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Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes?

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Abstract

Cationic antibacterial peptides are produced in all living organisms and possess either selective activity toward a certain type of cell or microorganism, or a broad spectrum of activity toward several types of cells including prokaryotic and mammalian cells. In order to exert their activity, peptides first interact with and traverse an outer barrier, e.g., mainly LPS and peptidoglycan in bacteria or a glycocalix layer and matrix proteins in mammalian cells. Only then, can the peptides bind and insert into the cytoplasmic membrane. The mode of action of many antibacterial peptides is believed to be the disruption of the lipidic plasma membrane. Therefore, model phospholipid membranes have been used to study the mode of action of antimicrobial peptides. These studies have demonstrated that peptides that act preferentially on bacteria are also able to interact with and permeate efficiently anionic phospholipids, whereas peptides that lyse mammalian cells bind and permeate efficiently both acidic and zwitterionic phospholipids membranes, mimicking the plasma membranes of these cells. It is now becoming increasingly clear that selective activity of these peptides against different cells depends also on other parameters that characterize both the peptide and the target cell. With respect to the peptide's properties, these include the volume of the molecule, its structure, and its oligomeric state in solution and in membranes. Regarding the target membrane, these include the structure, length, and complexity of the hydrophilic polysaccharide found in its outer layer. These parameters affect the ability of the peptides to diffuse through the cell's outer barrier and to reach its cytoplasmic plasma membrane.

Keywords: Antimicrobial peptides; LPS; Peptidoglycan; Glycocalix layer; Model phospholipids membranes; Peptide–lipid interactions

1. Introduction

Interactions between bioactive peptides and cell membranes play an important role in many cellular processes. A major group within these peptides includes a large number of membrane-active peptides, termed antimicrobial peptides, used as the first chemical barrier between all organisms and microbes. These peptides are activated, in response to bacterial infection, by a regulatory process and are produced by almost all species of life, ranging from microorganisms and plants to animals, including humans [1–4].

Antimicrobial peptides are produced predominantly in the animal's most exposed tissues (e.g., skin, eye, and lungs) which are most likely to come in contact with microorganisms (for a recent review, see [5]). In higher organisms they are produced mainly on epithelial surfaces and in phagocytic cells. They are rapidly synthesized at low metabolic cost, easily stored in large amounts, and readily available shortly after microbial infection to kill a broad spectrum of microbes. At present, more than 700 such peptides have been reported (see a complete list at http://www.bbcm.univ. trieste. it/∼ tossi/pag1. htm). These peptides vary significantly in their sequences, length, and structures. Many of them are linear whereas others are cyclic due to the existence of one or more disulfide bridges. Some adopt α-helical, β-sheet or both β-sheet and α-helical structures [1,6–9]. Despite the different folding characteristics, most peptides seem to adopt an amphipathic arrangement with opposing hydrophobic and positively charged faces when they are in contact with the bacterial membrane. However, recent studies revealed that it is possible to mimic the basic properties of native antimicrobial peptides, namely, a threshold hydrophobicity, a net positive charge and amphipathicity (not necessarily an amphipathic α-helical or β-sheet structures) by introducing D-amino acids into lytic peptides [10–13] or by using non-natural amino acids [14–18], or even polymers and oligomers of a repeating unit [19,20].

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Interestingly, many antimicrobial peptides that share similar structures differ considerably in their spectrum of activity; they can be classified into two major groups: (i) peptides that are highly potent against bacteria but not against normal mammalian cells and fungi; e.g., insect cecropins, which are active only on Gram-positive bacteria [21], magainins [22], dermaseptins [23], and short model diastereomeric peptides (containing only Leu and Lys) [13], which are active on both Gram-negative and Gram-positive bacteria; (ii) peptides that are toxic to both bacteria and normal mammalian cells, such as melittin [24], pardaxin [25], gramicidins [26], bombinins H 3–5 [27,28], and the human cecropin-like LL-37 [29,30]. Since fungi and mammalian cells are both eukaryotic cells, peptides that are active on mammalian cells have the potential to also kill fungi. Examples include pig cecropins [31–33], the amphibian buforins [34], human histatin [35], indolicidin isolated from cattle [36], tachyplesin II isolated from horseshoe crab [7], protegrin I from pig [37,38], mammals alpha and beta defensins [31] and insect defensins [39,40]. However, there are several antimicrobial peptides that are active solely on fungi and not on bacteria or mammalian cells [33,41]. Examples include drosomycin from fruit fly [7], and the plant defensins [42]. The question is, therefore, how can different cells have different susceptibilities toward a certain antimicrobial peptide?

