# Molecular Mechanisms of Membrane Perturbation by Antimicrobial Peptides and the Use of Biophysical Studies in the Design of Novel Peptide Antibiotics

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**Abstract**: Antibiotic resistant bacterial strains represent a global health problem with a strong social and economic impact. Thus, there is an urgent need for the development of antibiotics with novel mechanisms of action. There is currently an extensive effort to understand the mode of action of antimicrobial peptides which are considered as one alternative to classical antibiotics. The main advantage of this class of substances, when considering bacterial resistance, is that they rapidly, within minutes, kill bacteria. Antimicrobial peptides can be found in every organism and display a wide spectrum of activity. Hence, the goal is to engineer peptides with an improved therapeutic index, i.e. high efficacy and target specificity. For the rational design of such novel antibiotics it is essential to elucidate the molecular mechanism of action. Biophysical studies have been performed using to a large extent membrane model systems demonstrating that there are distinctive different mechanisms of bacterial killing by antimicrobial peptides. One can distinguish between peptides that permeabilize and/or disrupt the bacterial cell membrane and peptides that translocate through the cell membrane and interact with a cytosolic target. Lantibiotics exhibit specific mechanisms, e.g. binding to lipid II, a precursor of the peptidoglycan layer, either resulting in membrane rupture by pore formation or preventing cell wall biosynthesis. The classical models of membrane perturbation, pore formation and carpet mechanism, are discussed and related to other mechanisms that may lead to membrane dysfunction such as formation of lipid-peptide domains or membrane disruption by formation of non-lamellar phases. Emphasis is on the role of membrane lipid composition in these processes and in the translocation of antimicrobial peptides.

**Keywords**: Amphipaticity, membrane mimetic, pore formation, carpet mechanism, lipid-peptide domains, non-lamellar, combinatorial libraries, SAR.

## NEED FOR NOVEL ANTIBIOTICS - THE PROBLEM OF ANTIBIOTIC RESISTANCE

Nowadays, we face a world-wide rapid increase in pathogenic bacteria that are multi-resistant to commercially available antibiotics. This alarming situation has various origins being largely due to the excessive and often inappropriate use of antibiotics in human and animal health care for the treatment and prevention of bacterial infections [1]. The numerous warnings of the World Health Organization emphasize the relevance and importance of this serious global health problem and the pressing need to develop new classes of antibiotics with novel mechanisms of action. Since bacteria have evolved to present multiple resistances to a large number of existing antibiotics, a new class of compounds is more likely to delay the emergence of bacterial resistance. Several strategies have emerged in the last decade to combat infectious diseases [2]. Strong progress has been made in the field of microbial genomics and in finding new targets. Thereby, the development of molecular biology tools brought forth a number of strategies for creating novel antibiotics as e.g. the next-generation macrolide antibiotics. Furthermore, the accumulating knowledge on immuno-stimulatory properties of DNA as well as on adjuvant candidates such as detoxified derivatives of bacterial enterotoxins and recombinant bacterial vaccine vectors will allow the design of novel vaccines. Studies on host-pathogen interactions have also opened a new avenue for developing adhesin-based vaccines.

Another emerging strategy is based on host defense peptides, effector molecules of innate immunity that can provide a first line of defense against a substantial array of pathogenic microorganisms. These peptides have evolved in nature to contend with invaders as an active system of defense [3,4]. Within the last decade the number of such peptides being isolated and characterized increased immensely and are compiled in the Antimicrobial Sequences Database (http://www.bbcm.univ.trieste.it/~tossi/antimic.html). Furthermore, a list of available three-dimensional high-resolution structures with PDB deposited coordinates has been reported by Powers and Hancock [5], showing a wide variety of structures ranging from α-helical, β-sheet, extended to loop among the different peptides. These antimicrobial peptides (AMPs) usually exhibit a high specificity towards their target cell, i.e. demonstrating toxicity which is restricted to microorganisms. Such AMPs represent therefore novel sources for the development of therapeutic agents with which to overcome antimicrobial resistance, because bacteria have not been very successful in resisting the action of AMPs considering the time span over which such mechanisms could have evolved in nature [6]. The main advantage of this class of substances, when considering bacterial resistance, is that they rapidly, within

