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Review

Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides

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Abstract

Permeation of the cell membrane leading to cell death is a mechanism used by a large number of membrane-lytic peptides. Some are linear, mostly helical, and others contain one or more disulfide bonds forming β-sheet or both β-sheet and α-helix structures. They are all soluble in solution but when they reach the target membrane, conformational changes occur which let them associate with and lyse the membrane. Some lytic peptides are not cell-selective and lyse different microorganisms and normal mammalian cells, while others are specific to either type of cells. Despite extensive studies, the mode of action of membrane-lytic peptides is not fully understood and the basis for their selectivity towards specific target cells is not known. Many studies have shown that peptide-lipid interactions leading to membrane permeation play a major role in their activity. Membrane permeation by amphipathic α-helical peptides has been proposed to occur via one of two general mechanisms: (i) transmembrane pore formation via a 'barrel-stave' mechanism; and (ii) membrane destruction/solubilization via a 'carpet' mechanism. This review, which is focused on the different stages of membrane permeation induced by representatives of amphipathic α-helical antimicrobial and cell non-selective lytic peptides distinguishes between the 'carpet' mechanism, which holds for antimicrobial peptides versus the 'barrel-stave' mechanism, which holds for cell non-selective lytic peptides.

Keywords: Antimicrobial peptides; Peptide-membrane interaction; Lytic peptides

Contents

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1. Introduction

Membrane-lytic peptides are cytolytic peptides that serve in the vertebrate and invertebrate world for both offensive and defensive purposes. They have been isolated from insects, amphibians, and mammals. Lytic peptides can be classified into three major groups: those which are active selectively against eucaryotic cells, procaryotic cells or both eucaryotic and procaryotic cells. Antimicrobial peptides are the largest group within these families. They were found initially in invertebrates [1], but later on also in vertebrates, including humans [2,3]. They are used as a cell-free defense mechanism in addition to, or complementary to, the highly specific cell-mediated immune response [4]. This secondary, chemical immune system provides organisms with a repertoire of small peptides that are promptly synthesized upon induction, and act against invasion by occasional and obligate pathogens [1,5–7]. So far, more than 400 different antimicrobial peptides have been isolated and characterized. Most of the antimicrobial peptides are composed of L-amino acids, with defined α-helix or β-sheet secondary structures. Some are linear, mostly helical, without cysteines, while others contain one or more disulfide bonds, forming β-sheet or both β-sheet and α-helix structures [1,8]. In most cases, the peptides' mode of action appears to be by direct lysis of the pathogenic cell membrane. A second native group of peptides antibiotics, although smaller, is composed of both L- and D-amino acids. This group includes gramicidins, actinomycins, bacitracin, polymyxins, lantibiotics and bombinins H 3–5 [9–11]. The co-existence of L- and D-amino acids gives rise to unique structures and properties that are completely different from the all-L-peptides. One important example is the group of linear gramicidins [12], peptide antibiotics composed of alternating L- and D-amino acids, that form β-type helices with a hydrogen bonding pattern of the backbone similar to that in β-sheets. In these β-helices the amino acid residues are pointing outward and the carbonyl moieties are alternately pointing upward and downward in the interior of the helix, providing the helices with a hydrophilic pore.

Membrane-lytic peptides have been studied extensively in order to understand general aspects related to peptide-lipid interactions, as well as the relation of these interactions to the biological function of these peptides. The largest and most studied group out of those described above includes short linear polypeptides (≥40 amino acids) which are devoid of disulfide

bridges (see reviews in [5,6,13]). These polypeptides vary considerably in chain length, hydrophobicity and overall distribution of charge, but share a common α-helical structure when associated with phospholipid membranes [13]. Some of these peptides are not cell selective (e.g. the bee venom melittin [14,15]), the Moses sole fish lytic peptide pardaxin [16,17], and the human cathelicidin-like LL-37 [18], being able to lyse both bacterial and mammalian cells. Others are selective either to mammalian cells but not to bacteria (e.g. δ-hemolysin from *Staphylococcus aureus*) [19] or vice versa, i.e. cytotoxic to various pathogenic microorganisms but not to normal mammalian cells (e.g. cecropins, isolated from the cecropia moth [20] and from the hemolymph and cuticular extracts of other Lepidopteran and Dipterian insects (for a review, see [21,22]), magainins [23] and dermaseptins [24–26], both isolated from the skin of frogs. Each antimicrobial peptide has a broad but not identical spectrum of antimicrobial activity, providing the host maximum coverage against a rather broad spectrum of microbial organisms.

Despite extensive studies, the mode of action of this group of antibacterial and cytolytic polypeptides is not fully understood and the basis for their selectivity towards specific target cells is not known.

2. Peptide-membrane interaction

2.1. *Phospholipid head group charge and peptide charge distribution dictate the target for membrane-lytic peptides*

