membrane leaflet, integrating into the membrane and causing the thinning of the outer leaflet itself. Phase transition would occur at this stage, with the formation of transient 'wormhole' pores. Transport of lipids and peptides into the inner leaflet would then take place, with the eventual diffusion of peptides onto intracellular targets. At this stage, the membrane would collapse into fragments [2].

If this coarse-grain picture is accepted by most authors because it explains, in an intuitive manner, the membrane activity of AMPs, the fine details of peptide-lipid interactions are much less understood, and a matter of much investigation and debate. The main fact behind this is that AMPs are a highly diversified ensemble, and even those belonging to the same structural class may interact with membranes following different mechanisms. A clear example of this has recently been provided by a set of detailed studies conducted by the group of Paulo Almeida at the University of North Carolina at Wilmington (NC, USA). Picking cecropin A, a 37-residue α-helical peptide isolated from the insect *Hyalophora cecropia* and analysing the kinetics of dye release from large unilamellar lipid vesicles as a function of bound peptide concentration and vesicle lipid composition, authors were able to depict a clear and exact model that accounts for the all-or-none release of vesicle content that this peptide causes. In this model, cecropin A binds reversibly to vesicles, causing membrane thinning and a positive curvature strain due to accumulation of the peptide on the outer membrane leaflet. At a certain point, the membrane enters an unstable state and breaks into a pore that relieves the strain and causes a complete leakage of content, precisely what is expected from an all-or-none mechanism [18]. Intriguingly, these authors used the same experimental approach to show that the 26-residue bacterial peptide δ-lysine and the cell-penetrating peptide transportan 10, both of which adopt an amphipathic α-helical structure when bound to membranes, cause a graded release of lipid vesicle content, and thus behave very differently from cecropin A [19, 20].

A number of other experimental settings are routinely used by many research groups in their efforts to outline the mechanism of action of different AMPs. Lipid monolayers spread at an air/water interface, for example, provide a simple, sensitive model for mimicking biological membranes and to assess membrane insertion of peptides [21, 22]. This technique has recently been used to explore the mechanism of temporins, an interesting class of short (10–14 residues) peptides originally isolated in the skin extracts of the European red frog *Rana temporaria* and later found in many ranid amphibians and also in wasp venom [23, 24]. Since the combination of membrane and peptide physico-chemical characteristics lay at the basis of target-cell specificity of AMPs, the importance of understanding these parameters as key to the development of efficient peptide-based drugs for therapeutic applications may not underestimated.

To fold, not to fold, when to fold

One of the main tenets concerning the mechanism of action of AMPs is that they become structured upon interaction with cytoplasmic membranes and/or with components of outer bacterial membranes [25], a process sometimes referred to as interfacial folding and better rationalized in the case of α-helical peptides [26]. The human cathelicidin LL-37, for example, is converted from a disordered structure in water to an α-helix conformation in the presence of solvents, and the extent of α-helicity is positively linked to the antibacterial activity against Gram-positive and Gram-negative bacteria [27]. In the case of the bovine AMP indolicidin, a 13-amino acids peptide unusually reach in tryptophan and proline, circular dichroism (CD) measurements revealed unordered conformations in aqueous and bulk organic solutions, and a somewhat more ordered, but not α-helical conformation in SDS micelles and lipid bilayers [28]. Subsequent NMR determination of indolicidin structure when bound to dodecylphosphocholine (DPC) and SDS indicated that the peptide can fold into a unique membrane-associated, amphipathic structure [29]. In another study using a set of prevalently hydrophobic model peptides, molecular dynamics simulations have shown that the peptides first become localized at the membrane/solvent interface. There, they eventually form a significant helical structure via a helix-turn-helix motif that inserts the central hydrophobic residues into the membrane interior, after which a stable helical structure is formed throughout the peptide, which then moves across the membrane starting from its N-terminus [30].