It is generally assumed that most antimicrobial peptides disrupt and permeate the target cell membrane, which result in an irreversible damage that is hard to fix [41,43,44]. Lehrer et al. [45] reported, for the first time, that the mechanism of killing in intact bacteria includes the membrane permeation step. They showed that human neutrophil peptide defensin [HNP]-mediated bactericidal activity against *Escherichia coli* is associated with sequential permeabilization of the outer and inner membranes, and that inner membrane permeabilization appears to be the lethal event. In this study they utilized the ability of the normally impermeable substrate *o*-nitrophenyl galactoside (ONPG) to be hydrolyzed by a cytoplasmic enzyme L-galactosidase as a test of increased permeability. Therefore, many studies were focused on the bacterial phospholipid membrane as the main target of these peptides. The inner membrane of Gram-negative bacteria and the single membrane of Gram-positive bacteria are composed of negatively charged phospholipids. In contrast, the phospholipids comprising the outer surface of the membrane of normal mammalian cells are composed predominantly of zwitterionic phospholipids and cholesterol. Therefore, the net positive charge, which is the most conserved property of antimicrobial peptides, allows them to bind preferentially to the bacterial negatively charged membrane [46–49].

In line with this, many studies were performed at the level of model membranes mimicking the phospholipid membranes of bacteria and mammalian cells, with an attempt to predict the connection between the properties of the membrane and biological activity. However, it is clear that there is a large difference between living cells and model membranes. This is mainly because peptides need to cross the cell wall, an external barrier, before reaching the cytoplasmic membrane, a process that is dependent on several properties of the peptides such as structure and oligomeric state. Many pathogens including bacteria, yeast, and fungi are surrounded, in addition to the plasma membrane, by an external barrier, which contains mainly polysaccharide compounds.

This review summarizes recent studies dealing with the role of the cell wall in preventing potent membrane-active antimicrobial peptides from reaching the cytoplasmic target membranes, as well as the role of peptide structure and assembly in traversing these cell walls and to bind and increase the permeability of the cytoplasmic membrane.

2. Peptide-membrane interaction and antimicrobial activity: studies with model membranes

A common characteristic of most antimicrobial peptides is their net positive charge. Many studies demonstrated that they bind and increase preferentially the permeability of negatively charged phospholipid membranes, which constitute the main component of the bacterial cytoplasmic membrane. Overall, there is a correlation between the capacity of antimicrobial peptides to bind and permeate phospholipid membranes and their biological function. Non-hemolytic antimicrobial peptides bind strongly and permeate efficiently negatively charged phospholipid membranes that mimic the bacterial membrane much better than zwitterionic membranes [8,9,50–52], which are the major constituents of the outer leaflet of erythrocytes [53]. Some examples include magainins, dermaseptins, cecropins, cathelicidins, protegrins, defensins, temporins and many others [21–23,54–56]. In addition, the biological activity of many non-cell-selective antimicrobial peptides can be predicted, based on their ability to interact and disrupt both negatively charged and zwitterionic membranes. A few examples will elaborate this issue:

(i) The cecropin-like human peptide LL-37 is an amphipathic α-helical antimicrobial peptide that belongs to the cathelicidin family and is highly toxic toward bacteria, but it also possesses lytic activity toward mammalian cells [57,58]. The peptide binds and permeates efficiently both zwitterionic and negatively charged phospholipid vesicles, and is very resistant to proteolytic degradation when bound to these model membranes [59]. That LL-37 does not distinguish between zwitterionic and negatively charged lipid vesicles suggests the involvement of hydrophobic interactions between this peptide and the vesicles. Indeed, LL-37 reaches and stays in phosphatidylcholine membranes as oligomers that contain bundles of hydrophobic N-terminals that bind strongly to model membranes independent of the charge of the phospholipid headgroups [59]. (ii) Pardaxin is a shark repellent lytic neurotoxin, isolated from the Red Sea Moses Sole *Pardachirus marmoratus* and from the Peacock Sole of the western Pacific *Pardachirus pavoninus* [25,60,61]. Pardaxin is highly toxic to both bacteria

and mammalian cells [62–64]. Studies with model membranes revealed that it binds and permeates efficiently both zwitterionic and negatively charged phospholipid vesicles. Structural and functional studies with various pardaxin analogs and truncated forms showed that the C-terminal part in its sequence is important for its assembly within the zwitterionic membrane milieu [62,64]. In line with this, the C-terminal-deleted analogs of pardaxin preserved the antimicrobial activity of the intact pardaxin, but their hemolytic activity was significantly reduced [62]. (iii) That hemolytic and membranolytic activities are both influenced by peptide assembly within membranes was recently illustrated by covalently linking peptide molecules into undissociable peptide oligomers. This was done by using diastereomeric (containing both L- and D-amino acids) cationic peptides with variable lengths (13, 16, and 19 amino acids long) and their covalently linked pentameric bundles (oligomers) [65]. Whereas the monomeric peptides were practically devoid of hemolytic activity, the pentameric forms were highly active on human erythrocytes. Concomitant with the biological function, all the monomeric peptides, regardless of their length, displayed a low affinity toward zwitterionic membranes. In contrast, all the bundles bound strongly and irreversibly to these membranes and disrupted them. Furthermore, peptide assembly seemed to affect its structure, as was observed from its increased α-helical and β-sheet contents, which also enhanced acyl chain disruption in PC/cholesterol membranes. In addition, there was a correlation between the antibacterial activity of the peptides and their ability to depolarize the transmembrane potential of *E. coli* spheroplasts (that lack the bacterial LPS and peptidoglycan), as well as the ability to bind negatively charged vesicles and to induce leakage of calcein from them. (iv) Studies with dermaseptins, antimicrobial peptides isolated from frog's skin [23], revealed that mutants that were able to assemble in solution could better permeate zwitterionic membranes, concomitant with their increased hemolytic and antifungal activity [66,67]. Truncated forms and mutants of dermaseptin S4 demonstrated that the hydrophobic N-terminal is responsible for its oligomerization and cytotoxicity toward erythrocytes.