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minutes, destroy bacteria. This fast killing rate of bacteria - "a hall-mark of peptide-based defense" [7] - enables an effective defense being faster than the growth rate of bacteria, which in the right environment can double every 20 min. Furthermore, due to the nature of their target, i.e., mostly the cell membrane rather than a specific receptor, bacterial resistance is less likely to occur rapidly since substantial modification of the lipid composition would affect bacterial cell viability. Degradation of AMPs by proteases may be a concern, but also imposes problems, as most peptides are created from nondescript sequences of amino acids and hence lack unique epitopes as recognition site of proteases. In addition, multicellular organisms attack bacteria with multiple peptides of different structural classes, and therefore, degradation of one peptide might not be sufficient to combat such an attack [6]. Both lipopolysaccharides of the outer membrane of Gram-negative bacteria and the peptidoglycan-teichoic acid network forming the cell wall of Gram-positive bacteria, which confer a negative charge to the cell surface, can convey some protection against AMPs. A few instances of bacterial resistance to cationic AMPs have been reported [reviewed in 8], mostly via reduction of the negative surface charge of the bacterial cell envelope with positively charged substituents [9]. A role of the surface charge in protection of bacteria against cationic AMPs was suggested from experiments with staphylococcal mutants that were characterized by a different degree of D-alanine esterified teichoic acids. Such mutation resulted in an increased net negative surface charge as compared to the wild-type [10]. These mutants were more sensitive than the wild-type to a number of AMPs such as defensins, protegrins or magainin 2. Resistance to AMPs could also be achieved by modifying the negatively charged phosphatidylglycerol (PG) in *S. aureus* with charged L-lysine that reverses the lipid charge presumably resulting in repulsion of cationic peptides [11].

The impact of surface charge for resistance mechanism(s) has also been shown for Gram-negative bacteria, which in addition can stabilize its outer membrane by an increased degree of acylation of the lipid A moiety [12]. Electrostatic interactions result in the accumulation of AMPs at the LPS layer forming a peptide/LPS complex as described for e.g. tachyplesin [13], magainin [14] and cecropin A [15]. Studies on the interactions between the antimicrobial peptide NK-2, derived from mammalian NK-lysin, and various LPS from *Salmonella enterica* also suggested the involvement of hydrophobic interactions [16]. These results agreed with the observed improved binding capacity of lipophilic analogs of a human lactoferrin fragment, LF11, to LPS derived from *Salmonella typhimurium* [17]. Hancock and coworkers proposed that the peptide transfer across the outer membrane to the periplasmic space is mediated by the so-called self-promoted uptake system, which involves the direct binding of the cationic peptides to LPS [reviewed in 18]. In agreement X-ray and CD measurements using oriented LPS layers revealed that at physiological relevant concentrations magainin and protegrin penetrated transmembrane in the LPS layer, which implies that these AMPs readily penetrate the LPS monolayer of the outer membrane [19]. One premise would then be that reducing or blocking peptide binding to LPS has implications on the peptide transfer to the periplasmic space, and in turn in the resulting interactions with the inner membrane, where the lethal effect is assumed to occur. Hence, modification of LPS could result in resistance to AMPs, as indicated for polymyxin B-resistant strains of *S. typhimurium* and *E. coli*. Modifications in both the fatty acid profile and degree of