Clearly, the fact that the local environment at the bacterial outer surface and cell membrane may induce AMPs to acquire an amphipathic conformation that will, in turn, assist peptide attachment and insertion into the membrane, fits very well with the general view of AMPs' mechanism of action, at least for that part that has membranes as central stage. However, recent observation suggests that things might be more complicated than previously believed. Indeed, MD simulations – coupled to CD spectroscopy, studies with model membranes, and antimicrobial assays – have shown that, at least for some peptides, a significant correlation exists between the conformation adopted by the peptide in solution, i.e. *before* the interaction with membranes, and its antimicrobial activity. In one of these studies, two well-characterized members of the temporin family, namely temporin A (FLPLIGRVLSGIL-NH_2) and temporin L (FVQWFSKFLGRIL-NH_2) were selected to test the validity of this hypothesis [31]. Temporin A is preferentially active against Gram-positive bacterial strains, has a moderate haemolytic activity and efficiently kills the human parasitic protozoan *Leishmania*; temporin L, on the other hand, displays the highest antimicrobial potency among tested temporins and strong affinity for lipid membranes and also for the LPS component of the Gram-negative outer membrane. CD spectra determined that temporin L has a higher propensity to acquire a stable α-helix conformation in solution with respect to temporin A [31]. Confirming and extending this experimental finding, long time-scale MD simulations aimed to evaluate the free energy conformational landscape of both peptides and their folding propensity in water solution (in the absence of typical helical structure stabilizers such as trifluoroethanol) revealed interesting differences for the two temporins. In particular, α-helix formation free energy for temporin L was found to be constantly lower than that of temporin A, indicating that the former possesses a higher, intrinsic propensity (negative free energy of folding) to form an α-helix in water. Although these peptides do not show any well defined α-helix conformational state in water (i.e., formed by at least six residues organized in a helix structure), both temporins appear not to be in a completely random coil conformation and, in particular, temporin L seems to exist basically in a ''semi-folded'' (low-entropy) state characterized by a 4(5)-residue helix [31]. Thus "(it) is conceivable that the greater antimicrobial and haemolytic activity exhibited by temporin L could be ascribed to its higher propensity to assume a folded conformation. In other words, the presence of a partially folded structure in aqueous solution may plausibly facilitate, both thermodynamically and kinetically, the peptide folding in

the microbial membrane." [31]. Overall, this example shows that looking for built-in conformational characteristics could well help to rationalize – not only in the case of temporins but most likely also for other cationic α-helical peptides – the different spectrum and level of activity recorded on membrane-enveloped targets.

More recently, to further test the hypothesis that a positive correlation between folding propensity of peptides in dilute solution and their biological/pharmacological activities is needed, we have focused our attention on a novel 13-amino-acids peptide, Vitr-p-13 (YPIVGQELLGAIK-NH_2), derived from the position 95–107 of the dimeric haemoglobin of the bacterium *Vitreoscilla* (VHb) [32]. The physiological role of VHb is not known precisely, but it is generally thought that the protein may act as a scavenger of oxygen or NO radical species, or as an oxygen-delivering protein (myoglobin-like) that favours oxygen diffusion towards terminal oxidases. VHb is known to reversibly bind cyclopropanated fatty acids and phospholipids within the active site and also to interact readily with both lipid monolayers and bilayers [33]. Vitr-p-13, when tested in artificial membranes, was unable either to insert into phospholipids monolayers or to induce calcein leakage from calcein-loaded liposomes, and was also found devoid of significant antimicrobial activity. Confirming CD experiments, MD simulations indicated that aqueous Vitr-p-13 does not spontaneously adopt an α-helix folding, which is completely lost within a few ns of simulation, but rather it is preferentially found in β-hairpin-like conformations. Our MD long-time-scale simulation has also shown that Vitr-p-13 presents a 'topological-trigger', characterized by residues 7 to 10, exactly as in temporins, which initiates α-helix folding. The difference with temporins is that, in the case of Vitr-p-13, such a process in water at 300°K is energetically very demanding (+10 kJoule/mole) [32]. Results obtained with Vitr-p-13 thus served as a negative control, reinforcing the idea that interaction with lipid membranes is somehow precluded to those peptides unable to adopt a partially-folded structure when in aqueous solutions (from a different perspective, they also indicate that this region of VHb does not mediate protein-membrane interactions).