Similarly to LL-37, oligomerization of dermaseptin S4 would form a bundle of hydrophobic N-terminals that could initiate binding to the zwitterionic membranes of erythrocytes, thus causing their lysis.

Recently, attempts to decrease hemolytic activity without affecting antimicrobial activity resulted in the synthesis of diastereomeric peptides (containing both L- and D-amino acids in their sequence). These include diastereomers of both native and de novo-designed peptides, such as melittin [68], pardaxin [10] and Lys and Leu-containing peptides [11]. These diastereomers had higher affinities toward negatively charged membranes compared to zwitterionic model membranes. Several all L-amino acid peptides containing only Lys and Leu have shown previously to be both antimicrobial and hemolytic [69]. It was suggested that the introduction of D-amino acids within the sequences of the parental amphipathic α-helical peptides would make it more difficult for them to form an amphipathic structure unless they are located very close to the hydrophobic membrane. Since they are positively charged they are better attracted to negatively charged membranes via electrostatic interactions. Nevertheless, if the peptides are highly hydrophobic, the incorporation of D-amino acids does not prevent their binding to both zwitterionic and negatively charged membranes, and therefore, they are less selective to bacteria [70]. High hydrophobicity should force a strong partition of the peptide into the hydrophobic core of the lipid bilayers, regardless of the phospholipid head group, thereby permeabilizing membranes of both eukaryotes and prokaryotes [70]. Another interesting group of diastereomeric peptides was recently reported [71]. This group includes six- and eight-residue cyclic D- and L-amino acid-containing alpha-peptides that act preferentially on Gram-positive and/or Gram-negative bacterial membranes compared to mammalian cells. These peptides were reported to assemble in phospholipids membranes to form nanotubes. Recently, Deber and coworkers also reported on antimicrobial peptides derived from transmembrane segments of membrane proteins with double D-amino acid replacements and a few lysins at both termini. Their antimicrobial activity was dependent on the position of the D-amino acids [72].

A correlation between antimicrobial activity and binding and disrupting model membranes was also found in a new family of peptides which contained β, instead of α, amino acids. Beta-17, for example, was found to promote negative curvature and reduce bilayer formation in negative membranes [73]. Studies on its ability to permeate liposomes indicated a different mode of membrane interaction compared to the α-amino acid peptide magainin 2. However, both leakage studies, membrane-binding experiments, and biological activity assays showed that beta-17, similar to magainin 2, had a strong affinity to negatively charged membranes and had selective antimicrobial activity [15,73,74].

In contrast to what has been previously discussed, several antimicrobial peptides bind and permeate both zwitterionic and negatively charged membranes and yet they are not hemolytic [75–77]. Furthermore, it seems that a negatively charged surface is not sufficient for cell wall disruption because human erythrocytes, for example, contain a large number of highly negatively charged sialic acid-containing carbohydrate moieties in the form of glycoproteins and glycosphingolipids, which form their outer glycocalix layer. Despite this, erythrocytes are not affected significantly by most naturally occurring antimicrobial peptides. A possible explanation is that the peptides are probably attached first to the glycocalix layer via electrostatic interaction. However, because most cationic non-hemolytic antimicrobial peptides have low affinity to zwitterionic membranes, it makes it difficult for them to be released from the anionic glycocalix barrier and to be partitioned into the cell phospholipid membrane.

All the studies just described suggest that the lethal step in most cationic antimicrobial peptides is the disruption of the cytoplasmic membrane. This process is accomplished in two steps: membrane binding and membrane insertion/permeation. Therefore, understanding the parameters involved in the mechanism of membrane permeation is crucial for the development of antimicrobial peptides as future antibiotics.

