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Review

Antimicrobial peptide mimics for improved therapeutic properties

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ABSTRACT

The relatively recent recognition of the major role played by antimicrobial peptides (AMPs) in sustaining an effective host response to immune challenges was greatly influenced by studies of amphibian peptides. AMPs are also widely regarded as a potential source of future antibiotics owing to a remarkable set of advantageous properties ranging from molecular simplicity to low-resistance swift-kill of a broad range of microbial cells. However, the peptide formula per se, represents less than ideal drug candidates, namely because of poor bioavailability issues, potential immunogenicity, optional toxicity and high production costs. To address these issues, synthetic peptides have been designed, reproducing the critical peptide biophysical characteristic in unnatural sequence-specific oligomers. Thus, the use of peptidomimetics to overcome the limitations inherent to peptides physical characteristics is becoming an important and promising approach for improving the therapeutic potential of AMPs. Here, we review most recent advances in the design strategies and the biophysical properties of the main classes of mimics to natural AMPs, emphasizing the importance of structure–activity relationship studies in fine-tuning of their physicochemical attributes for improved antimicrobial properties.

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Abbreviations: AMP, antimicrobial peptide; OAK, oligo [acyl-lysine]; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; LB, Luria Bertani; CFU, colony-forming unit; PBS, phosphate-buffered saline; LC–MS, liquid chromatography – mass spectrometry; SPR, surface plasmon resonance; MIC, minimal inhibitory concentration; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance

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1. Introduction

The discovery of the ubiquitous occurrence of antimicrobial peptides (AMP) and their role as major host defense effectors was greatly motivated by the emergence and spread of resistance to antibiotics, while their potential use in fighting resistance has been stimulating AMPs research for over two decades. A most peculiar finding is the realization that AMPs are present in multiple isoforms with a common set of properties enabling their grouping as families. While the significance of this finding raised intriguing questions, some of which unanswered to date, these isoforms have certainly helped in identifying the importance of charge and hydrophobicity as the most crucial elements responsible for antimicrobial properties. Thus, AMPs made in the skin of Hylidae and Ranidae frogs and belonging to the dermaseptin family, display a remarkable duality, presenting both common yet distinct structural and functional attributes which moreover are rather typical to the vast majority of AMPs of any origin. Therefore, while their study as families has revealed most fascinating findings of both practical and evolution significances (see P. Nicolas, in this issue, for an authoritative review), their structure activity relationships (SAR) studies have helped revealing new strategies for specific targeting of microorganisms and shed new light on the roles of various parameters such as amphipathy, supramolecular organization and conformational flexibility, on the interaction with microbial targets, as detailed below.

1.1. Antimicrobial peptides and antimicrobial resistance

It is now widely recognized that the AMP concept could play a promising role in fighting the presently raging microbial resistance to conventional antibiotics [1,2]. Historically, resistance to antimicrobial agents actually predates the introduction of the first antibiotic (Penicillin) into clinical usage [3]. Because the first antibiotics, excluding the synthetic sulfa drugs, were all identified or derived from natural products, resistance determinants had already accumulated in the environments from which these agents originated. It was only a short period of time before selection pressures allowed these environmental resistance determinants to become incorporated into the pathogenic bacteria that were being treated with the new antibiotics [3]. In addition, medical and agricultural practices of the past 50 years have promoted resistance development and spread in both human and animal pathogens, compromising effective chemotherapy of infectious diseases [4]. Today, many important pathogens are resistant to multiple antimicrobial classes, covering most, sometimes all, clinically useable antimicrobials. Infections caused by these so-called multidrug resistant (MDR) organisms are costly to treat while the treatment is increasingly prone to failure [5,6].

Bacterial resistance to antimicrobials comes in many flavors, relying typically on drug inactivation [7] or target site modification/mutation [8]. Reduced drug accumulation owing to limited uptake or enhanced efflux is also an important resistance mechanism for certain classes of antimicrobials [9]. Another mechanism is that of phenotypic resistance owing to specific growth modes (e.g., biofilms) during infection. It is prevalent amongst bacterial pathogens and likely plays a major role in in-vivo resistance and treatment failure despite indications of in-vitro drug susceptibility [10]. Overall, the clinical impact of resistance is immense, characterized by increased cost, length of hospital stay and mortality.

Thus, it was time to consider new classes of antibiotics such as the AMPs [11] whose mode of action promises both low susceptibility to MDR mechanisms and high activity against a vast range of microorganisms [12]. AMPs are produced by most species of life, from prokaryotes [13,14] to plants [15,16], insects [17–20], amphibians [21–25] and mammals including humans [26–28], as part of the organism's host defense mechanism. As major effectors of the innate immune system, AMPs complement the highly specific but relatively slow adaptive immune system [29,30]. Unlike the acquired immune mechanisms, endogenous AMPs, which are constitutively expressed or induced, provide fast and effective means of defense. Most of these gene encoded peptides are mobilized shortly after microbial infection and act rapidly to neutralize a broad range of microbes [31].

1.2. Modes of action

Many AMPs are defined as membrane-active molecules. While their lytic activity is generally not mediated by a chiral center [32], the exact mechanism behind this activity is not fully understood. Nevertheless, based on the available data, all proposed modes of action have implicated the cationic and hydrophobic nature of the AMP in its initial interaction with the negatively charged lipids in bacterial membranes [33,34] while some variations are expected in non-bacterial targets. Presumably, the amphipathic structural arrangement of AMPs plays an important role in this mechanism. Due to their cationic nature AMPs are electrostatically attracted to negatively charged microbial surfaces such as lipopolysaccharide (LPS) in Gram-negative bacteria [35] and teichoic and teichuronic acids in Gram-positive bacteria [36]. Binding of AMPs to these most external elements is generally not directly implicated as solely responsible for bacterial death. On contrary, this binding affinity maybe required for preferential accumulation of AMPs in bacteria. Binding is eventually used as a "jumping-board" to insinuate into inner layers reaching for the cytoplasmic membrane as elegantly illustrated in the self-promoted uptake hypothesis [33].