Other theories exist that challenge more radically the dominant conceptual model for membrane insertion of helical peptides, which, as mentioned, postulates complete peptide folding within the interfacial zone followed by insertion into the lipid bilayer core. In a thought-provoking study, Angel Garcia and colleagues performed all-atom MD simulations of the interactions of a synthetic 16-amino acids WALP peptide with a solvated dipalmitoylphosphatidylcholine (DPPC) bilayer, showing that an alternative route for peptide insertion might be viable [34]. Indeed, the simulation results suggested that the model peptide would first spontaneously insert deep into the bilayer, and that folding to a transmembrane α-helix would take place only *inside* the membrane interior. "The composition of the peptide and lipid are certain to modulate the large enthalpic and entropic terms driving insertion, making insertion mechanisms more variable than have been suggested," wrote Garcia and colleagues. "In this regard, we suggest that membrane proteins, like globular proteins, may have multiple folding routes best described as motion on a multidimensional free energy surface." [34].

Peptide oligomerization may also play a non-marginal role in modulating peptide-membrane interactions. Distinctin, an AMP purified from skin extracts of the southern-American frog *Phyllomedusa distincta*, presents an uncommon structure consisting of two different polypeptide chains linked by a disulfide bond [35]. In water, the peptide undergoes non-covalent homodimerization, giving rise to a symmetrical full-parallel four-helix bundle, with a well-secluded hydrophobic core and a rather uniform surface distribution of basic residues. Distinctin dimers were found to permeabilize planar lipid bilayers and, although no structural details in a membrane-like environment were provided, it is likely that the peptide would require some grade of molecular rearrangement to penetrate the lipid core. What is probably most interesting is the observation that the distinctin homodimer is more resistant to protease degradation with respect to melittin, magainin II and the distinctin monomer, indicating that dimerization (or oligomerization) can significantly stabilize peptide structure and should thus be taken into account as a potentially important feature for the design of enhanced synthetic analogues of AMPs [35] (see also next chapter). If preassembly is possibly an important factor driving membrane activity of peptides, aggregation of AMPs *into* the membrane is also a crucial step in their functioning. In many cases, however, determination of the oligomerization state of membrane-bound peptides is technically difficult to assess. By choosing the β-hairpin peptide protegrin-1 – an 18-amino acids disulfide bridged cationic AMP from porcine leucocytes – in palmitoyloleoylphosphatidylcholine (POPC) membranes as a model system, recent work by Mei Hong and colleagues showed that ^{19}F spin diffusion magic-angle-spinning NMR might be suitable to determine the modes of association of peptides directly in the lipid bilayer [36]. Together with other solid NMR approaches this technique may thus help in better defining the membrane permeabilization process which, especially in the case of short peptides – unable