It is clear that peptide-lipid interactions, rather than receptor-mediated recognition processes, play a major role in the function of most membrane-lytic peptides. This has been demonstrated with the cell non-selective lytic peptide melittin and the antimicrobial peptides cecropin and magainin, in which their analogues composed entirely of D-amino acids (enantiomers) possess lytic activity indistinguishable from that of the parent molecules [27–29]. These enantiomers preserved the amphipathic α-helical structure of the wild-type peptides, a structure proposed to be a prerequisite for their function. However, the finding that diasteriomers of lytic peptides (containing both L- and D-amino acids) are able to lyse bacteria similarly to the all-L-amino acid parent peptide, despite the fact that their amphipathic structure was altered, suggests that even the α-helical structure is not necessary for activity [30–32]. Another common feature found in most native membrane-lytic peptides is that the distribution and the amount of the net charge correlate with their biological function; peptides with a low net negative charge (e.g. δ-hemolysin), or with a low net positive charge spread along their helix backbone (e.g. pardaxin, melittin) are mostly lytic to mammalian cells or to both mammalian and bacterial cells. On the other hand, native non-hemolytic antibacterial peptides contain high net positive charge contributed by a large number of basic amino acids which are distributed along the hydrophilic face of the amphipathic α-helix. This feature has been proposed to account for their preferential activity against bacteria and not normal mammalian cells. Bacterial membrane is rich in acidic phospholipids [33]. Therefore, the net positive charge of the antibacterial peptides facilitates their perturbing activity towards bacterial membrane. In contrast, the outer membrane of human erythrocytes (representatives of normal mammalian cells) is composed predominantly of zwitterionic phosphatidylcholine (PC) and sphingomyelin phospholipids [34]. Indeed, studies on the interaction of antimicrobial peptides with model phospholipid membranes revealed low affinity to zwitterionic phospholipids compared to acidic phospholipids. This has been demonstrated with cecropins [35,36], magainins [37–41], dermaseptins [42,43] and others [30–32,44]. In addition, the outer surface of Gram-negative bacteria contains negatively charged lipopolysaccharides (LPS). The role of the outer membrane and LPS in the interaction between magainin 2 and the Gram-negative cell envelope was studied by FTIR spectroscopy [45]. Magainin 2 alters the thermotropic properties of the outer membrane-peptidoglycan complexes from wild-type *Salmonella typhimurium* and a series of LPS mutants which display differential susceptibility to the bactericidal activity of cationic antibiotics. LPS mutants show a progressive loss of resistance to killing by magainin 2 as the length of the LPS polysaccharide moiety decreases. While disruption of outer membrane structure most likely is 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, outer membrane molecular architecture, and the presence or absence of the O-antigen side chain. Nevertheless, there is not always a direct correlation between binding to LPS and antimicrobial activity. For example, it has been suggested that the reduced antimicrobial activity of gramicidin S analog families is the result of their increased affinity to outer membrane components of Gram-negative microorganisms, and therefore compete for binding to the inner membrane [46]. It should be noted that although electrostatic interactions between cationic peptides and negatively charged bacterial cell envelope components may be important, 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 the glycocalx layer. However, binding and penetration are less efficient in the eucaryotic clycocalx.

The low affinity of antimicrobial peptides for zwitterionic membranes might explain their inability to lyse erythrocytes. However, there are exceptions. Functional studies with dermaseptin S4 [47] and the human-like cecropin, LL-37 [48], demonstrated that they bind and permeate efficiently both zwitterionic and negatively charged phospholipid vesicles, compared to other native antibacterial peptides, which permeate efficiently only negatively charged membranes. The high affinity of these peptides towards zwitterionic PC phospholipid membranes is surprising in light of their high net positive charge. This may suggest the involvement of hydrophobic interactions between the peptides and the zwitterionic membranes. LL-37 and dermaseptin S4 reach PC membranes as oligomers. Since the N-terminus of both peptides is hydrophobic, a bundle of N-terminal regions could initiate binding to the membrane.

2.2. *Proposed mechanisms for the mode of action of amphipathic α-helical membrane-lytic peptides*

Two alternative mechanisms were proposed to describe the detailed steps involved in membrane permeation by amphipathic α-helical lytic peptides. These models differ conceptually from each other. In the first one, the 'barrel-stave' model [49], amphipathic α-helices insert into the hydrophobic core of the membrane and form transmembrane pores. In the second one, the 'carpet' model [42], the peptides, which do not need necessarily to adopt amphipathic α-helical structure, are in contact with the lipid head group during the whole process of membrane permeation and do not insert into the hydrophobic core of the membrane. A major question is whether a particular mechanism can be assigned to a particular biological function (i.e., antibacterial activity versus cytotoxicity to mammalian cells).

The purpose of this review is to summarize several studies on the mode of action of lytic peptides with relation to the above question. The list of lytic peptides studied, which is by no means exhaustive, includes several linear naturally occurring all-L-amino acids peptides, as well as their diasteriomers, in which several L-amino acids were substituted with their D-amino acids. Most of these studies were performed with model phospholipid membranes and several ambiguities which might arise using these model systems have been described in the relevant articles. Nevertheless, since phospholipid head groups have a major contribution to the activity of lytic peptides, they should be the best model system rather than negatively charged aliphatic compounds such as phosphatidic acid.