3. Mechanisms of membrane disruption by antimicrobial peptides

Two general mechanisms were originally proposed to describe the process of phospholipid membrane permeation by membrane-active peptides. These were the "barrel-stave" [78] and the "carpet" [79,80] mechanisms. The major difference between these two mechanisms is that in the barrel stave model only a few molecules can pinch a pore in the membrane, whereas in the carpet mechanism the membrane needs to be covered before permeation appears. Accumulating data suggest that the permeating pathway depends on both the peptide and the membrane. More specifically, according to the "barrel-stave model", membrane-bound peptides recognize each other, oligomerize, and form transmembrane pores. Theoretically, such pores can be formed from as few as three molecules. To allow pore formation, the inserted molecules should have distinct structures, such as an amphipathic α-helix, hydrophobic α-helix, β-sheet or both α-helix and β-sheet structures. Peptides that act via this mechanism should presumably kill bacteria below the experimentally observed micromolar concentrations, becoming lethal once they penetrate into the phospholipid membrane of the target cell. On the other hand, according to the "Carpet model" [44,79,80,81], peptides bind the phospholipid membrane surface until a threshold concentration is reached, and only then permeate it in a detergent-like manner. The detailed permeation/disruption mechanism can vary between the different peptides, and could be disintegration in a detergent-like manner [44], torroidal pores [82,83], or channel aggregates [84]. In contrast to the barrel stave mechanism, the carpet mechanism requires neither a specific peptide structure nor the formation of structured channels. It should be emphasized that, according to the carpet mechanism, the peptide's positively charged amino acids are spread along its chain, and are continuously in contact with the lipid head group during the process of membrane permeation. Since channel formation requires the penetration of the peptides into the hydrophobic core of the membrane, the interaction of these peptides with the membrane is predominantly governed by hydrophobic interactions. Therefore, peptides that act via the barrel stave mechanism can interact strongly with both zwitterionic and negatively-charged membranes, and therefore are non-cell-selective [44].

Very recently, surface plasmon resonance (SPR) was used to study the interaction between lytic peptides with both lipid monolayers and bilayers with different lipid compositions [20,85,86]. These studies allowed differentiating between the above proposed mechanisms. The peptides investigated were from two major families: (i) the bee venom melittin, as a model of a non-cell-selective peptide that forms transmembrane pores; and (ii) magainin and a diastereomer of melittin (four amino acids were replaced by their D-enantiomers), as models of bacteria-selective and non-pore-forming peptides. Fitting the SPR data to different interaction models allowed differentiating between two major steps: membrane binding and membrane insertion. It was found that melittin binds to PC/cholesterol ∼450-fold better than its diastereomer and magainin, mainly because it is inserted into the inner leaflet (2/3 of the binding energy), whereas the other two peptides are not. In contrast, there was only a slight difference in the binding of all the peptides to negatively charged PE/PG mono- and bilayer membranes (in the first and second steps), indicating that the membrane's inner leaflet contributes only slightly to their binding to PE/PG bilayers. Furthermore, the 100-fold stronger binding of the cell-selective peptides to PE/PG, compared with PC/cholesterol, resulted only from electrostatic attraction to the negatively charged headgroups of the membrane's outer leaflet. Indeed, these results could clearly differentiate between the two general mechanisms: pore formation by melittin only in zwitterionic membranes, and a detergent-like effect (carpet mechanism) for all the peptides in negatively charged membranes.

A difference between the affinities of the peptides to lipid monolayers compared to bilayers indicates the contribution of the membrane's inner leaflet to the binding process. The ratio $K_{\text{A bilayer}}/K_{\text{A monolayer}}$ can give an indication on the depth of penetration into the membrane core. A ratio of ∼1 indicates that the peptide is surface localized, and a ratio above ∼10 indicates that the peptide inserts into the lipid bilayer [20] (and unpublished results). Very recently, experiments performed with all L-amino acid model peptides and their diastereomers (containing both L- and D-amino acids) demonstrated that the binding of the diastereomers to PC/cholesterol monolayers is similar to their binding to bilayers, indicating that the diastereomers are not influenced by the inner layer in agreement with the carpet mechanism [87]. On the other hand, their binding to PE:PG monolayers is approximately three-fold lower than to bilayers. Compared to the diastereomers, their all L-amino acid parental peptides and the two pore forming peptides pardaxin and melittin, bind 15–25-fold stronger to bilayers compared to monolayers [20] (and unpublished results) (Fig. 1).