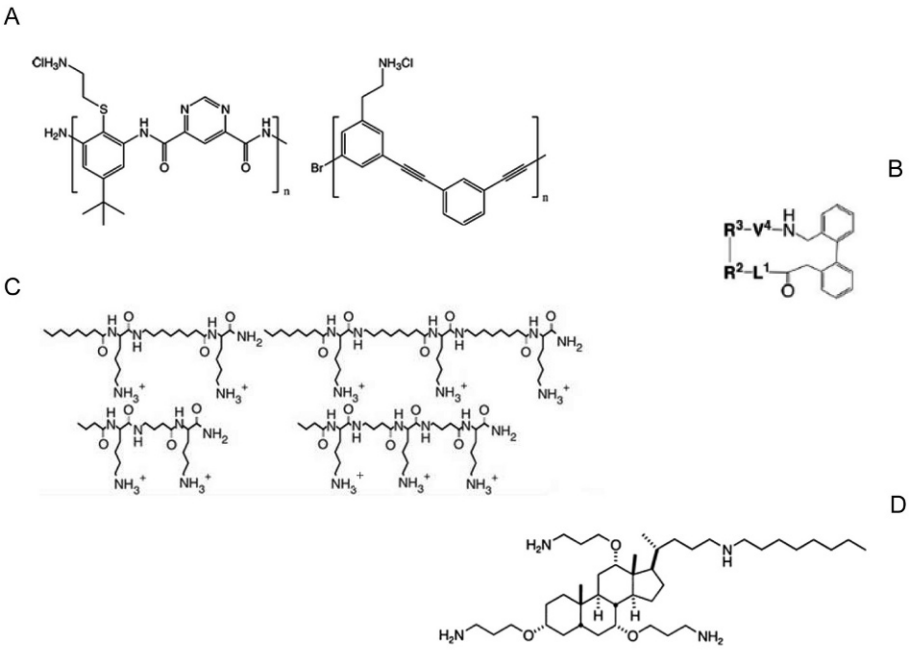

Figure 2. Chemical structures of some peptidomimetics described in the text: (*A*) facially amphiphilic structures of non-natural arylamide (left) and phenylene ethynylene (right) oligomers, [38]. (*B*) A small macrocyclic peptidomimetic incorporating the *o,o*-biphenyl template [43]. (*C*) A peptide-mimetic compound, called oligo-AKs (OAKs), where A and K stand for, respectively, an acyl (fatty acid) chain and the charged amino acid lysine [44]. (*D*) CSA-13, a cationic steroid antibiotic belonging to the Ceragenin family [53, 54].

to span the bilayer and form a classic barrel-stave pore, as discussed above – is hard to rationalize. Another technique with an elevated potential to address several issues pertaining to the association of AMPs and lipid membranes is interface-sensitive X-ray scattering, as recently reviewed by Tim Salditt and colleagues [16].

Peptidomimetics: better by design

Antimicrobial peptides have all the potential of being a viable alternative to conventional antibiotics. Unfortunately, due to their peptidic nature, they suffer from poor bioavailability and poor proteolytic stability, two features that have severely hampered clinical progress to date. To overcome these disadvantages, several modifications have been introduced to develop synthetic analogues that mimic the properties of AMPs, opening a vast research area specialized in the development of an expanding array of peptide and lipopeptide mimetics. These modifications are mainly modelled after AMPs found in nature and are focused on their main physico-chemical and membrane activity-related properties, including positive charge and amphiphilic nature.

The incorporation of D- and non-natural amino acids, for instance, represents a common approach to creating peptidomimetics endowed with potent biological activity and resistance to proteolysis, and it has been successfully applied in the field of AMPs. P113D, just to pick a fresh example, is an all D-amino acid derivative of a 12-residues cationic peptide based on histatins, naturally occurring AMPs present in the human saliva [37]. P113D was demonstrated to be less amenable to enzymatic degradation and maintained antimicrobial activity of the parent compound against the pathogenic fungus *Candida albicans*; currently, it is on track to initiate a Phase IIb dose-ranging study by Pacgen Pharmaceuticals (Canada, www.pacgenbiopharm.com).

The goal of other synthetic approaches is to maintain the physico-chemical properties of AMPs within a very simple framework of polymers and oligomers. Modifications of a panel of inexpensive non-peptidic oligomers and polymers with two different aromatic backbones resulted in the identification of smaller facially amphiphilic phenylene ethynylene AMP mimics with antimicrobial activity and with molecular weights ranging between 690 and 1,100 Da (Fig. 2, A) [38]. These compounds were designed around the belief that the non-natural backbone without amide or ester functionality would not undergo proteolytic degradation. A unique member of this family showed broad activity against antibiotic resistant bacteria, a significantly decreased induction of resistance development when compared to fluoroquinolone, and a good therapeutic index calculated *in vitro* [39]. In a very recent study, the same authors reported the *in vitro* activity and structure-function relationships of a novel peptide mimetic, meta-phenylene ethynylene (mPE), modelled after the structural characteristics of magainin [40]. mPE exhibited the same ability as magainin to bind both membrane-bound and free