Once the cytoplasmic membrane is reached, AMPs interact with negatively charged groups of the external leaflet. At this stage, linear AMPs re-organize and assume an optimal amphipathic conformation where the hydrophilic face interacts with the phospholipid head groups whereas their hydrophobic face is inserted in the bilayer core. Note, a similar interaction with peptide mimics is depicted in Fig. 6. Such interactions can lead to a wide variety of structural distortions/damages to the membrane architecture and can result from various possible mechanisms: 1) peptide molecules accumulate parallel to the surface and affect the membrane in a detergent-like manner, a process known as a carpet-like mechanism [35]. 2) self association of peptide molecules to form pores [37–39]; 3) in the "toroidal-pore model" insertion of peptides into the membrane is envisioned to induce lipids to bend contiguously around peptide aggregates until a water core is formed, lined by both the inserted peptide and the lipid headgroups [40]. Solution NMR studies have played an important role in providing atomistic-level structures of AMPs. Along with solid-state NMR these studies have provided high-resolution data for different types of mechanisms by AMPs such as barrel-stave pores by pardaxin [41], carpet/toroidal type mechanism by LL37 [42] and MSI peptides [43]. A recent study of the toroidal-pore model, conducted with the magainin analog MG-H2 and a model phospholipid bilayer, showed that the pore is stabilized by a diffuse distribution of peptides which bind to the rim of the pore, adopting a largely parallel orientation to the membrane plane, rather than the previously described perpendicular orientation [44].

Lipid composition alone might account for the preferential activity against bacteria. Whereas microbial membranes are enriched in anionic lipids, mammalian cell membranes usually contain fewer anionic lipids which moreover are mostly found in the inner leaflet [36,45,46]. The presence of sterols in the target membrane also can account for selectivity. Cholesterol can reduce AMPs potency due to its stabilization effect on the lipid bilayer or to its interaction with the AMP [2,47,48]. Differences in transmembrane potential ($\Delta\psi$), electrochemical gradient determined by extents and rates of proton flux across the membrane, were also implicated in selectivity of AMPs [46]. In practice however, highly hydrophobic AMPs are capable of interacting with both microbial and mammalian membranes [49]

and were shown to induce hemolysis at relatively low concentrations [50–53]. This fact points to the relatively higher importance of the hydrophobic forces at play, compared with those of charge.

AMPs interactions with membranes are thus known to lead to a variety of damages ranging from anions segregation and cell surface topology alterations [54,55] to cancellation of the membrane-barrier function via its depolarization and permeation, eventually. In some cases however, "harmless" interactions of AMPs with the plasma membrane were proposed to affect cell viability following internalization and interaction with intracellular targets [2,56]. Translocation to the cytoplasm enables AMPs to target a variety of additional molecules such as DNA [57], RNA and enzymes [58,59] or inhibit their synthesis [60–64]. For example, buforin II binds to DNA and RNA and alters their electrophoretic mobilities in agarose gels [57]. A hybrid AMP, designed from the flounder pleurocidin and the frog dermaseptin, was shown to inhibit *E. coli* nucleic acid and protein synthesis at the lowest inhibitory concentration, without damaging the cytoplasmic membrane [63].

1.3. Antimicrobial peptides as drug candidates

Owing partly to the above described properties, it is now well recognized, that the AMP specific sequence by itself is not crucial in defining antimicrobial activity, which rather depends upon physicochemical attributes including charge, hydrophobicity and structure [36]. Consequently, although non-specific modes of action might indeed decrease pathogens abilities to develop resistance mechanisms to AMPs [65,66], common characteristics between microbial and mammalian cell-membranes seem to be responsible for relatively low selectivity of AMPs (certainly compared with conventional antibiotics). As drug candidates, AMPs present various additional disadvantages including reduced activity in presence of salts and divalent cations [67–70], susceptibility to pH changes [70,71] and to proteases and other plasma components [72]. AMPs can also suffer from poor pharmacokinetic issues and high production costs [46,73–75]. Thus, although the use of peptides composed of D-amino acids only is sometimes contemplated owing to their high resistance to proteolysis [76,77] this option often significantly increases the product costs while there is no published pharmacokinetic evidence for enhanced bioavailability of D-peptides.

There is thus, a clear need for alternative strategies to overcome AMPs drawbacks, particularly as potential systemic therapeutic agents. A wide range of SAR studies aiming at better understanding the mechanisms of action of natural AMPs, have considerably enhanced our appreciation of the main factors influencing their actions, leading in recent years, to increasing attempts to derive this kind of information from systematic variations of model peptide sequences using several approaches: combinatorial libraries [78–80], minimalistic approach [81,82] and sequence templates [36,83,84] or from design of non-natural peptide mimics. The rational being that reproduction of critical peptide biophysical characteristics in an unnatural, sequence-specific oligomer, should presumably be sufficient to endow antibacterial efficacy, while circumventing the limitations associated with peptide pharmaceuticals [85–87].

2. Peptidomimetics

The term peptidomimetic is used here in a broad sense, referring to essentially any oligomeric sequence designed to mimic a peptide structure and/or function but whose backbone is not solely based on α-amino acids (representative chemical structures are shown in Fig. 1). Peptidomimetics can thus be based on polymers that mimic a peptide primary structure through the use of amide bond isosteres and/or modification of the native peptide backbone, including chain extension or heteroatom incorporation. Typical classes of antimicrobial peptidomimetics will be briefly outlined (for recent reviews see references [88,89]), then we shall focus on oligomers of acylated lysines. Other interesting "mimic" systems such as ceragenins [90] and diastereoisomers [91] will not be included.

2.1. Peptoids

Poly-N-substituted glycines (peptoids) differ from peptides only in that peptoid side chains are attached to the backbone amide nitrogen rather than to the α-carbon [92], which among other attributes, renders them protease-resistant [93]. The poly-N-substituted glycine structure of peptoids prevents both backbone chirality and intrachain hydrogen bonding; nevertheless, peptoids can be driven to form helical secondary structures via a periodic incorporation of bulky, α-chiral side chains [94,95]. Incorporation of homochiral side chains can give rise to polyproline type-I-like helices with a periodicity of approximately three monomers per turn [96,97]. The threefold periodicity of the peptoid helix facilitates the design of facially amphipathic structures similar to those formed by many AMPs; for example, the trimer repeat $(X–Y–Z)_n$ forms a peptoid helix with three faces, composed of X, Y, and Z residues. *in vitro* activities of certain antimicrobial peptoids are similar to those of many AMPs, they exhibit