4. Disrupting the bacterial inner membrane is not the only killing mechanism of antimicrobial peptides

It has been reported that some antimicrobial peptides are active against one bacterial species but not against another, although their inner membranes have similar phospholipid compositions [13]. This could be due to differences in the

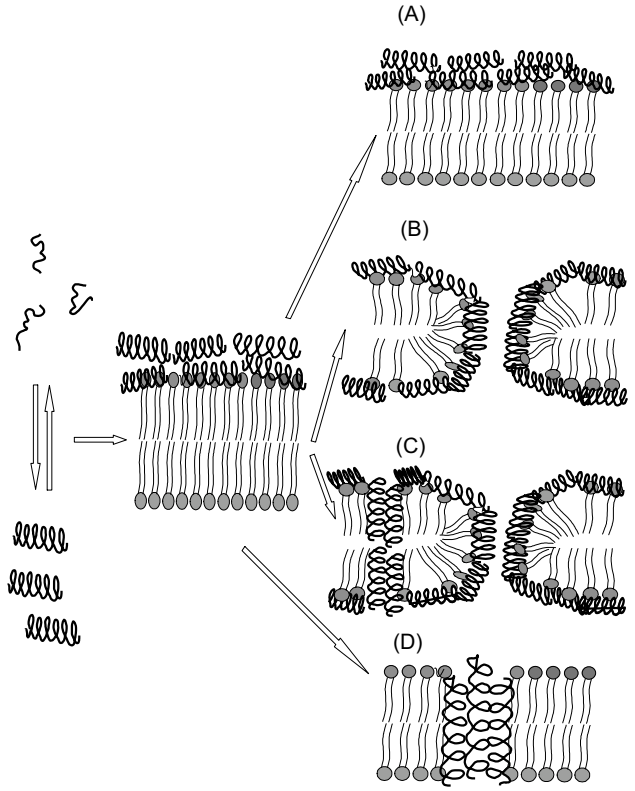

Fig. 1. Schematic representation of the different mechanisms of membrane lysis by the peptides. In the first step (binding step), which is mainly governed by electrostatic interactions, all the peptides align parallel to the outer membrane bilayer surface. In contrast, the following step (the insertion step) is different for each peptide; in the case of the diastereomers interacting with PC/cholesterol membrane (panel A), the peptides align parallel to the outer membrane surface without disturbing the membrane structure. In the case of the all-L model peptides and their diastereomeric counterparts in PE:PG membrane (panel B), the peptides align parallel to the outer membrane surface disturbing the membrane structure. During the whole process, the peptides remain in contact with the head group region of the lipid bileyer [79,80,99,100]. In the case of the all-L model peptides and pardaxin binding to zwitterionic and anionic lipids, respectively, it is possible that both 'carpet like' and 'pore-forming' mechanisms are involved (panel C). Finally, in the case of pardaxin in PC/cholesterol membrane (panel D), the peptide insert deeply into the hydrocarbon region of the target cell membrane and forms transmembrane pores by the assembly of several monomers via a pore-forming ('barrel stave') mechanism. The exterior of the formed helical conformation contacts with the hydrocarbon region of the bilayers [78].

outer membranes of the various strains. These membranes are the first barrier the peptides encounter and through these membranes the peptides need to traverse in order to reach the inner cytoplasmic membrane. In addition, whereas Gram-negative bacteria contain two discrete membranes with different compositions, Gram-positive contain only one. The outer membrane of Gram-negative bacteria is an asymmetric membrane with glycolipid lipopolysaccharides (LPS) covering more than 90% of the cell surface in its outer leaflet and phospholipids with a composition similar to that of the cytoplasmic membrane in its inner leaflet. Initially, the peptides interact with the polyanionic LPS exterior and competitively dislocate the divalent cations that partially neutralize the LPS (for more details, see Hancock's review in [32]). This causes the disruption of the outer membrane, and it is through these disrupted outer membranes that the peptides translocate. In the next step, the peptides reach the inner phospholipid membrane, bind to it and finally disrupt and disintegrate it. Disintegration of this membrane will eventually kill the cell. Therefore, predicting the biological potency of antimicrobial peptides from their activity on model phospholipid membranes that do not contain this barrier is not straightforward. Parameters that contribute to the ability of the peptides to cross the cell wall include their secondary and tertiary structures, as well as their oligomeric state. The larger the molecule, the more difficult is it to cross this barrier.

A few examples will elaborate this issue. (i) A cyclic analog of magainin was synthesized by incorporation of cysteins at both the N- and C-termini of the linear peptide and formation of disulfide bond [88]. It was found that at similar molar ratios of bound peptide:lipid, both linear and cyclic magainins showed similar membrane permeation activity in both zwitterionic and negatively charged phospholipid membranes, indicating that linearity is not required for membrane permeation. However, the finding that cyclic magainin was less active than linear magainin in the killing of bacteria, points to the importance of linearity in this case in reaching the target antibacterial inner phospholipid membrane. To reach this membrane, peptides must cross the outer barriers, a process that should be easier for the linear molecule compared to the bulky cyclic one. Cyclic magainin should have a larger volume and less flexibility compared with the linear version. Therefore, it is probably more difficult for the cyclic analog than for the linear magainin to cross these barriers. In addition, the five positively charged amino acids in magainin are distributed along its amphipatic helix. Upon cyclization, all the positive charges, including the N-terminal-free amine, are distributed throughout the ring, and the peptide thus becomes less helical. These changes can induce more efficient binding of the cyclic magainin analog to the negatively charged cell wall components, and hence make it more difficult for the cyclic form to diffuse into the inner target phospholipid membrane. (ii) A group of model amphipathic all L-amino acids and their diastereomers were synthesized. The sequence of the peptides was $KX_3KWX_2KX_2K$, where X = Gly, Ala, Val, Ile, or Leu. Functional studies showed that peptides in which X = Leu, Ile or Val are highly active in the permeation of negatively charged vesicles [89]. However, only the Leu-containing peptide was bactericidal, whereas the Ile and the Val-containing peptides had no antimicrobial activity. Studies on their oligomeric state showed that in contrast to the Leu-containing peptide that formed predominantly dimers in SDS–PAGE, the Ile- and the Val-containing peptides formed high-order oligomers. The large size of these oligomers prevent them from penetrating through the bacterial outer wall into the bacterial