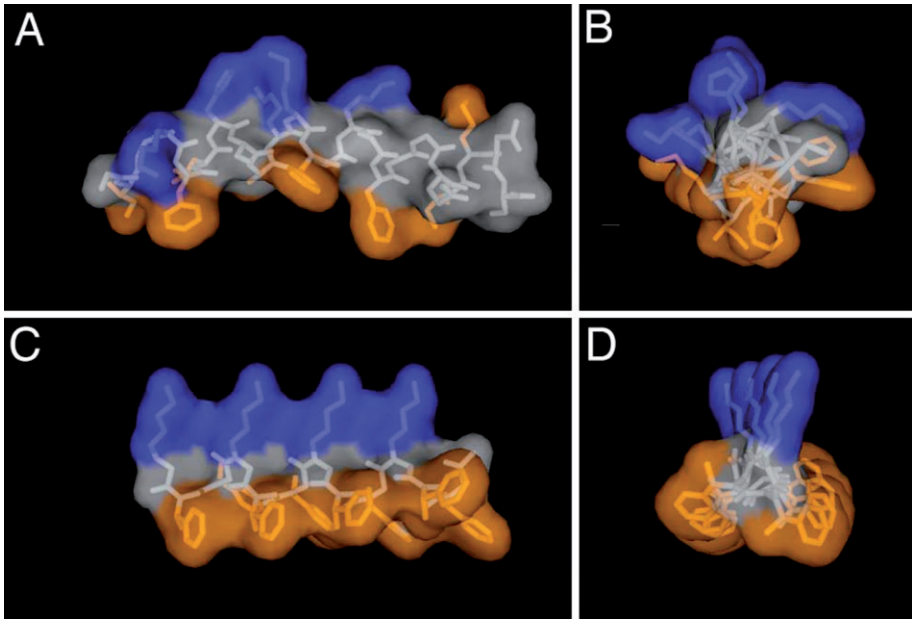

Figure 3. Antimicrobial peptoids ('ampetoids') mimicking the structure of helical AMPs. NMR structure of magainin-2 in dipalmitoylphosphatidylcholine (DPPC) micelles, parallel (*A*) and perpendicular (*B*) to its helical axis. (*C* and *D*) Similar views of a model structure of an ampetoid composed of *N*-(4-aminobutyl) glycine (*N*Lys), a peptoid analog of lysine, and (*S*)-*N*-(1-phenylethyl) glycine (*N*spe), a peptoid analog of phenilalanine. The ampetoid depicted in C and D is a dodecamer [H-(*N*Lys-*N*spe-*N*spe)4-NH_2], and because *N*Lys is achiral, the structure of the ampetoid is expected to be significantly dynamic in solution. Residues are color coded: cationic, blue; hydrophobic, orange; all others, grey. From ref. [62], reproduced with permission.

LPS, and antimicrobial activity in the nanomolar range against a variety of bacteria and *Candida* species found in the oral cavity and against biofilm formation by *Streptococcus mutans*, an etiological agent of dental caries. *In vitro* data support its development as an inexpensive anti-infective against oral infections.

Small mimetics of AMPs may have several advantages over their naturally occurring counterparts due to their increased stability, tissue distribution, and the possibility to fine-tuning their potency and safety. Following this banner and starting from its computational engine out-licensed by the University of Pennsylvania, Polymedix (USA, www.polymedix.com) is developing a *de novo*-designed series of polymeric, oligomeric and small molecule mimetics of host defense peptides (PMX series). In particular, the lead compound PMX 30063 is now under preclinical development for broad spectrum systemic infections. Other potential uses include ophthalmic and oral applications. PepTx (USA, www.peptx.com) is developing another series of peptidomimetics, the PTX series, based on the β-sheet domain of human neutrophil bactericidal protein (BPI) [41]. All the compounds tested so far have been shown to be effective against a large panel of antibiotic resistant bacteria and, in particular, drug candidates PTX002 and PTX005 were able to neutralize LPS, proving to be effective in an animal model of *Pseudomonas aeruginosa*-induced sepsis [42].