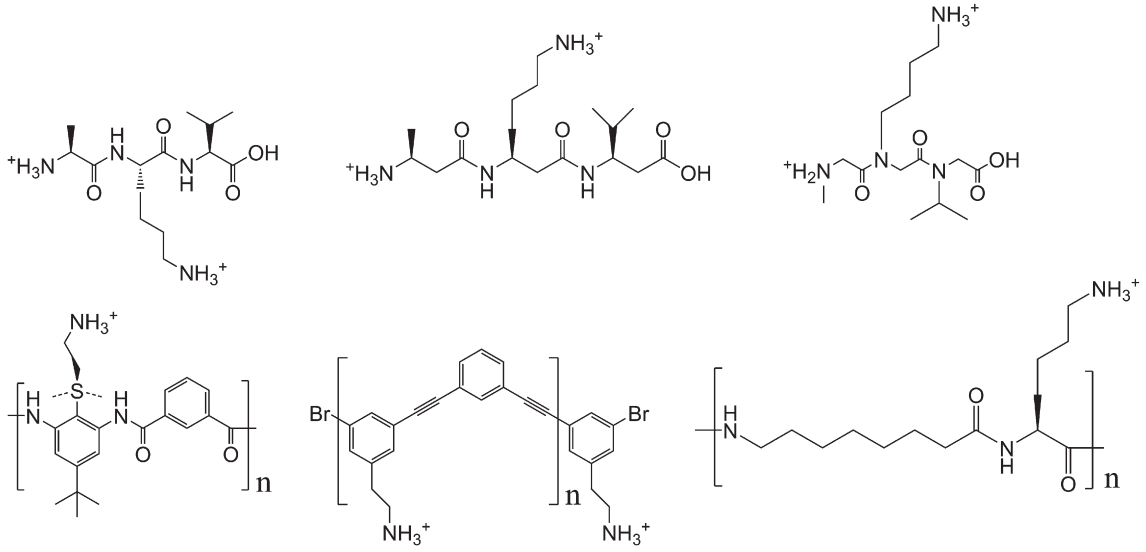

Fig. 1. Chemical formula of representative AMP-mimics. Upper structures (from left to right): An α-peptide sequence and its β-peptide and peptoid counterparts. Lower structures: Arylamide, phenylene ethynylene and acyl-lysyl oligomers (brackets define the building blocks).

broad-spectrum antibacterial activity and low mammalian cytotoxicity, for example SAR studies on peptoids revealed a twelve residue compound termed 1-pro$_6$ which had potent antibacterial activity (MIC values of 3.1 and 1.6 μM against *E. coli* and *B. subtilis*) and low hemolytic activity (LC_{50} of >110 μM against hRBC) [98].

2.2. β-Peptides

DeGrado et al. first reported de novo design of antibacterial β-peptides, short polymers of β-amino acids [99]. This class of polyamides folds into turns, helices, and sheet-like structures, analogous to the secondary structures found in proteins composed of α-amino acids but display higher resistance to enzymatic degradation. These compounds were highly active against *E. coli* but also against erythrocytes. For example the longest peptide, which consisted of 18 β-amino acids, induce 50% hemolysis (LC_{50}) at 80 nM which is considerably greater than that of melittin (0.5 μM) [99]. Extension of this work resulted in analogs with significantly reduced hemolytic activity [100]. The authors recognized that the lack of selectivity of their original β-peptides arose from excessive hydrophobicity.

Other studies demonstrated that "β^2/β^3" type peptides, which are composed of a mixture of β^2 (β-amino acid were the side chain is on the α-carbon) and β^3 (β-amino acid were the side chain is on the β-carbon) amino acids, can display selectivity [101,102]. For example the "mixed" β^2/β^3-nonapeptide **1** (composed of 9 amino acids) showed MIC range of 6–100 μM against a panel of bacteria with an LC_{50} of 175 μM against hRBC. The pharmacokinetic profile of an α/β peptide termed "β^3/α tetrapeptide 2" indicating stability in-vivo after *i.v.* and *p.o.* administrations (1 and 2 mg/kg, respectively) displayed similar rates of clearance in the blood (about 90% per hour) [103].

Studies comparing activities against a panel of bacteria of two helix-forming compounds, β-17 and a synthetic magainin derivative, revealed comparable antibacterial activities (e.g., MIC of 6.2μM against *E. coli*) but β-17 was less hemolytic [104]. Further studies of β-peptides with flexible acyclic residues incorporation revealed that variation in helical propensity did not significantly affect antibiotic activity [105]. A recent work on oligomers containing a 1:1 pattern of α- and β-amino acids showed that having a globally amphiphilic helical conformation is not a requirement for selective antibacterial activity [106,107]. In contrast to β-peptides, whose activity was clearly correlated with amphiphilic helical conformations [105] studies with α/β sequences revealed that scrambled versions can manifested stronger antibacterial activity than the globally amphiphilic most favorable helical conformations, suggesting that stable folds do not necessarily determine antibacterial potency. Interestingly, the trend in hemolytic activities among these compounds was quite different from the trend in antibacterial activity [106] suggesting that stable folds might determine hemolytic potency.

2.3. Arylamide oligomers

Tew et al. later reported a class of facially amphiphilic arylamide oligomers that utilized hydrogen bonding to produce conformationally stiff backbones [108]. Arylamide oligomers which were designed with the aid of molecular dynamics simulations, exhibited a broad spectrum antibacterial activity [108] with a mechanism of action resembling native antimicrobial peptides. For example a typical oligomer (composed of two subunits) displayed a MIC of 25 μM against *E. coli* and induced calcein release from phosphatidylserine/phosphatidylcholine bilayers, suggesting membranolytic mode of action. Modifications to the initial oligomers using various hydrophobic and hydrophilic side chains or by switching benzene with pyrimidine, have yielded several potent and moderately selective compounds [108–110]. For example compound 10 (with a single pyrimidine in the backbone) showed ~10 fold improvement in antibacterial activity (MIC of ~1 μM) compared to the benzene ring analog (compound 11) which has a MIC of ~18 μM. Both compounds displayed LC_{50} against hRBC at ~20 μM [109]. Thus, unlike β-peptides, these studies seem to suggest that rigidifying the conformation of arylamide oligomers could lead to improved antibacterial activity and selectivity.