Table 1
Designations and sequences of de novo designed L-amino acids and their corresponding diastereomers (taken from [13])

Peptide designation	Sequence[a]
L-aa peptides	
Amphipathic-1L	L K L L K K L L K K L L K L L-NH_2
Scrambled-4L	K L K L L K L L K L L K L L K-NH_2
Segregated-5L	K K K L L L L L L L L L K K K-NH_2
Diastereomers	
Amphipathic-1D	L K ***L*** L K ***K*** L ***L*** ***K*** K L L ***K*** L L-NH_2
Amphipathic-2D	L L ***K*** K L ***L*** K ***L*** ***L*** L K L ***L*** K K-NH_2
Amphipathic-3D	L L ***K*** L L ***K*** K ***L*** ***L*** K K L ***L*** K L-NH_2
Scrambled-4D	K L ***K*** L L ***K*** L ***L*** ***K*** L L K ***L*** L K-NH_2
Segregated-5D	K K ***K*** L L ***L*** L ***L*** ***L*** L L L ***K*** K K-NH_2
Segregated-6D	L L ***L*** L L ***K*** K ***K*** ***K*** K K L ***L*** L L-NH_2

[a] Italicized and bold amino acids are D-enantiomers. All the peptides are amidated in their C-terminus.

cytoplasmic phospholipid membrane. However, in a model membrane system the peptides interact directly with the exposed lipid membrane and therefore oligomerization of the peptides should not prevent them from direct interaction with the membrane. (iii) Recently a series of short lytic peptides composed of only leucines and lysines (15 amino acids) and their diastereomers (containing five D-amino acids in their sequence) were synthesized and functionally and structurally investigated (see list in Table 1) [13]. Among the diastereomers, three were designed to fold into an ideal amphipathic α-helix in their all L-amino acid form but with a different distribution of the D-amino acids along the hydrophobic and hydrophilic faces; the fourth one had a scrambled sequence of hydrophobic and hydrophilic amino acids, and in the other two peptides the hydrophobic and hydrophilic amino acids were grouped (in the primary structure). It was found that all the peptides had similar potencies in increasing the permeability of negatively charged vesicles (containing phosphatidylethanolamine, PE, and phosphatidylglycerol, PG), and also induced similar activities on *E. coli* spheroplasts (which do not have LPS and peptidoglycan in their outer surface). These results were not expected because of the major alteration in the sequence and structure of these peptides. In contrast, the spectrum of antimicrobial activity of the peptides was different from that of the segregated peptides, which were less active than the amphipathic or the scrambled ones (see Table 2). This suggests that the differences in the potencies of the diastereomers result from their different abilities to diffuse through the bacterial wall to reach the cytoplasmic membrane, which depend on their different structures. However, once the diastereomers cross this barrier, they behave similarly in the permeation of the cytoplasmic membrane.

5. The role of the outer membrane and lipopolysaccharide (LPS) in antimicrobial activity—studies with intact bacteria

The influence of bacterial LPS on the peptides biological function was demonstrated in the case of the bee venom melittin known as a non-cell-selective lytic peptide. Although it is believed that melittin kills bacteria by directly interacting and permeating the phospholipid's membrane, bacteria sensitivity toward melittin has been shown to vary among different bacterial species. It was found that LPS caused high resistance to melittin-induced cell leakage, indicating that LPS in Gram-negative bacteria offers strong protection against the lytic effects of melittin. This resistance is due in part to the tight packing of the lipid acyl chains in the LPS layers. Moreover, the addition of bacterial phospholipids to LPS bilayers increased their sensitivity to melittin. This is in line with the finding of different sensitivities to melittin of deep rough bacteria compared to smooth phenotypes [90]. There are at least two major differences in the lipids in the outer

Table 2
Minimal inhibitory concentrations of the peptides (μM) and their hemolytic activity (taken from [13])