Macrocyclization and introduction of linkers or templates provide a further strategy to produce improved AMPs mimetics and influence their conformation, biological activity and selectivity (Fig. 2, B). In this context, biaryls occur in many peptide natural products and antibiotics, such as the glycopeptide vancomycin, and have been studied as a semi-rigid template to mimic the amphiphilic β-hairpin structure of protegrin I [43]. The mimetic retained much of the antimicrobial activity of protegrin-I along with a significantly reduced haemolytic activity. This strategy has been pursued by Polyphor (Switzerland, www.polyphor.com) which is developing POL7080 for treatment of multidrug-resistant *Pseudomonas* infections. The synthesis of peptidomimetic acyl-based oligomers (Fig. 2, C) endowed with stability in mouse and human plasma, high activity *in vivo* and a good safety profile has recently been reported [44]. This panel of compounds, consisting of alternating acyl chains of variable length and lysines, has been designed to retain the amphipathic nature of AMPs while preventing the formation of a stable secondary structure. The library provides a tool to dissect the role of incremental variations that modulate charge and hydrophobicity with antimicrobial activity, and a template to design novel antibiotics for systemic use. Belonging to this family of molecules is BL2060, a synthetic compound comprising fatty acid and lysine copolymers, which is under development by BiolineRx (Israel, www.biolinerx.com) to combat resistant bacterial infections.

Lipopeptides are products of bacterial and fungal cells, where they are synthesized through the non-ribosomal pathway. They are composed of a specific lipophilic moiety attached to a linear or cyclic short (six to seven amino acids) peptide sequence, and are active against both bacteria and fungi. The lipopeptides family is a large one and includes both cationic and anionic peptidic molecules, generally constrained

by cyclization, such as polymyxins (polymyxin B and colistin), lipopeptaibols, echinocandin, laspartomycin, daptomycin (marketed under the trade name CUBICIN® by Cubist Pharmaceuticals, USA, www.cubist.com), and many others [45]. It has been postulated that fatty acid acylation of proteins is necessary to increase their membrane association and sorting into specific sub-cellular localizations [46]. Several studies have revealed that mimicking of natural lipopeptide antibiotics by the attachment of an aliphatic chain to the N-terminus of native or designed short peptides can result in an enhancement of their activity. Lipidation applied to otherwise inactive short cationic peptides made up of four L and D amino acids, produced a panel of so-called 'ultra-short lipopeptides', economically affordable antibiotics active both against bacteria and fungi [47]. The specificity of these molecules proved to be tunable through variability in the length of the aliphatic acid and the type of the hydrophobic amino acid in the peptidic portion. In another case, lipopeptide MSI-843, consisting of a short helical stretch made of the non-standard amino acid ornithine (Oct–OOLLOOLOOL–NH_2) and an octyl chain at the N-terminus, showed activity against bacteria and fungi at micromolar concentrations, the ability to permeabilize the outer membrane of *Escherichia coli* and an anionic model membrane mimicking bacterial inner membrane [48]. MX-2401, a semi-synthetic compound based on lipopeptide amphomycin, is currently under preclinical development for the treatment of serious nosocomial Gram-positive infections by Migenix (Canada, www.migenix.com). The semi-synthetic approach has also been used by Cubist Pharmaceuticals to produce over 800 next-generation daptomycin analogues, several of them currently being evaluated for preclinical development [45]. Lipophilic modification at the C-terminus of a peptide based on the amphipathic α-helical region (residues 21–31) of human lactoferrin has resulted in enhanced antimicrobial activity against Gram-negative and Gram-positive bacteria [49]. A range of acyl modifications have been prepared with N-terminal peptide fragments of dermaseptin derivatives, linear cationic peptides isolated from amphibian skin [50]. Those lipopeptides exhibited fast bactericidal activity against biofilms of oral pathogens such as *S. mutans* and *Actinomyces viscosus* [51]. Generally speaking, the aliphatic chain can greatly influence both the stability and the pharmacokinetic properties of an AMP. From a structural point of view, a lipidic tail is not only an appendage, but it rather participates actively in the formation of peptide-membrane interaction. It can either increase the membrane binding affinity by inserting deeply and allowing a small peptide, otherwise too short to span the bilayer, to form a transmembrane channel [47, 48], or may actively participate in the formation of a defined tertiary structure by forming a lipophilic cluster together with hydrophobic peptide residues, as demonstrated by solution NMR for the lauryl-LF11 lipopeptide [52].