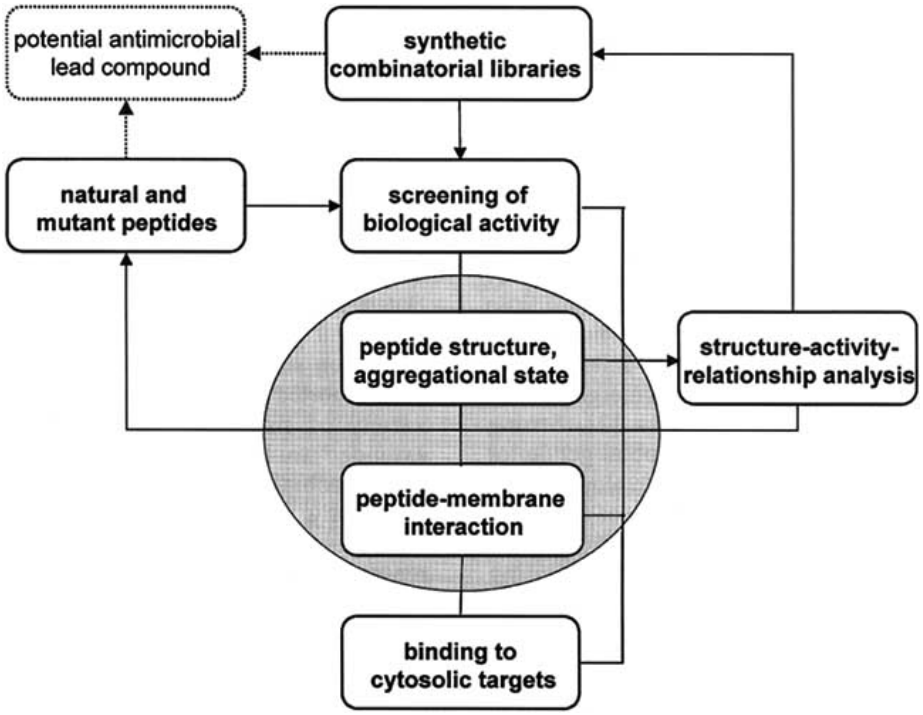


**Fig. (1)**. Main contribution of biophysics (dashed area) in the design of potential antimicrobial lead compounds derived from natural and synthetic peptides.

acylation of lipid A were detected in such resistant bacteria [20, 21]. For example, these resistant strains showed extensive esterification of the lipid A monophosphate by arabinose as well as of the LPS diphosphates by aminoethanol [22, 23]. Other escape mechanisms to AMPs involve the direct degradation of the peptides by outer membrane-associated proteases [24, 97] or the involvement of efflux pumps as shown for the Gram-negative human pathogen *Neisseria gonorrhoeae* [25]. However, such instances remain scarce and clinical studies on the magainin derivative MSI-78 demonstrated that the peptide did not cause emergence of resistance supporting the assumption that AMPs are promising candidates for new antibiotics [26].

## STRATEGIES TOWARD THE DEVELOPMENT OF ANTIMICROBIAL PEPTIDES AS NOVEL ANTIBIOTICS

The ultimate goal of this research area has been to engineer AMPs of high therapeutic index in terms of improved antimicrobial activity. For the rational design of such novel peptide antibiotics it is essential to elucidate the molecular mechanisms of action of AMPs and their cell target specificity. This can only be achieved by an interdisciplinary approach taking especially advantage of state-of-the-art methods of biophysics, which have strongly contributed to unravel the mode of action of AMPs. The general strategy "Fig. (**1**)" to design a lead compound with improved and selective interaction with bacterial strains has been largely based on screening of natural AMPs or fragments thereof by using a mutation strategy. The latter is facilitated if the structure of the peptide is known. Biophysics and structural biology have strongly contributed to elucidate the structure of the peptides as well as their state of aggregation. Both may be affected by the interaction with membranes, the target of most antimicrobial peptides. Studies regarding the interaction of AMPs and membranes on a molecular level have been mostly performed using membrane model systems, i.e. liposomes, lipid monolayers and oriented lipid layers. Again biophysical techniques have played a predominant role in these interaction studies. Correlation of these data with antimicrobial activity as well as hemolytic and cytotoxic activity forms the basis for an efficient structure-activity-relationship (SAR) analysis and modeling to guide further peptide design.

A novel and more recent approach toward the search for antibiotic lead compounds is based on the use of synthetic combinatorial libraries. Combinatorial library approaches enable hundreds to thousands of times more compounds to be synthesized and screened in shorter periods of time relative to traditional approaches [reviewed in 27]. In particular, non support-bound combinatorial libraries have the primary advantage of allowing the screening of compounds free in solution in a cell suspension environment, without the interference of a secondary solid support [28]. Combinatorial libraries have been generated either as large numbers of individual compounds by parallel synthesis methods or as mixtures of compounds from a given chemical class (summarized in Table 1). The latter are prepared by incorporating at a given synthetic step a mixture of incoming reagents (e.g., amino acids, aldehydes, carboxylic acids, etc.) at a predetermined ratio to ensure equimolar insertion of the corresponding chemical moiety into the pharmacophore backbone [29]. While the majority of reported antimicrobial and antifungal peptides are derived from mixture-based libraries, individual compound arrays are generally preferred for the generation of small organic molecules. The selection between individual arrays and mixture-based libraries is generally based on the chemistry, the number of compounds within the library, and/or capability to screen a large number of samples. Thus, mixture-based libraries allow the screening of a large number