Peptide designation	*E. coli* ATCC 25922	*E. coli* D21	MRSE[a] LT1324	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 19606	Micrococcus luteus	Staphylococcus simulans	*S. aureus* II ATCC 6538P	Percent hemolysis at 100 μM
L-aa peptides									
Amphipathic-1L	13	13	4	25	1	2	2	3	100
Scrambled-4L	3	3	1.5	6	2	2	1.5	6	82
Segregated-5L	50	50	32	>50	50	16	32	50	66
Diastereomers									
Amphipathic-1D	7	4	1	3	13	0.5	1	13	0
Amphipathic-2D	22	6	0.5	6	11	0.5	1	25	0
Amphipathic-3D	11	5	1	3	10	0.5	1	45	0
Scrambled-4D	6	3	1	6	15	0.4	1	25	0
Segregated-5D	44	14	1	50	50	0.3	1	>50	3
Segregated-6D	>56	7	1	8	50	0.5	1	>50	0

[a] Methicillin resistant Staphylococcus epidermidis.

monolayers of the outer membranes of these different mutants. The first difference is in the structure of the LPS molecule, in particular, the length and complexity of the hydrophilic polysaccharide region. A second difference between these mutants is that the phospholipid:LPS ratio in the outer monolayer of the outer membrane of deep rough bacteria is larger than that of smooth mutants, making rough bacteria more sensitive to melittin. Overall, in this specific case there was no correlation between biological activity and interaction with the negatively charged phosphatidylglycine (PG). Note that the actual lipid composition of the bacterial membrane also includes about 70% phosphatidylethanolamine (PE) which might give different results compared to PG alone.

The role of the length of the polysaccharide of outer membrane on antimicrobial activity was also investigated by using magainin 2 [91]. This peptide was found to modify the thermotropic properties of the outer membrane-peptidoglycan complexes derived from wild-type *Salmonella typhimurium* and a series of LPS mutants, which displayed a different susceptibility toward this cationic peptide. LPS mutants showed a progressive loss of resistance to killing by magainin 2, as the length of the LPS polysaccharide moiety decreased. Although disruption of the outer membrane structure is most likely not the primary factor leading to cell death, the susceptibility of Gram-negative cells to magainin 2 has been proposed to be associated with factors that facilitate the transport of the peptide across the outer membrane, such as the magnitude and location of LPS charge, the concentration of LPS in the outer membrane, the outer membrane molecular architecture, and the presence or absence of the O-antigen side chain.

The inability to predict biological function from the bacterial outer membrane alone has been shown also by Hancock and coworkers, who investigated three structural variants of the horseshoe crab cationic antimicrobial peptide polyphemusin I with improved amphipathic characters [92]. All the peptides showed similar abilities to bind bacterial LPS. Consistent with their high affinity to LPS, polyphemusin I and its variants were able to increase the permeability of the bacterial outer membrane to a similar extent, as shown by using the N-phenylnaphthylamine uptake assay. Nevertheless, in terms of their activity against intact Gram-negative bacteria, the variants were moderately less active in vitro but more effective in animal models. However, a direct correlation was found between the inability of the peptides to bind zwitterionic lipids and their lower hemolytic activities. Furthermore, compared with polyphemusin I, all variants showed reduced ability to interact with anionic lipids, in agreement with their lower efficacy to depolarize the cytoplasmic membrane of *E. coli*, as assessed using a membrane potential sensitive fluorescent dye 3,3-dipropylthiacarbocyanine (diSC(3)5) assay. Note also that the ability of the mutants to interact and permeate membranes did not correlate with their killing mechanism. This was demonstrated by the finding that depolarization of the bacterial cytoplasmic membrane potential by polyphemusin I occurred prior to lethal damage to cells [92].

6. Multi-drug resistant (MDR) proteins and membrane-binding domains can influence the peptides activity and specificity toward bacteria

The association of an antimicrobial peptide with the bacterial membrane's phospholipids is only a partial process among the overall interactions between the peptide and the living microorganisms or cells. Recent reports suggest that bacteria produce multi-drug resistant (MDR) proteins that inhibit by different ways the action of some antimicrobial peptides. Note also that some antimicrobial peptides use a receptor located on the target cells, even though their killing mechanism in presumably via membrane destabilization. A few examples illustrate these cases: (i) Several isolates of the human pathogen *Streptococcus pyogenes*, which include virulent strains of the M1 serotype, secrete the protein SIC [93]. This protein is found to inactivate human neutrophil α-defensin and LL-37, known to be involved in bacterial clearance in humans. The fact that SIC is found to be secreted in large amounts by *S. pyogenes* and in many variations, can explain the high frequency of *S. pyogenes* infections caused by the M1 serotype. (ii) It has been suggested that the apparent ineffectiveness of several plant antimicrobials peptides is largely due to the MDR proteins expressed in many plant pathogens. In agreement with this, a recent study showed that the activities of plant antimicrobial peptides were considerably greater against bacteria in which their MDR proteins were inhibited [94]. (iii) The first identified peptide that used a receptor-mediated mode of action against its target cells was nisin Z. This peptide is active at nanomolar concentrations and has a receptor-binding domain as well as a membrane-interacting and insertion domain. It is hypothesized that the receptor-binding site helps to increase its affinity to the membrane and only then the peptide can act on the membrane by increasing its permeability similarly to other cationic antimicrobial peptides. More specifically, nisin Z uses the membrane-anchored cell wall precursor Lipid II as a receptor [95]. It is not clear, however, how the pore-forming domain permeates the target membrane. Further studies revealed that when the receptor-binding domain is removed, the peptide becomes active at a micromolar concentration toward several bacteria similar to most antimicrobial peptides that act via a non-receptor mediated mechanism. (iv) Mesentericin Y also uses a receptor for its function. This receptor is located only on the specific bacteria *listeria* [96]. Similarly to nisin, removal of the receptor-binding domain in mesentericin resulted in a loss of bacteria-specific selectivity. The resultant analog (that lacks the binding domain) became active toward this and other bacteria at a micromolar range.