Expanding the view over a larger set AMPs mimetics, some interesting non-peptidic molecules are also coming to light. Ceragenins, originally developed by Paul Savage at Brigham Young University [53] and exclusively licensed to Ceragenix (USA, www.ceragenix.com), are a new class of compounds belonging to this guild. They are membrane-active cationic steroid antibiotics that display cationic facial amphiphilicity with charges arranged on one side and hydrophobic residues on the other, able to target bacterial membranes of both Gram positive and Gram negative bacteria without a significant induction of resistance [54]. Noteworthy, the lead compound CSA-13 (Fig. 2, D) demonstrated to be less susceptible to inactivation by DNA or F-actin – polyanions present in high concentration in cystic fibrosis airway fluid – than classical AMPs like LL-37 or synthetic WLBU2 and HB71 [55]. Ceragenix is planning to apply its technology platform to the development of novel systemic and topical antibiotic (in particular MRSA), burn infections and coating materials [42].

Several studies have recently introduced the concept of 'multivalency' in the design of novel antibiotic molecules mimicking the membrane perturbing activity of natural AMPs [56]. The well proven increased activity of multimeric peptides compared to their monomeric counterparts may be related to the higher local concentration of bioactive units that would remove the assembly hurdle required by certain AMPs to form pores or transient defects on the bacterial membrane. Lysine cores (Multiple Antigen Peptide system) have been used as synthetic scaffolds for the attachment of two to eight copies of a tetrapeptide (R4) or an octapeptide (R8), both representing a combination of basic and lipophilic amino acids that have been found in natural AMPs such as protegrins and tachyplesins [57], or four copies of a rationally modified synthetic decapeptide, originally isolated by phage display technology [58]. It has been hypothesized that the increased proteolytic stability of multimeric peptides is probably due to the steric hindrance of the branching core that would limit the cleavage rates of plasma peptidases, thus increasing their pharmacokinetics properties [59, 60].

The last two peptidomimetic examples will we quote here are peptoids and random-sequence copolymers. In the first case, Annelise Barron and colleagues have shown that poly-*N*-substituted glycines (peptoids) – protease-resistant compounds in which side chains are

attached to the backbone amide nitrogen rather than to the α-carbon – can be moulded into a new interesting class of AMPs mimics [61, 62]. Recently, these authors have created a library of antimicrobial peptoid oligomers, or 'ampetoids', with helical structures and biomimetic sequences [62]. Some of the synthesized molecules have shown remarkable antimicrobial activities coupled with low cytotoxicity against mammalian cells, and the complex of obtained structure-activity data revealed striking structural, functional, and probably mechanistic similarity to natural AMPs (Fig. 3). Flexible sequence-random polymers containing cationic and lipophilic subunits that act as functional mimics of host-defense peptides look like another interesting venue to the preparation of low-cost anti-infective agents [63, 64]. For example, compounds such as cationic and lipophilic β-lactams have been ring-opened and copolymerized, obtaining substances with antimicrobial properties matching or exceeding those of many natural AMPs [63]. Besides being fully tunable from a structural point of view, the convenience and ease of preparing random copolymers rather than synthesizing sequence-specific oligomers offers another clear advantage. To explain the activity reported for random copolymers, it has been proposed that bacterial membranes would induce globally amphiphilic but irregular conformations in these molecules [63, 64].

Perspectives

That which has briefly been outlined above, coupled to the huge repertoire of other existing design-and-synthesis approaches not discussed here [e.g., 65], indicates that the peptidomimetic route will likely be increasingly privileged in the future. Hopefully, endowing AMPs with improved pharmacokinetic properties and lower manufacturing costs will open up new ways to fully exploit their therapeutic potential. Investigating the various modes of peptide-membrane interactions at a closer level of inspection may well provide key pointers to achieve this important goal.

Acknowledgements. Conflict of interest. The authors declare competing financial interests. A. Giuliani is an executive board member and minor shareholder of SpiderBiotech S.r.l. which develops antimicrobial peptides. G. Pirri is chief scientific officer and minor shareholder of SpiderBiotech S.r.l.

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