2.4. Phenylene ethynylenes

Tew et al. reported selective antibacterial activity of phenylene ethynylene oligomers, with a strictly hydrocarbon backbone [111,112]. The most active compound, composed of three phenyl rings (compound 1), showed potent antimicrobial activity against a large panel of pathogenic bacteria with a MIC range of 0.4–14 μM and LC_{50} of 153 μM against hRBC. Compound 1 also did not induce resistance in *S. aureus* [112]. Interestingly, this compound was found to inhibit also LPS-mediated activation of macrophages at nM concentrations [113]. The pore forming properties of these oligomers have been investigated using various biophysical techniques including, Langmuir films at the air–water interface [114,115], grazing incidence X-ray diffraction [116], small angle X-ray scattering [117], sum frequency generation vibrational spectroscopy [118], and dye leakage experiments from model membranes [117]. Thus, when the ethylamine group of compound 1 was shortened to a single methylene, activity against both bacteria and RBCs was lost. In contrast, when the ethylamine group was extended to propylamine it showed activity against both bacteria and RBCs. Furthermore, compound 1 induced maximal dye leakage from vesicles reconstituted from *E. coli* lipid extract. Unlike its propylamine analog, leakage was reduced when supplemented with phosphotidylcholine, suggesting that compound 1, induced membrane damage by a mode of action that resembles many AMPs, whose selectivity is dependent on anionic lipids concentration.

Collectively, such AMP mimics were shown to overcome one of the main shortcomings of α peptides – susceptibility to enzymatic degradation – suggesting improved antibacterial properties in vivo. However, to the best of our knowledge, there are no such studies published as yet, for any of the above mentioned peptide-mimics.

3. Introduction to OAQs: SAR studies of dermaseptins

The AMPs mode of action has been traditionally investigated using sequence specific modifications or de-novo design of AMP analogs. Many of these studies were based on the assumption that the secondary structure played a key role and consequently attempted to improve antimicrobial properties via improving structural aspects. We among others, have long been studying structural effects on antimicrobial properties using the frog dermaseptin family as model AMPs [51,119–122]. However, we eventually realized that while enhancing the helical content did in fact enhance antimicrobial potency, it also reduced the peptide's ability to discriminate between target cells. For instance, sequence manipulations of dermaseptin S4 led to the analog K_4K_{20}-S4, a highly structured peptide with potent and large spectrum antimicrobial activity [120,123]. This analog however, was among the most hemolytic peptides ever described in the literature, with obvious in-vivo toxicity [124]. We suspected therefore, that this strategy had a dead end that severely limited the potential use of AMPs, especially towards systemic applications.

We therefore turned to new alternatives, seeking at first to improve selectivity via short (truncated) analogs of natural dermaseptins. This strategy also turned out to be disappointing although not devoid of some interest. Thus, elimination of flexible peptide domains indeed led to short α-helical derivatives with reduced hydrophobicity but with improved selectivity [120,125]. N-terminus acylations – which represented a convenient means, from the synthetic point of view, to compensate/modulate for hydrophobicity loss – revealed to significantly affect the peptide amphipathic organization and activities. Thus, acylated short derivatives that maintained α-helical conformation displayed improved selectivity [51,119]. Interestingly,

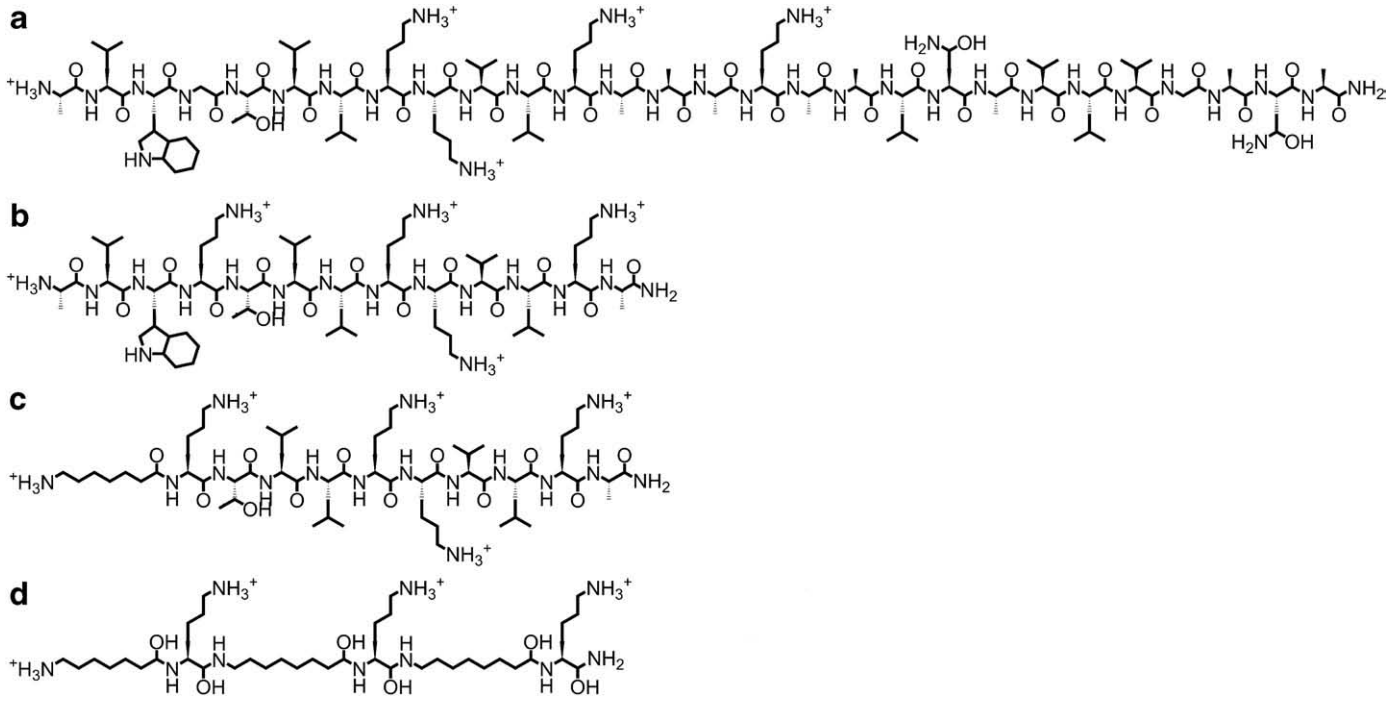

Fig 2. Dermaseptin to OAK evolution. (a) Full length native dermaseptin S4(1–28) [141]; (b) Truncated derivative K4-S4(1–13) [125]; (c) Lipopeptide version of the truncated derivative, animoheptanoyl-lysyl-S4(5–13) [126]; (d) OAK version, animoheptanoyl-lysyl-2α_8 [130].

extension of this study revealed that the N-terminal acyl could substitute for considerably lengthy N-terminal sequences, maintaining some helical conformation but displaying a highly narrow spectrum of activity, which included practically only pseudomonal species [126]. These attempts nonetheless, raised the possibility that acyl moieties might be able to substitute extensive sequences within the peptide backbone (i.e., the OAQ concept), suggesting that the consequence of losing the secondary structure might be rather advantageous, especially in terms of selectivity (as detailed below). The structural evolution from a native dermaseptin to an OAK is illustrated in Fig. 2.