7. Anitimicrobial peptides need to cross the glycocalix layer in order to partition into the zwitterionic plasma membrane of erythrocytes

The phospholipids comprising the membrane of normal mammalian cells are asymmetrically distributed; the outer leaflet is composed predominantly of zwitterionic phosphatydilcholine (PC) and sphingomyelin (SM) phospholipids, whereas the inner leaflet is composed of negatively charged phosphatydilserine (PS) [53]. This is the reason for the use of PC-containing liposomes in most studies to mimic the outer surface of mammalian cells. However, human erythrocytes, for example, contain also a large number of highly negatively charged sialic acid-containing carbohydrate moieties in the form of glycoproteins and glycosphingolipids, which form their outer glycocalix barrier. Therefore, cationic antimicrobial peptides need first to cross this layer in order to reach the cytoplasmic PC membrane. The peptides' hydrophobicity is an important factor in this process. For example, studies with two closely related magainin analogs with enhanced hydrophobicity, magainin-H1 and magainin-H2, showed similar activities toward model zwitterionic membranes. However, the more hydrophobic peptide, magainin-H1, was more active against intact erythrocytes [97]. An increase in hydrophobicity, which was the result of an increase in its helical content, increased the ability of the H1 analog to diffuse through the glycocalix barrier of the cells and to better partition into their zwitterionic plasma membrane.

In another study, a group of short model diastereomeric lytic peptides containing D-amino acids at different positions along their sequence were found to be non-hemolytic, despite the fact that all of them were able to bind strongly and increased the permeability of zwitterionic membranes similarly to negatively charged membranes [13]. A possible explanation is that the peptides bound strongly to the negatively charged glycocalix layer, probably similar to many cationic antimicrobial peptides. However, because of the incorporation of D-amino acids within their sequences, it is hard for them to adopt an ampiphatic structure, which is required for efficient binding to phospholipid membranes. Therefore, they do not partition well into the cytoplasmic zwitterionic membrane of the erythrocytes but rather, stick within the gligocalix layer.

Although selectivity of antimicrobial peptides for bacterial membranes may result, in part, from the preferential display of anionic residues in these membranes, the inability to interact with or bind to zwitterionic phospholipids offers no guarantee that the peptide will lack considerable toxicity toward normal mammalian cells. For example, recently, Epand and coworkers investigated the properties of two amphipathic peptides with identical size and amino acid composition: one formed an α-helical structure and the other a β-sheet structure [98]. They found that both of them were highly hemolytic. Nevertheless, only the α helical peptide was a potent promoter of negative curvature in zwitterionic membranes. Moreover, this peptide could bind and permeate both zwitterionic and anionic liposomes, whereas the β-sheet peptide interacted only with the anionic ones. In other words, although having similar activities against intact erythrocytes, the helical peptide was much more lytic than the β-peptide against zwitterionic PC liposomes, and against liposomes composed of lipids extracted from either sheep or human erythrocytes.

In summary, the ability of most cationic antimicrobial peptides to interact and permeate model phospholipids membranes is crucial for their biological function. However, interaction with model membranes cannot guarantee that the peptide will be biologically active, since biological activity depends also on other parameters that characterize both the peptide and the target cell. These include the oligomeric state of the peptide in solution and membranes, the structure of the peptide, the stability of the active structure (i.e., the energy required to change its conformation from an unordered structure in solution to an amphipatic structure in the lipidic membrane), the overall distribution of the positive charges and hydrophobic amino acids along the peptide chain, and the type and width of the cell wall covering the cytoplasmic phospholipid membrane of the cell. Studies that will take into consideration all of these parameters might better address the actual mode of action of antimicrobial peptides and assist in the development of this group of antimicrobial peptides that act via a new mode of action, as future therapeutic agents to combat bacterial and fungal infections.

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