**Table 1. Strategies Linked to Non-Support Bond Combinatorial Libraries**

| Library type | Strategy to identify active compounds | Advantages | Disadvantages |
|---|---|---|---|
| Single compound arrays | Synthesize and screen compounds individually | - Ensure that the most active compound is identified<br>- Provide complete SAR information<br>- Does not require additional steps to identify the active compounds | - Require high throughput screening<br>- Limited to manageable number of compounds to be synthesized |
| Mixture-based libraries | Synthesize and screen pools of soluble compounds<br>1. Iterative format:<br>Synthesis and screening of iterative sets of mixtures with the identification of individual active compounds as last step of iteration<br>2. Positional scanning format:<br>Identification of individual active compounds by synthesis and screening of all combinations of building blocks defining active mixtures<br>3. Orthogonal format:<br>Identification of individual active compounds by synthesis and screening of compounds common to active mixtures | - Speed and low cost of synthesis and screening<br>- Large number of compounds can be easily generated<br>- No requirement for high throughput screening | - Does not ensure that the most active compound is identified<br>- Does not provide complete SAR information<br>- Require additional step(s) to identify the active compounds<br>- Analytical characterization of mixtures not easily accessible |

of compounds without the requirement of high-throughput screening facility. Such methods have therefore been preferred by academic groups and in the case of very large libraries. The main advantages of individual compound arrays over mixture-based libraries however are that 1) no additional synthetic steps or deconvolution processes are required to identify the most active compounds from the library, and 2) since every single compound is tested separately, one can ensure that the most active compound will be identified. It is fundamental that mixture-based libraries are synthesized using chemical pathways that afford high yield with minimal side reactions and high reproducibility. While peptide chemistry is nowadays robust and straightforward, the generation of small organic molecules as mixtures may require extensive optimisation of reaction conditions prior to the synthesis of the library. While such prerequisites may have favoured the development of small molecule arrays, the recent development of more robust synthetic pathways have now allowed the preparation of such libraries in a mixture format. Thus, a variety of mixture-based libraries of heterocyclic and small organic compounds have now been reported [27].

The majority of combinatorial libraries developed to date are built around a given pharmacophore that is unrelated to any of the known antibiotics or other biologically active compounds. These generic libraries provide a broad chemical diversity and large number of compounds for each class of structures being generated. Such libraries are anticipated to result in a greater probability of identifying ligands having chemical characteristics that are different from native ligands and therefore more prone to circumvent existing drug-resistance than analogs of existing antibiotics. Such an approach has proven useful for the identification of numerous biologically active compounds including several antimicrobial agents [27]. For example, generic peptide combinatorial libraries were originally generated and screened for the identification of novel short AMPs. Such studies lead to the discovery of tetra- to decapeptides having low micromolar inhibitory activities against a variety of Gram-positive and Gram-negative bacteria as well as fungi [30-33]. Most of these peptides exhibited no or little cytolytic activity towards erythrocytes and/or mammalian cells. While these novel peptides were discovered through an entirely non-biased approach, i.e., all possible peptide sequences were screened for activity, most if not all short L-peptides that were identified from combinatorial libraries show the characteristic features of endogenous AMPs, i.e., cationicity (rich in arginine and/or lysine residues) and high hydrophobicity. These results further support the general belief that net positive charge and hydrophobicity are fundamental factors for antimicrobial activity to occur. These short peptides derived from generic libraries combined with the breath of information that can be extracted from screening such large diversity are greatly valuable toward a better understanding of the mode of action of AMPs. Thus, these peptides represent readily available new tools to dissect the factors involved in the lysis of bacteria and the lytic specificity of AMPs. Of note, such library approaches greatly reduced the effort toward identification of sets of active analogs and may suggest a minimum core structure for biological activity. This knowledge together with biophysical studies and biological activity data will provide a basis for a profound structure-activity-relationship analysis that may define a scaffold for the further development of antimicrobial peptides or peptidomimetics with improved activity.

Combinatorial chemistry was also applied to the generation of libraries of small organic molecules with the aim to develop bioavailable antimicrobial compounds. For example, aryl hydrazine urea-based antibacterial agents were identified from a 10,000 semi-carbazone library [34]. Those agents act specifically against Gram-positive bacteria via disruption of the bacterial cell wall biosynthesis pathway. Agents acting on cell wall biosynthesis and exhibiting broad spectrum antimicrobial activity were also obtained from a library of 1,300 disaccharides [35]. Other examples of novel antimicrobial and antifungal compounds derived from combinatorial libraries include oligomers of N-alkylglycines (known as peptoids) [36, 37], 1,5-dialkylamino-2,4-dinitrobenzenes [38], N-benzyl aminocyclic ureas [39], and bicyclic guanidines [40].