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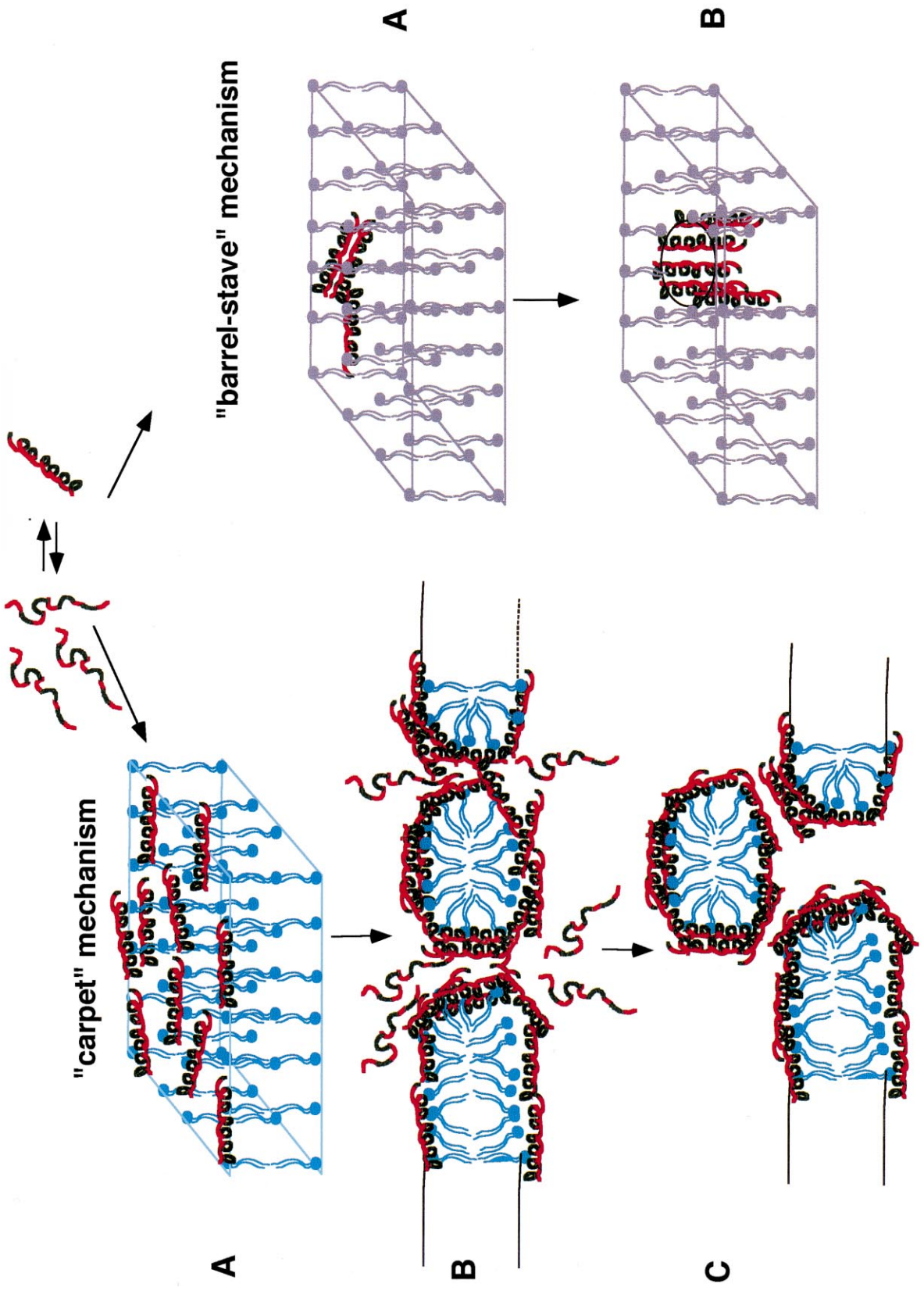
Fig. 1. A cartoon illustrating the 'barrel-stave' (to the right) and the 'carpet' (to the left) models suggested for membrane permeation (modified from [48]). In the 'carpet' model the peptides are bound to the surface of the membrane with their hydrophobic surfaces facing the membrane and their hydrophilic surfaces facing the solvent (step A). When a threshold concentration of peptide monomers is reached, the membrane goes into pieces (steps B and C). At this stage a transient pore is formed. In the 'barrel-stave' model peptides first assemble in the surface of the membrane, then insert into the lipid core of the membrane following recruitment of additional monomers.


"barrel-stave" mechanism
A
B
"carpet" mechanism
A
B
C

2.3. 'Barrel-stave' model for transmembrane channel/pore formation

The 'barrel-stave' mechanism [49] describes the formation of transmembrane channels/pores by bundles of amphipathic α-helices, such that their hydrophobic surfaces interact with the lipid core of the membrane and their hydrophilic surfaces point inward, producing an aqueous pore (Fig. 1, right panel). The following criteria must be fulfilled in order that a molecule would form a transmembrane pore: (i) monomers bind to the membrane in an α-helical structure; (ii) monomers recognize each other in the membrane-bound state already at low surface density of bound peptide; (iii) helices insert into the hydrophobic core of the membrane; and (iv) progressive recruitment of additional monomers occurs to increase the pore size. A very crucial step in this mechanism is the assembly of monomers on the surface of the membrane before the peptide is inserted, since it is energetically unfavorable for a single amphipathic α-helix to traverse the membrane as a monomer. In the latter case, the low dielectric constant and inability to establish hydrogen bonds will not allow the fatty acyl region of a lipid bilayer to be in a direct contact with a polar surface of a single amphipathic α-helix. Based on these criteria, it is unlikely that the helices will be highly homogeneously charged because their hydrophilic surfaces are facing each other while forming a transmembrane pore. Furthermore, it is reasonable to assume that since these peptides can insert into the hydrophobic core of the membrane, their interaction with the target membrane is driven predominantly by hydrophobic interactions, and as a consequence they should bind to phospholipid membranes irrespective of their charge.

2.4. 'Carpet' model for membrane disruption

The 'carpet' model was proposed for the first time to describe the mode of action of dermaseptin S [42], and later on was used to describe the mode of action of other antimicrobial peptides, such as dermaseptin natural analogues [43,47], cecropins [50,51], the human antimicrobial peptide LL-37 [48], caerin 1.1 [52], Trichogin GA IV [53], and diasteriomers of lytic peptides [30–32]. The model describes a situation in which lytic peptides are in contact with the phospholipid head group throughout the entire process of membrane permeation. According to this model, lytic peptides initially bind onto the surface of the target membrane and cover it in a carpet-like manner. Membrane permeation occurs only if there is a high local concentration of membrane-bound peptide. High local concentration on the surface of the membrane can occur either after all the surface of the membrane is covered with peptide monomers, or alternatively, after there is an association between membrane-bound peptides, forming a localized 'carpet'. In the 'carpet' model, contrary to the 'barrel-stave' model, peptides are not inserted into the hydrophobic core of the membrane (left panel in Fig. 1), neither do they assemble with their hydrophilic surfaces facing each other. Furthermore, a peptide that permeates the membrane via this mechanism does not necessarily require the adoption of a specific structure upon its binding to the membrane. Initial interaction with the negatively charged target membrane is electrostatically driven, and therefore peptides are positively charged. The four steps proposed to be involved in this model are: (i) preferential binding of peptide monomers to the phospholipid head groups; (ii) alignment of the peptide monomers on the surface of the membrane so that their hydrophilic surface is facing the phospholipid head groups or water molecules; (iii) rotation of the molecule leading to reorientation of the hydrophobic residues toward the hydrophobic core of the membrane; and (iv) disintegrating the membrane by disrupting the bilayer curvature. An early step before the collapse of the membrane packing may include the formation of transient holes in the membrane. Holes like these may enable the passage of low molecular weight molecules prior to complete membranal lysis. The formation of such holes was proposed to describe the mode of action of dermaseptin [54] and magainin [55–57], and named a toroidal (or wormhole) model (step B in left panel of Fig. 1). As seen in Fig. 1, these holes may allow the passage of peptide molecules from the outer membrane into the inner membrane of, for example, Gram-negative bacteria in a process which may be referred to as 'self-promoting uptake' [58–61].