3.1. OAQs design

To investigate the link between antimicrobial- and folding-properties of AMPs, we recently designed a library of peptide-like sequences where lack of a defined secondary structure was promoted by an acyl chain bridging between two cationic amino acids, according to the general formula: **$[A_iQ]_n$** where A and Q represent an acyl (a fatty- or amino fatty-acid of variable length (i)) and a charged amino acid, respectively. The following descriptions will refer to oligomers of acylated lysine (AK), i.e., a particular case of OAQs where Q = lysine (K). The aminoacyl-lysyl building block is termed α_i subunit while lysyl-aminoacyl-lysyl is termed β_i subunit, where i defines the number of carbon atoms in the acyl chain (for example, α_4 is an aminobutyryl-lysine subunit). While providing both hydrophobicity and positive charge features, these molecules are expected to limit formation of defined secondary structures because of the optimal rotational freedom of the carbon atoms in an acyl chains (the particular case of long acyls will be discussed below). This method therefore enables a structure-independent and systematic tool for a gradual control of molecular hydrophobicity (by changing acyl chain length). Similarly, using this approach, comparisons between α- and β-OAKs can reveal the importance of charge distribution (by changing the nature, the position and/or the number of cationic residues). An OAK library including over one hundred sequences was produced and characterized [127]. As detailed in the following paragraphs, analysis of the obtained data enabled to draw pertinent conclusions as to the physico-chemical properties required for potency and selectivity of OAKs.

3.2. Relationships between HQ values and antibacterial properties

The OAKs physical and antibacterial properties (assessed against *E. coli*) are summarized in Fig. 3 which also highlights the fact that they cover a wide range of hydrophobicity (H = up to 60%) and charge (Q = up to +11) values. Three major trends have emerged:

1) The OAK biophysical properties are dictated by the nature of the N-terminal acyl [127]. For instance, in OAKs that lack an N-terminal acyl, the H value readily increased with increasing backbone length,

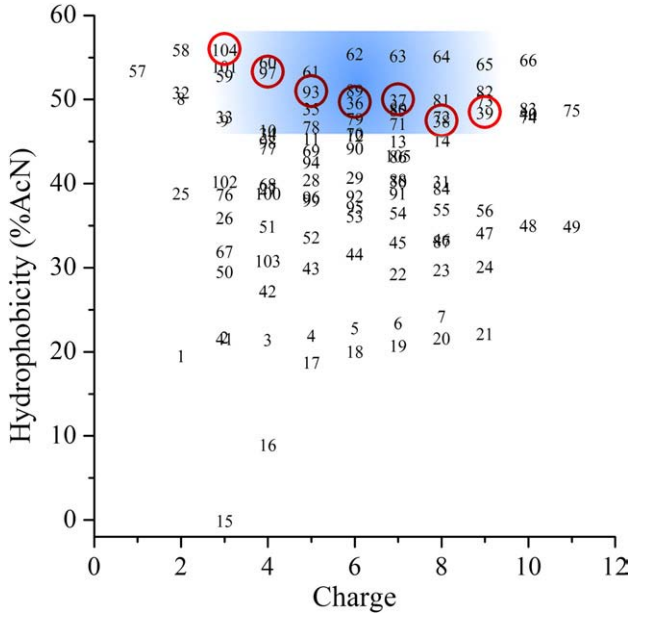

Fig. 3. HQ map of 105 OAKs. Shown are the HQ values of each OAK (numbered from 1 to 105). Most sequences (103) are listed in [127]. New OAKs are: C_{16}K-1β_{12} (number 104, $H = 56$, $Q = 3$) and C_{10}K-3β_{12} (number 105, $H = 44$, $Q = 7$). Highlighted (red circles) are potent OAKs (displaying MIC ≤ 3 μM). These were found to concentrate in a narrow band of HQ values (highlighted in blue).

Table 1
Biophysical properties of typical short OAKs

OAK[a]	Q[b]	H[c] (%)	CAC[d] (μM)	MIC[e] (μM)		LC_{50}[f] (μM), RBC
				E. coli	*S. aureus*	
$C_{12}K\text{-}2\alpha_{12}$	3	52.9	<1	>50	>50	8 ± 1
$C_{12}K\text{-}2\beta_{12}$	5	51.0	11.2 ± 2.5	3.1	6.3	88 ± 3
$C_{12}K\text{-}1\beta_{12}$	3	54.0	9.6 ± 1.6	25	3.1	29 ± 9
$C_{12(\omega7)}K\text{-}1\beta_{12}$	3	50	50	>50	3.1	100 ± 8

[a] Designation, $C_{12}K$ = lauryl-lysyl; α_{12} = aminolauryl-lysyl; β_{12} = lysyl-aminolauryl-lysyl.
[b] Molecular charge at physiological conditions.
[c] Hydrophobicity, defined the percent acetonitrile eluent on a C_{18} HPLC column.
[d] Critical aggregation concentration evaluated by linear extrapolation of light scattering.
[e] Lowest OAK concentration that fully inhibited bacterial growth (>99%) after 24 h culture at 37 °C.
[f] OAK concentration that induced 50% hemolysis (1% hematocrit) after 3 h incubation; values represent the mean ± standard deviation of at least two independent experiments.

whereas an N-terminal acyl essentially determined the H value, which remained practically constant regardless of the number and type of subunits. This hydrophobicity stabilization often allows focusing on effects specific to charge upon subunit variations.

2) Antibacterial activity emerged when the OAK attained an optimal window of HQ values [127]. Potent antibacterial activity (MIC ≤ 3 μM) required a relatively narrow window of HQ values and disregarded the specific OAK sequence (Fig. 3). For *E. coli*, these values were: H = 50 ± 2 and Q = 6.5 ± 2 (mean and SD calculated from the H and Q values of the most active OAKs). Note however that different HQ windows were obtained for different bacteria and although these might be grouped in terms of gram-negative and gram-positive bacteria, an overlapping zone seems to emerge that includes essentially non-specific antibacterial OAKs. This suggested the possibility that the OAK system might be able to uncover specific compounds per groups of bacteria.

3) Self-assembly properties can often explain discrepancies such as why the "active HQ window" contains seemingly inactive OAKs. Presumably, large supramolecular structures are prohibited from reaching internal targets that might be located beyond the bacterial cell wall. By using EDTA (which increases external membrane permeability) we showed that aggregated OAKs within the HQ window are potentially active but due to their aggregated state, are excluded by the bacterial cell wall [127]. Thus, this assay allows the distinction between "truly" inactive OAKs (i.e., those that lack the HQ properties required for activity) and potentially active OAKs. This is not to say that all types of aggregates are necessarily inactive, especially considering that an aggregated compound can be inactive against a given microorganism but active against another. An interesting example is represented by recent findings showing that short OAKs displayed potent antibacterial properties at concentrations above the CAC value ([128] and Table 1). Another example is represented by previous findings showing that various additional aggregated OAKs that were inactive against bacteria displayed potent antiplasmodial properties [129], supporting the notion that interactions of aggregates with eukaryotic (as opposed to prokaryotic) cells are governed by different rules, if only due to lack of a cell wall.

3.3. Relationships between 3D structure and hemolytic activity

As exemplified in Fig. 4, butyl- and octyl-based OAKs were consistently devoid of hemolytic activity at least up to 150 μM (a concentration that represents ~100-folds the MIC value) whereas their circular dichroism (CD) profiles in PBS were characteristic of

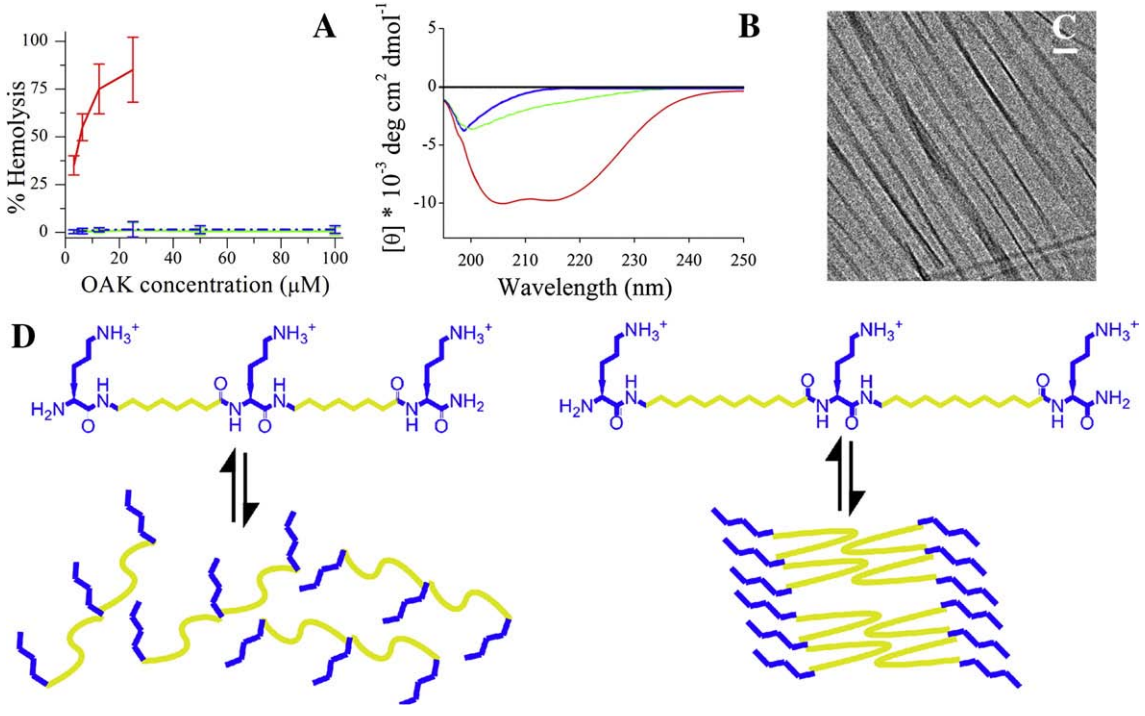

Fig. 4. Relationships between hemolysis and organization in solution. Panel A shows the hemolytic activity of three representative OAKs (with identical N-terminus and number of subunits but varying in length of intercalating acyls) determined against Human RBC (1% hematocrit, 3 hour incubation at 37 °C in PBS). Error bars represent ± 1 SD. Colors: $C_{12}K\text{-}7\alpha_{12}$, red; $C_{12}K\text{-}7\alpha_{8}$, green; $C_{12}K\text{-}7\alpha_{4}$, blue. Panel B compares the circular dichroism spectra of the same OAKs (100 μM) in PBS. Panel C, shows a cryo-TEM image of a representative α_{12}-OAK at 1 mg/ml (Bar = 50 nm) showing its self-assembly in aqueous solution [128]. Panel D depicts a hypothetical model for organization of representative OAKs. Upper line shows two OAK sequences based on C8 and C12 acyls (left and right, respectively). In aqueous solution, only long enough acyl residues can fold into amphipathic structures that can subsequently associate to form micron-long multimers (panel C). Such structures would be stabilized primarily by hydrophobic interactions and eventually (if packed tightly enough) by hydrogen bonds between backbone amides within each molecule and in adjacent layers.

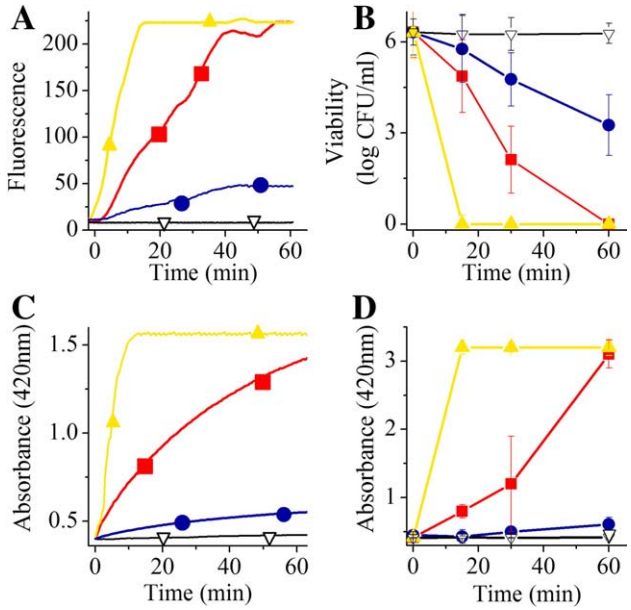

Fig. 5. Assessment of the mode of action. (A) Cytoplasmic membrane depolarization of *E. coli*. Test compounds were added to cell suspensions at 6xMIC and changes in fluorescence were recorded online. Symbols (same for panels A–D): $C_{12}K\text{-}5\alpha_8$, circles; $C_{12}K\text{-}7\alpha_8$, squares; dermaseptin S4(1–16), triangles; bacterial suspension without treatment, upside triangles. Note: Panels A and C depict continuous recordings, symbols were added for clarity. (B) Viability of EDTA-treated bacteria. Aliquots from bacterial suspensions described in A, were plated for CFU count after overnight incubation. (C) Kinetics of ONPG hydrolysis in *E. coli* ML35 measured by incubating bacteria with test compounds at 6xMIC. (D) Leakage of *E. coli* ML35 intracellular β-galactosidase to the surrounding medium following exposure to the test compounds at 6xMIC, assessed by monitoring enzymatic activity upon mixing ONPG with the suspension's supernatant.