2.5. Steps involved in peptide-membrane interaction which define a particular mode of action

Based on the criteria described above, an attempt to determine the mechanism by which a particular membrane-lytic peptide interacts with and induces leakage of molecules from phospholipid membranes needs to address several questions with regard to the steps involved in the process.

(a) Is the binding of the peptide to the membrane electrostatically driven or does it result from hydrophobic interactions?

(b) Does the peptide reach the membrane as monomers or oligomers?

(c) Is there an association between membrane bound peptide monomers before or after a threshold concentration occurs?

(d) Is there a cooperativity in the peptide binding process?

(e) Does the peptide insert into the hydrophobic core of the phospholipid membrane or lie on the surface?

(f) What is the structure of the peptide in the membrane-bound state?

3. Different methods used to measure membrane-binding properties of lytic peptides may yield different conclusions

The ability of cytolytic peptides to bind to phospholipid membranes has been investigated using a variety of methods. Most of these methods utilized small or large unilamellar vesicles composed of lipids with different compositions. These methods can be classified into three major groups: (a) methods that monitor the initial steps involved in membrane binding (i.e., a step in which one or very few molecules bind to one vesicle), and as a result, the morphology and integrity of the vesicles is not altered (e.g. fluorescence studies with highly sensitive fluorescence probes, as well as studies with radiolabeled peptides); (b) methods that are not sensitive enough to follow the initial steps of binding but vesicle morphology is preserved or slightly altered (e.g. fluorescence studies with tryptophan-containing peptides, equilibrium dialysis and circular dichroism (CD) spectroscopy); (c) methods that correlate peptide binding to membrane leakage, i.e. membrane integrity is altered. The latter method might be suitable only if membrane binding correlates directly with membrane leakage, since peptides can bind strongly to vesicles but not cause leakage of their contents [62].

Using different methods to investigate binding of a particular peptide to membranes may sometimes lead to conflicting conclusions. For example, the positive cooperativity of membrane permeabilization by magainin observed in different systems suggested that the oligomerization of lipid-bound magainin is important for its activity. The formation of active associated structures was indicated by the positive cooperativity of membrane permeabilization and cytotoxic activity [63]. Positive cooperativity was also observed for magainin binding to lipid bilayers, derived from calcein leakage studies of negatively charged lipid vesicles, and the dependence of the fluorescence quantum yield of bound tryptophan-containing magainin on surface concentration [64,65]. These results suggested the existence of preformed associates which were expected to facilitate pore formation [64,65]. The pore model suggested by Matsuzaki et al. [55,65] assumed a side-by-side dimerization of magainin helices. However, a direct study of Schumann et al. [66], examining the state of association of magainin analogues using fluorescence energy transfer, revealed that the experimentally determined transfer efficiency was lower than that predicted for monomeric magainin analogues randomly distributed exclusively at the outer leaflet of lipid vesicles. Furthermore, for tryptophan-containing magainin and for a diasteriomer of magainin in which two D-amino acids substituted two L-amino acids, an identical energy transfer efficiency was observed, although the non-helical double D-amino acid-substituted analogue should have a significantly reduced association tendency resulting in decreased fluorescence energy transfer (FET). Disturbance of the amphipathic helix of magainin by double D-amino acid substitution should also significantly reduce the formation of magainin associates and consequently decrease FET caused by association. The conclusion of this study was that magainin is not associated in its membrane bound state. These two conflicting results can be accommodated in the carpet model (Fig. 1) as follows: magainin binds initially to the surface of the membrane as monomers and only after a threshold

concentration has been reached, transient pores are formed which allow the leakage of fluorescent marker.

4. Initial steps in the binding, insertion and organization in membranes of lytic peptides

The interfaces, defined by the distribution of the water associated with the phospholipid polar head groups, are each about 15 Å thick and consist of a complex and thermally disordered mixture of water, head groups, glycerol, carbonyl, and methylenes from the edges of the hydrocarbon core [67]. These regions are rich in possibilities for non-covalent interactions with peptides. Several studies, which are described in the following paragraphs, indicate that antimicrobial peptides are located at the interface, parallel to the surface of the membrane, without deeply inserting into the acyl chain region, whereas cell non-selective lytic peptides tend to insert into the hydrophobic core of the membrane.

4.1. The binding process

Tryptophan fluorescence has been used in many studies to determine binding and environmental changes in lytic peptides. Tryptophan fluorescence is sensitive to the environment and increases upon interaction with membranes. However, in many cases the quantum yield of tryptophan is not sufficient to follow the initial steps in the binding process, especially if the binding is weak. It has been reported that tryptophan fluorescence is strongly affected by the surrounding amino acids. Eight side chains of naturally occurring amino acids can quench tryptophan fluorescence: the amide groups of glutamine and asparagine, the carboxyl groups of glutamic and aspartic acids, the lysine-amino group, tyrosine phenol, cysteine sulfhydryl, and histidine imidazole [68]. Therefore, results may be sometimes confusing. For example, incorporation of tryptophan-containing peptides into membranes may result in fluorescence decrease rather than increase. This may happen if the lytic peptide forms oligomers in solution and the tryptophan is buried within a hydrophobic core of the oligomers. In addition, Yau et al. [69] have shown that tryptophan prefers membrane surfaces