random structures and remained so despite interaction with liposomes and other hydrophobic media [130]. Thus, unlike most AMPs, antibacterial activity of these AMP-mimetic OAKs can be highly dissociated from both stable conformation and hemolytic activity. Lauryl-based OAKs however, were often hemolytic, highly aggregated and displayed CD profiles of stable folds reminiscent to those of α-helix or β-sheet structures [131]. The fact that non-random CD profiles were observed only with aggregated OAKs suggests that these CD profiles rather reflect supra-molecular organizations. In fact, as exemplified in Table 1, attempts to limit aggregation either by increasing backbone charge (e.g., using β-subunits [127] or through the use of unsaturated acyls [128] lead, in both cases, to simultaneous loss of hemolytic activity and "normalization" of the CD profile.

The correlation existing between aggregated OAKs and hemolytic activity provides a strong support to the hypothesis underlying OAKs design [130], proposing a link between stable folds (i.e., rigid structures) and hemolytic properties of AMPs. According to this hypothesis, rigid structures (if hydrophobic enough) can interact with RBC membrane in a manner that perturbs its architecture and lead to hemoglobin leakage, whereas, due to their high backbone malleability (i.e., non-rigid structures) OAKs interactions with RBC membrane would be inefficient in perturbing the membrane. Indeed, the data indicate that incorporation of fatty acids within a peptide backbone can suppress the formation of a stable molecular fold — if the intercalating acyl chains are not too long (i.e., must be shorter than twelve carbons). Conceivably, unlike shorter acyls, lauryl residues are long enough to bend and create rigid amphipathic structures, hence their propensity to stabilize in aqueous solutions through self-assembly (aggregates) and their hemolytic activity, reflecting their ability to reorganize within membranes in presence of RBC. Fig. 4 depicts a drawing of such potential aggregates that can interact with RBC membrane and perturb its architecture, much like amphipathic conventional AMPs [51,125,132].

3.4. OAKs can affect bacterial viability by a variety of distinct mechanisms

The first OAK investigated was found to exert antibacterial activity through membrane disruption [130]. Further investigations however, revealed that the OAK library contains additional antibacterial OAKs, sometimes very closely related, that acted by different mechanisms of action. For instance, various analogous OAKs revealed drastically different time-kill curves, e.g., minutes versus hours [133]. The differential killing rates of these OAKs was proposed to reflect different targets such as cytoplasmic membrane disruption and interference with intracellular processes. This view was supported by several studies of a pair of analogous OAKs, the octamer $C_{12}K\text{-}7\alpha_8$ and the hexamer $C_{12}K\text{-}5\alpha_8$ [133,134]. On one hand, the OAKs displayed similar properties in many respects, suggesting a similar mode of action: Both OAKs exerted a bactericidal effect, displayed high binding affinities to model membranes, similar stability in blood while bacteria were unable to develop resistance to either OAK [133]. Moreover, in-vivo studies also showed similar pharmacokinetics and similar abilities to protect mice from a lethal bacterial infection [133].

However, additional probing experiments revealed a different picture, rather arguing for distinct mechanisms. Thus, differential time-kill rates of the analogous OAKs correlated with rates of depolarization of the bacterial membrane potential and with rates of leakage of an intracellular enzyme (Fig. 5). These results indicated that only $C_{12}K\text{-}7\alpha_8$ induced bacterial death through disruption of the cytoplasmic membrane (Fig. 6). In contrast, only $C_{12}K\text{-}5\alpha_8$ displayed the ability to translocate across the cytoplasmic membrane and interact with nucleic acids [133]. The incapacity of $C_{12}K\text{-}7\alpha_8$ for a similar behavior is probably (at least partly) linked to it stronger interaction with cell wall components (e.g., LPS) as well as with cytoplasmic membrane phospholipids. Once $C_{12}K\text{-}7\alpha_8$ reaches the cytoplasmic membrane and disrupts it, the membrane potential is canceled and so is the driving force for translocation. Conversely, $C_{12}K\text{-}5\alpha_8$ reduced positive charge is likely to comparatively reduce all interactions on the way to the cytoplasmic membrane where the trans-membrane potential difference can be used as the driving force for internalization, thereby promoting its interaction with intracellular targets.

A recent study using ITC to compare the analogs interaction with model phospholipid membranes, confirmed the higher binding affinity of $C_{12}K\text{-}7\alpha_8$ (as observed using SPR [133]) and further DSC and NMR studies indicated that only $C_{12}K\text{-}7\alpha_8$ had the ability to induce the clustering of anionic lipids [134]. This clustering effect is believed to lead to the lateral segregation of domains rich in anionic or zwitterionic lipids, producing phase boundary defects that ultimately breach the permeability barrier of the cell membrane.

The finding that OAK analogs can use distinct mechanisms of action is therefore surprising although not unprecedented. While most conventional AMPs are believed to act by destabilizing the plasma membrane, there is a growing list of peptides such as PR-39 [60], buforinII [135], pyrrhocoricin [59], tPMP-1 and HNP-1 [136], all proposed to induce bacterial death through interaction with intracellular targets. Combined with the OAK data, these findings show that tiny differences (such as a single charge and/or backbone length) are required for an antibacterial compound to switch from one mechanism to another. Stretching this idea further, the data also raise the possibility that the simultaneous production of multiple closely related host defense peptides might be linked to an evolutionary advantage providing the producing cell/tissue with a highly efficient attack system that uses both synergistic strategies as previously proposed [137,138] and multiple mechanisms to combat a larger spectrum of pathogens.

3.5. Antiplasmodial properties of OAKs

Targeting of intracellular parasites provides a challenging model system for development of selectivity in "non-specific" antimicrobial

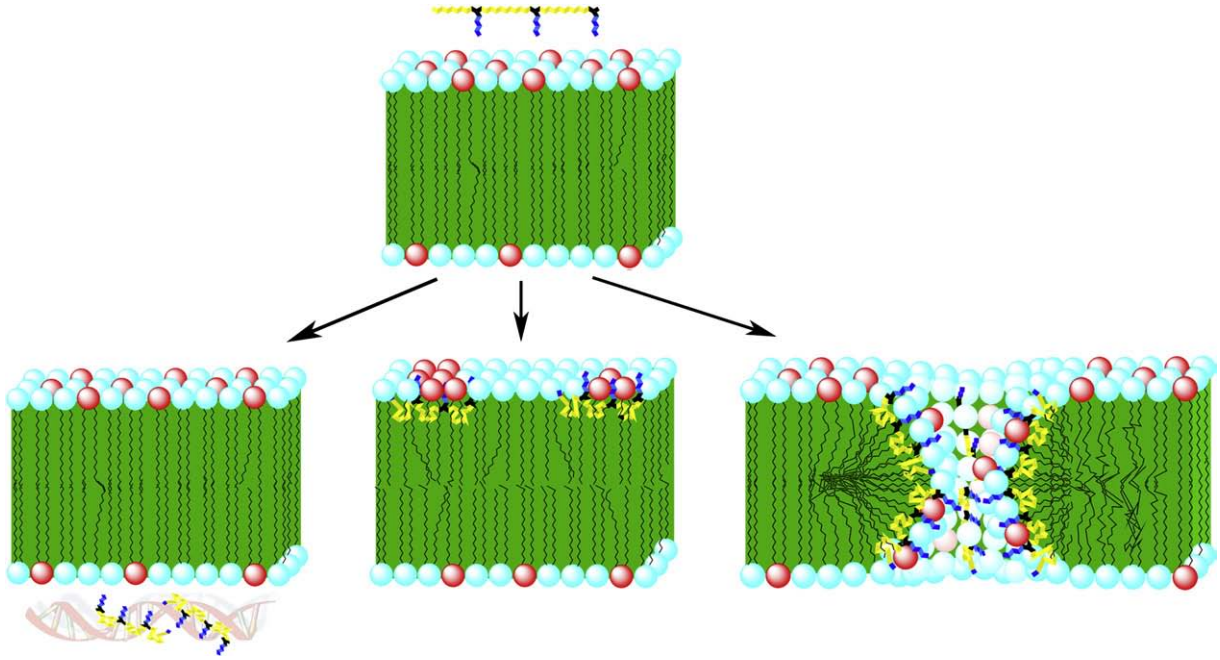

Fig. 6. Schematic diagram of OAKs antibacterial modes of action. Upper diagram depicts an OAK molecule composed of three acyls (yellow) and lysines (blue) about to interact with a model membrane composed of lipids with two different kinds of headgroups, represented by red and cyan balls. The lower diagrams depict three potential modes of action representing (from right to left) a drastic perturbation of the membrane structure (equivalent to punching holes in the membrane) which involve leakage of intracellular components, as observed with C_{12}K-7α_8 [130]; the middle diagram represents a milder damage to the membrane, as observed with C_{12}K-7α_8 [134], where the preferential interaction of the OAK with one of the lipids leads to phase separation of lipids. This creates phase boundary defects between domains enriched in either of the two lipids; The left diagram represents the trans-localization (internalization) of the OAK to the cytoplasmic compartment (without causing any noticeable membrane damage), followed by inhibition of DNA functions through (probably non-specific) high affinity binding to DNA, as observed for C_{12}K-5α_8 [133].

modes of action. Previous studies of the antimalarial properties of dermaseptins in cultures of *Plasmodium falciparum*, have shown that native dermaseptins kill intraerythrocytic malaria parasites through lysis of the host cells [123]. Acylation of the truncated derivative S4 (1–13) resulted in an amplified antiplasmodial effect [139]. Although increased hydrophobicity was generally associated with increased hemolysis, various derivatives, such as isobutyryl [139] and amino-heptanoyl [140] analogs displayed improved selectivity (i.e., were more effective antiplasmodials yet less hemolytic). These studies suggested that AMPs could be engineered to specifically target the intra-erythrocyte parasite and prompted the investigation of the antiplasmodial properties of OAKs.

Certain lauryl based OAKs were found to display a highly selective antiparasitic activity [129] where the ratio of LC_{50} (hemolysis) to IC_{50} (parasite growth inhibition) was >10,000 for the most selective OAK, composed of three α_{12} subunits (3α_{12}). In achieving this selectivity, we have circumvented the major handicap of dermaseptins and their derivatives observed before, i.e., their hemolytic activity [123,139,140]. The most active OAK, lauryl-lysyl-3α_{12}, displayed an IC_{50} of 0.08 μM and a selectivity ratio of >1,000. Although it was not possible to discriminate between the lysis of infected cells and the lysis of the uninfected cohort, the overall lysis was very small at the relevant antiplasmodial concentrations. These results indicated that the OAKs did not exert their antimalarial action by lysis of infected RBC, as was the cases with the parent dermaseptins [123] and pointed to the potential of the OAQ system to generate highly selective low-cost compounds that might be useful in fighting Malaria.

4. Conclusions

When examining nearly a thousand sequences of natural AMPs (http://www.bbcm.univ.trieste.it/~tossi), one can find neither a meaningful consensus sequence nor even a common secondary structure. The only common feature is the occurrence of both hydrophobic and positively charged amino acids, suggesting that in principle, the requirements for antimicrobial activity do not include a defined secondary structure. Moreover, while SAR studies of AMPs have pointed to probable sites of action they failed in providing an optimal resolution as to the individual importance of molecular hydrophobicity and positive charge. Such an opportunity was considerably approached via peptidomimetics. Namely, the inherently simple and incremental nature of the OAQ libraries, provide a systematic tool for the dissection of the relative importance of charge and hydrophobicity and pin down their respective role(s). In this respect, since the available data support the concept that a defined secondary structure does not necessarily play a determining role, activity can be made to depend solely on a subtle interplay between positive charge and hydrophobicity. One might anticipate therefore, that AMP-mimetics can be useful in better understanding the role and function of host defense peptides and might also considerably increases the potential of uncovering low-cost highly selective derivatives that will be beneficially utilized in various antimicrobial fields, including in systemic treatment of medical conditions associated with MDR pathogens.

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