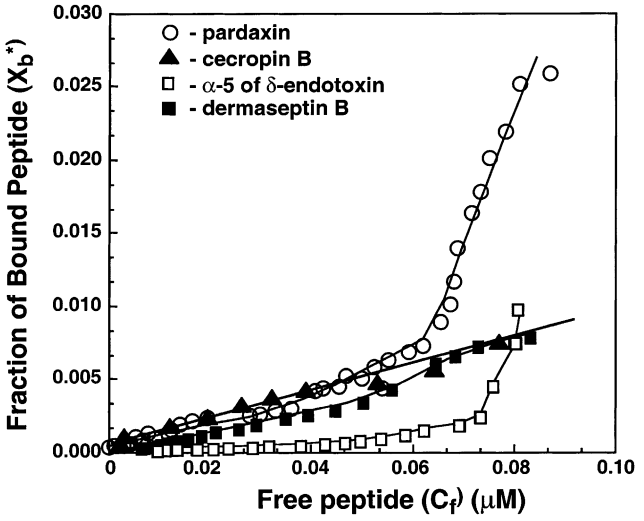


Fig. 2. Binding isotherms of NBD-labeled peptides to phospholipid vesicles. The binding isotherms were derived from binding curves of NBD-labeled peptides titrated with phospholipid vesicles, by plotting X_b* (molar ratio of bound peptide per 60% of lipid) versus C_f (equilibrium concentration of the free peptide in solution). Data were taken from [35,43,73,85].

and this preference is more likely dominated by tryptophan's flat rigid shape, that limits access to the hydrocarbon core, and its π electronic structure and associated quadrupolar moment (aromaticity), that favor residing in the electrostatically complex interface environment.

A highly sensitive spectrofluorometric approach has been developed to investigate the molecular mechanism by which cell-selective and cell non-selective membrane-lytic peptides permeate phospholipid membranes. Peptides were labeled with fluorescent probes having high extinction coefficients either at their carboxy or amino termini [17,35,42,43,70–74]. Various spectroscopic assays were then utilized to study the interaction of the labeled peptides with phospholipid membranes. This approach enabled the separate identification of the three steps involved in membrane permeation by lytic peptides: the binding process, surface/membranous localization of the monomers when bound to the membrane, and their assembly/non-assembly within the membrane.

The environmentally sensitive NBD (7-nitrobenz-2-oxa-1,3-diazole-4-yl) probe [75] was used as a marker for membrane binding [73,76] since its fluorescence can increase up to approx. 10-fold upon interaction with membranes. The higher wavelength

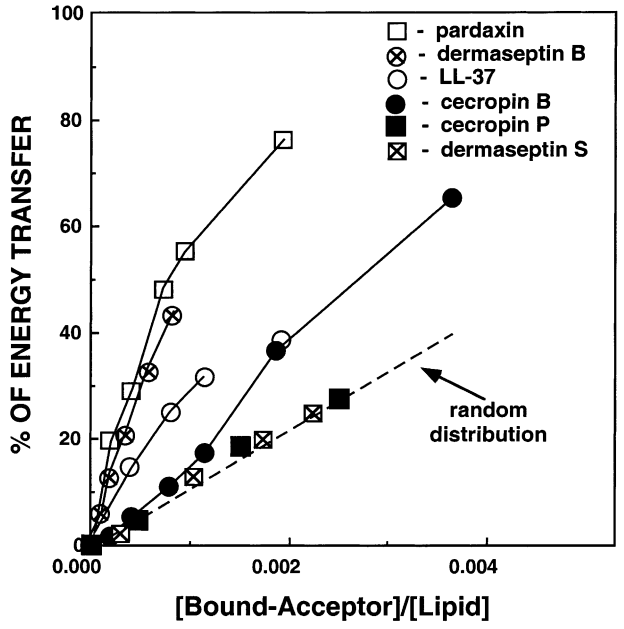


Fig. 3. Theoretically and experimentally derived percentage of energy transfer versus bound-acceptor/lipid molar ratios. The amount of lipid-bound acceptor (Rho-peptides), C_b, at various acceptor concentrations was calculated from the binding isotherms. First, the fractions of bound acceptor, F_b, were calculated for the various peptide/lipid molar ratios from their binding isotherms. The energy transfer expected for a random distribution of the monomers assumes $R_0 = 51.1$ Å as determined for membrane-bound NBD-Rho pair [74]. Data were taken from [42,74,35,43,50,48].

used for excitation (approx. 467 nm) and the high quantum yield reduce significantly the contribution of light scattering. Therefore, when attached to a particular polypeptide this probe can be used to determine whether this polypeptide binds to membranes and its localization therein. NBD-labeled peptides exhibit fluorescence emission maxima around 540 nm in solution, reflecting a hydrophilic environment for the NBD moiety [35,42,73,77]. However, upon addition of phospholipid vesicles, NBD-labeled membrane-lytic peptides exhibited increases in their fluorescence intensities and blue shifts of their emission maxima. This change reflects the relocation of the NBD group into a more hydrophobic environment. The depth of penetration can be evaluated from the value of the blue shift, which varies from approx. 520 nm (for membrane-inserted peptides) to approx. 530 nm (for peptides that are surface localized). The advantage of the use of the NBD moiety is that it allows the use of experimental conditions in which the lipid/peptide molar ratio ranges from $<100{:}1$ up to $>15\,000{:}1$. The lytic peptide pardaxin [73,74], and several antimicrobial peptides from the dermaseptin [42,43,47] and cecropin [35,50,78] families, including the human like cecropin, LL-37 [48], were labeled selectively at their N-terminal amino acid with the fluorophore NBD without altering significantly their biological function. In the presence of vesicles, NBD-labeled antimicrobial peptides exhibited increases in their fluorescence and blue shifts in the emission maxima towards 528–533 nm, which reflect a situation in which the NBD group is located at or near the surface of the membrane (emission maximum of approx. 530 nm) [42,77]. In contrast to these results, the channel-forming pardaxin had its emission maxima at approx. 520 nm, revealing interaction with the hydrophobic core of the membrane [73].

The NBD moiety also facilitates the calculation of partition coefficients of membrane-binding peptides at very low peptide concentrations (<0.1 μM) and very high lipid:peptide molar ratios (approx. $15\,000{:}1$). Compared to NBD, tryptophan fluorescence and CD spectroscopy studies are mostly carried out at a peptide concentration which is about 10–100-fold higher, respectively. Fig. 2 shows examples of binding isotherms obtained for pardaxin, the α-5 helix of δ-endotoxin (both channel/pore forming peptides), as well as for two antimicrobial peptides, cecropin and dermaseptin B. Such binding isotherms are obtained as described in detail elsewhere [35,42,73,79–81], using the following formula: $X_b{}^* = K_p{}^* C_f$, where $X_b{}^*$ is defined as the molar ratio of bound peptide in the outer leaflet of the vesicles (60% of total lipids), $K_p{}^*$ corresponds to the apparent partition coefficient, while C_f represents the equilibrium concentration of free peptide in the solution. Partition coefficients can be directly calculated from these curves. The binding isotherm of pardaxin [73] and the α-5 helix of δ-endotoxin [82] exhibit sharp increases in their slopes as the peptide/lipid molar ratio increases (high C_f value). This argues in favor of a process whereby peptides first incorporate into the membrane and then aggregate [79]. However, the binding isotherms of dermaseptin [42] and cecropin B [35] were found to be practically straight lines, indicating a simple binding process. However, straight

lines cannot exclude a situation whereby oligomers of small sizes are formed.

4.2. Oligomeric state, orientation and depth of penetration of membrane-lytic peptides

Cooperativity in the binding process may indicate whether a particular peptide can form large aggregates already at the initial step of binding. However, peptides can also assemble without cooperativity. The ability of lytic peptides to assemble in the membrane can be detected using FET between a donor and an acceptor pair. FET experiments were performed with NBD- or fluorescein (Flu)-labeled (energy donors) and rhodamine (Rho)-labeled (energy acceptors) peptides [35,42,74]. This pair of donor/acceptor permitted the measurement of peptide assembly in vesicles at lipid:peptide molar ratios of $>5000:1$, conditions in which as few as 2–10 peptide molecules are bound to a single vesicle. When the percentage of energy transfer was plotted versus the various molar ratios of 'bound-acceptor (Cb)'/lipid, the resulting curves always revealed high FET with cell non-selective pore-forming peptides such as pardaxin and the α-5 helix of δ-endotoxin. However, FET observed with non-hemolytic antimicrobial peptides resembled that of randomly distributed monomers, but sometimes increased after a threshold concentration occurred. Fig. 3 shows examples of FET versus acceptor:lipid molar ratios in negatively charged membranes, for the cell non-selective pardaxin, the slightly hemolytic human LL-37, and the non-hemolytic antimicrobial peptides cecropin B, cecropin P, dermaseptin S and dermaseptin B. Interestingly, dermaseptin B showed high FET, and the lipid:peptide molar ratio at which the peptide induced membrane permeation occurred before the entire surface of the vesicles was covered, which might suggest that it is not acting via the 'carpet' mechanism. However, dermaseptin B binds in a non-cooperative manner onto the surface of the membrane, has a weaker affinity to zwitterionic membranes compared to negatively charged membranes, and has lytic activity on bacteria similar to that of other antimicrobial peptides. In addition, a mean field method was used for simulation of the interactions of dermaseptin B with lipid bilayers, which included an approximate representation of the electrostatic effects of the head group region of the bilayer [83]. This study showed that the surface-associated orientation appears to be the most stable arrangement of this peptide. A possible interpretation of these results is that dermaseptin B associates when bound the surface of the membrane and forms a local 'carpet', but not a transmembrane pore via a 'barrel-stave' mechanism. The human LL-37 also oligomerizes in zwitterionic membranes. However, NBD fluorescence studies and polarized attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy revealed that the peptide is predominantly α-helical and oriented nearly parallel to the surface of zwitterionic lipid membranes, observations which do not support the channel forming hypothesis but rather the 'carpet' mechanism [48]. Similarly to dermaseptin B and LL-37, when tryptophan-containing magainin interacted with phosphatidylglycerol (PG) and phosphatidylserine (PS) membranes, a much lower peptide:lipid molar ratio was required to induce leakage of calcein from PG vesicles compared to PS vesicles [84]. These results where interpreted in favor of a non-'carpet' mechanism with PG membranes versus a 'carpet' mechanism for PS membranes.

ATR-FTIR was used to determine the secondary structure and orientation within phospholipid membranes of the mammalian cecropin P1 (CecP) [78]. The shape and frequency of the amide I and II absorption peaks of the peptide within acidic phosphatidylethanolamine (PE)/PG multibilayers in a 7:3 (w/w) ratio (a phospholipid composition similar to that of many bacterial membranes), indicated that this peptide is predominantly α-helical. Using polarized ATR-FTIR spectroscopy, it was found that the peptide is preferentially oriented nearly parallel to the surface of the lipid membranes. In addition, molecular dynamics simulations confirmed that, in response to a *cis* positive transmembrane voltage difference [78], CecP adopts an orientation parallel to the membrane surface and does not insert into the bilayer. ATR-FTIR and fluorescence spectroscopy were also used in the study of the human LL-37, indicating that similarly to other antimicrobial peptides, the peptide binds preferentially onto the surface of both negatively charged and zwitterionic membranes [48]. In contrast, ATR-FTIR spectroscopy revealed that the α-5 helix of the pore-forming peptide δ-endotoxin is inserted into the hydrophobic

core of the membrane rather than lying on the surface of the membrane [85].

Solid-state NMR measurement of ^{15}N-labeled magainin oriented in multilamellar membranes [86–89], Raman [38], fluorescence [65] and differential scanning calorimetry measurements [64], all indicated that at high and moderate lipid:peptide ratios, magainin is oriented parallel to the membrane surface, associated with the lipid head group, and does not significantly disturb the aliphatic chain region. For example, three analogues of magainin 2, each having a Trp residue substituted for Phe at the 5th, 12th, or 16th position, were synthesized and investigated [65]. The depths of the Trp residues, which were determined using the *n*-doxylphosphatidylcholine quenching technique, were about 10 Å from the bilayer center irrespective of the peptide aggregational state, suggesting that the orientation of the magainin 2 α-helix is parallel to the membrane surface.

4.3. The effect of membrane-lytic peptides on the acyl chain organization

Linear amphipathic α-helical antimicrobial peptides have little effect on the structure of the acyl chain of lipid bilayers when inserted in phospholipid membranes, whereas cell non-selective lytic peptides have a stronger effect. In a Raman study [38], the spectrum of the lipid acyl chain C-C stretching region was used to indicate the extent of acyl chain disorder induced by bound peptides. Magainin did not disrupt significantly the acyl chains of negatively charged and zwitterionic membranes, even at a high peptide-to-lipid ratio (w/w) of 1. The effect of magainin 2 on the gel to liquid crystalline phase transition of multilamellar vesicles was measured using differential scanning calorimetry [64]. At 3.5 mol% the effect of magainin on DPPG's (dipalmitoylphosphatidylglycerol) main transition seemed insignificant, leading to the conclusion that magainin does not penetrate deeply into the acyl chain region. In another study, ^{2}H-NMR was used to examine F16W magainin 2 incorporated into palmitoyloleoylphosphatidylglycerol (POPG)/deuterated palmitoyloleoylphosphatidylcholine (POPC) (1:3) MLV [90]. The results showed that the presence of the peptide only slightly decreased the order parameter of the double bond of the PC chain.

Polarized ATR-FTIR spectroscopy was used to determine the effect of cecropin P1 on the multibilayer acyl chain order [78]. The incorporation of cecropin P1 did not significantly change the order parameters of the acyl chain of PE/PG (7:3 w/w) multibilayers (that mimic the phospholipid composition of *Escherichia coli* [91]), suggesting that the peptide does not penetrate the hydrocarbon core of the membranes. Similar results were found with LL-37 [48]. Contrary to these results, significant effects on the acyl chain were observed with membrane-inserted hemolytic peptides such as melittin [92,93], δ-hemolysin [92], the α4-α5 hairpin helices from *Bacillus thuringiensis* δ-endotoxin [85], transmembrane segments of phospholamban [94,95] and colicin A [96].

4.4. Initial formation of secondary structure and insertion into the interface between the lipid head group and the acyl chain

The conformational changes that take place during the insertion process of membrane-lytic peptides into membranes are not clearly known. One model suggests that the secondary structure formation of an amphipathic α-helical structure is attained through sequential stages of interfacial binding in an unfolded state, secondary structure formation, and insertion of secondary structure units into the lipid bilayers, leading to disruption of the membrane [97]. This model is supported by a study that examined the conformational changes of the bee venom melittin upon insertion into phospholipids. The results showed that melittin adsorbed on the lipid layer surface contains less α-helix than its counterpart inserted into the lipid layer. As the penetration depth of melittin increased, more ordered structures (α-helix) appeared [98].

Before binding to membranes most antimicrobial peptides are present in an aqueous solution as monomers in an unfolded state. There are a few exceptions, such as dermaseptin S4 [47] and LL-37 [18,48], which are in equilibrium with oligomers in which the peptides exist in an α-helical conformation. The initial step of their insertion into the membrane is adsorption of the unfolded form on the surface of the negatively charged phospholipid membrane, as described above. Forces affecting the free energy cost of inserting unfolded peptide into phospholipid membranes include the hydrophobic

interactions between the non-polar amino acids and the phospholipid hydrocarbon layer. These forces are countered by the cost of partitioning the polar amino acids and the peptide bond (CONH) [99]. When bound to negatively charged membranes, the positive charges of antimicrobial peptides are partially neutralized by the negative charges of the phospholipid head groups, thus reducing the energy cost of adsorbing the peptide into the membrane. Subsequently, this may allow the intrinsic hydrophobic forces to manifest themselves by forming a stable α-helical structure, driving the peptide further into the interface [83]. However, with the zwitterionic phospholipids the rate limiting step appears to be the initial binding of the antimicrobial peptide to the surface, which is very low. As indicated previously, LL-37 and dermaseptin S4 bind strongly also to zwitterionic membranes. However, the N-termini of both peptides are hydrophobic and they form oligomers in solution. Therefore, a bundle of hydrophobic N-terminus regions can initiate binding to the membrane.

5. The membrane permeation process

5.1. Peptide-induced changes in the membrane curvature

The initial step involved in membrane permeation and micellization requires a change in the curvature of the membrane. Membrane-lytic peptides can be classified into two groups in terms of their effect on membrane curvature [100]. (i) Inducers of a negative curvature strain (class L peptides) [13]. A reciprocal wedge model was suggested by Tyler et al. [101] in order to explain the ability of class L peptides to lyse cells. In this model, a class L peptide folds into an amphipathic α-helical structure upon association with phospholipids, such that the polar face of the peptide is associated with the polar head groups of the phospholipids. The lipid-associated peptide, when viewed in cross-section, is an inverted wedge shape in which the hydrophobic face of the helix forms the apex. This structure is expected to force the alkyl chains of phospholipid apart, inducing a negative curvature on the membrane. (ii) Positive curvature inducers (class A peptides [13,102], cyclic tachyplesin I and magainin). Class A peptides and tachyplesin I have been shown to cause micellization of bilayers [103,104]. In a recent study the effect of magainin 2 on membrane curvature was examined [84]. Magainin 2 was found to significantly raise the T_H value of DPOPE (dipalmitoleoyl-L-α-phosphatidylethanolamine), suggesting that in the absence of electrostatic effects the peptide imposes positive curvature strain on the membrane. Phosphatidylserine, phosphatidic acid, and cardiolipin, as opposed to phosphatidylglycerol, are known to form the hexagonal II phase under conditions of reduced interlipid electrostatic repulsion [105–107]. Permeation of phosphatidylserine, phosphatidic acid, and cardiolipin by magainin 2 occurred at much higher P/L values than phosphatidylglycerol, indicating that lipids that impose higher negative curvature strain on membranes are more resistant to magainin cytolytic activity. Furthermore, addition of a sublytic concentration of cone-shaped LPC (palmitoyl-L-α-phosphatidylcholine), which imposes a positive curvature strain, facilitated magainin's leakage activity. A high positive curvature in a dimension perpendicular to the bilayer plane is required to stabilize a pore structure toward the formation of a micelle. Two-dimensional ^{1}H-NMR experiments in membrane mimetic environments showed that magainin 2 [108], cecropin A [109] and cecropin P1 [110] are composed of two helical regions with a flexible hinge in between. The ability of amphipathic antimicrobial peptides to induce positive membrane curvature may depend on this wedge-like shape. The role of flexible hinge in antibacterial activity has been previously demonstrated with a series of cecropin-like model peptides [111].

5.2. Antimicrobial peptides permeate membranes after a local threshold concentration has been reached

In order to form micelles, a peptide needs to reach high concentration at a certain area of the membrane. In accordance with this assumption, liposome leakage and cytotoxicity experiments suggested the existence of concentration-dependent changes in the membrane permeating activity of amphipathic antimicrobial peptides. In addition, these studies revealed that initial steps involved in micelle formation include transient holes in the membrane, and changes

in the orientation of the peptides. Below a critical concentration magainin causes only slight leakage, but at higher concentrations magainin causes widespread lysis [37,38,64,63,112–115]. A few examples may demonstrate this effect. (i) The kinetics of magainin 2-induced release of 6-carboxyfluorescein (CF) from phosphatidylserine liposomes, indicating that the fast release of dye is a transient effect resulting from transient destabilization of the bilayer upon initial interaction with the peptide [115]. No measurable CF release could be observed until a high level of bound magainin was achieved. (ii) The ability of magainin 2 to decrease the membrane potential in cytochrome oxidase liposomes was investigated by Juretic et al. [63]. At low concentrations the peptide was almost inactive, but activity was observed when a critical concentration has been reached. (iii) Neutron in-plane scattering detected pores formed by magainin 2 in membranes only when a substantial fraction of the peptide is oriented perpendicular to the membrane [57]. (iv) The interaction of magainin 2 and its analogue Ala^{19}-magainin 2 with lipid was investigated using two lipid photolabels. The results indicated a concentration-dependent insertion of the peptide into the lipid bilayer. Higher photolabeling at both shallow and deep photolabels was achieved at high peptide-to-lipid molar ratio [116].

Membrane permeation combined with membrane binding studies on dermaseptins [42,43,47] and cecropins [35,50] revealed that maximal activity was obtained when the peptides covered the surface of the liposomes. In addition, dermaseptins [42,43], cecropin B, cecropin P ([35,50]; unpublished results) and cecropin A [117] dissipated ion gradients at low concentrations of peptide, while substantially higher concentrations were required to release encapsulated calcein. Further support was obtained by Steiner et al. [118], who found that the stoichiometry of cecropin killing of bacteria suggested that amounts of cecropin sufficient to form a monolayer strongly modified the bacterial membrane. In another study, a survival assay was used in order to compare the concentrations of cecropin A required to kill bacteria to the concentration required to release cytoplasmic β-galactosidase [117]. The results clearly demonstrated that cecropin A is bactericidal at relatively high concentrations, where it increases membrane permeability to large molecules.

5.3. *Membrane permeation by antimicrobial peptides revealed by electron microscopy*

Examination of *E. coli* treated with cecropins at concentrations below the minimal inhibitory concentrations (MIC) by transmission electron microscopy revealed the formation of patches, whereas total lysis of the bacteria was observed at the MIC concentration [119]. These patches were similar to those obtained with non-cytolytic derivatives of pardaxin [120] and diasteriomers of lytic peptides [30,31]. When magainin was used at a concentration where bacterial viability had decreased about 30%, similar blebs were observed on the cell surface of the bacteria [121]. At higher concentrations magainin 2 produced string-like structures, which are considered to be cellular debris arising from cell lysis. Similar results were obtained when isolated *Heliothis* cecropin and cecropin B were incubated with *E. coli* K12 D31 [122]. Large parts of the cell envelope were missing from the bacteria and the outer membrane appeared to be dissociated from the cell. *E. coli* cells contained large lesions with an outer diameter of 9.3 nm and internal pore diameter of 4.2 nm. Scanning and transmission electron microscopy were used to study the morphological changes induced by cecropin B on the bacterial cell of *Klebsiella pneumoniae* [123]. The results showed that cecropin B causes bleb-like protrusions on the bacterial cell surface.

5.4. *A detergent-like effect in membrane permeation by antimicrobial peptides*

Comparison between the abilities of magainin 2 and the micelle-forming detergents Triton-100 and octyl glucoside to induced leakage of CF from PS indicated that their specific activities are similar. The onset of dye leakage took place at a magainin concentration of approx. 3 mole%. Similar results were obtained with Triton-100 and octyl glucoside [115]. The addition of magainin at a high concentration to phospholipid bilayers resulted in the appearance of large water-filled bilayer disruptions [57]. ^{31}P solid state NMR spectroscopy detected the optical clearing of dense suspensions and the formation of isotropic phases [124].

6. Summary and concluding remarks

Functional and structural studies with several membrane-lytic α-helical peptides presented in this chapter are in agreement with either the 'barrel-stave' model (right panel of Fig. 1) or some or all of the steps involved in the 'carpet' model (left panel of Fig. 1). Cell non-selective amphipathic α-helical lytic peptides permeate membranes predominantly by forming pores via a 'barrel-stave' mechanism. These peptides bind to both zwitterionic and negatively charged membranes, oligomerize at a very low peptide:lipid molar ratio, bind cooperatively to membranes, insert into the hydrophobic core of the membrane, form single channels at very low peptide concentration and cause leakage of vesicles content at very low peptide:lipid ratios. However, amphipathic α-helical antimicrobial peptides permeate membranes predominantly via the 'carpet' mechanism. In addition, despite the fact that linear antimicrobial peptides were developed by distant and diverse species during millions of years of evolution, they share common motifs, i.e. they are linear, positively charged and have a high propensity to adopt an amphipathic α-helical conformation in hydrophobic environments. The studies presented in this chapter have demonstrated the essential role of these properties in the different stages involved in membrane permeation according to the 'carpet' model.

This review focused mainly on cecropins, dermaseptins, magainins and LL-37 as representatives of the amphipathic α-helical antimicrobial peptides. The fact that these peptides vary with regard to their length, amino acid composition, and net positive charge, but act via a common mechanism, may imply that other linear antimicrobial peptides exhibiting the same properties, also share the same 'carpet' mechanism.

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