As an alternative to generic libraries, our laboratory and others took advantage of the strength and rapidity in developing novel biologically active compounds inherent to this approach to optimize the antimicrobial or antifungal activity of lead AMPs. Combinatorial chemistry allows the synthesis of thousands of analogs of a given compound in a time that could not be previously manageable using traditional chemical methodologies. For example, a series of 18-mer peptides having higher specificity towards bacteria versus erythrocytes were identified from a library built by randomizing the hydrophilic face of an amphipathic $\alpha$-helical AMP [41]. Of interest, those analogues contained at least one helix breaker residue (glycine or proline). A combinatorial approach was also used to extend the length of a short antifungal peptide (RwfIfH) resulting in the identification of a series of nonapeptides having up to one order of magnitude increased antifungal activity [42]. A combinatorial library of dimeric derivatives of vancomycin led to the discovery of potent agents up to 60-fold more active than vancomycin against vancomycin-resistant *Enterococcus faecium* [43]. Similarly, novel antimicrobial compounds with potent activity against methicillin-resistant *Staphylococcus aureus* were identified from a library of heterodimeric disulfide analogues of psammaplin, a natural antimicrobial agent isolated from the *Psammaplysilla* sponge [44].

## GENERAL ASPECTS REGARDING THE MODE OF ACTION OF AMPs

Despite the wealth of information and experimental data we still do not fully understand at a molecular level, how AMPs actually kill bacteria. As will be outlined below, hypotheses distinguish between processes that lead to membrane disruption and processes that are receptor-linked. It is generally assumed that most AMPs induce rapid depolarization of the normally energized bacterial membrane as demonstrated for magainin [45]. This results in membrane permeation as first shown for human defensins [46], which leads to an irreversible damage of the cell membrane [47-49]. However, it is still a matter of debate whether membrane depolarization is a lethal event per se. The molecular

mechanism(s) of membrane disruption are mostly discussed on the basis of two models, pore formation and carpet model. Alternative mechanisms of membrane damage are discussed in literature such as the complete breakdown of the bacterial membrane by formation of micellar aggregates [50, 51], induction of defect structures or non-lamellar structures [52], as well as by scrambling of the usual distribution of lipids between and within the leaflets of the bilayer that may result in membrane dysfunctions [53]. Finally, recent experimental data indicate that killing of bacteria can be mediated by binding to specific membrane lipid components [54] or by interference with essential intracellular targets after internalization of AMPs [5].

Independently on how AMPs kill bacteria, the peptides have to interact with the cell membrane resulting either in disruption or traversing this barrier. Therefore, understanding the parameters involved in the interaction with membrane lipids is crucial for the elucidation of the molecular mechanism of action. In addition it is obvious that discrimination between mammalian and bacterial cells, i.e. target cell specificity, must be at the level of the membrane. Steps to be considered in lipid-peptide interaction are schematically summarized in "Fig. (**2**)" and involve in general:

1. Solution structure of AMPs and changes upon interaction with the membrane including the state of association.
2. Binding of the cationic peptides to the negatively charged bacterial cell surface, i.e. to LPS of Gram-negative bacteria and teichoic acids of Gram-positive bacteria, respectively.
3. Translocation of the AMPs through these outer barriers to the cytoplasmic membrane.
4. Binding of the peptides to the cytoplasmic membrane, which with few exceptions is non-receptor mediated and electrostatically driven between the negatively charged membrane lipid components and the cationic peptides.
5. Insertion of the peptides, which is mainly governed by hydrophobic interactions, followed by membrane damage or translocation of the peptides into the cytoplasmic space. In the latter case, AMPs bind to cytosolic targets, such as the negatively charged bacterial DNA.

Obviously, membrane lipids are involved in all steps and their molecular properties exhibit a major impact on the consequent events. Therefore, it is essential to consider membrane architecture and lipid compositions in order to understand the molecular mechanism and target cell specificity of AMPs. Furthermore, this knowledge is also of importance for the design of the proper model system for *in vitro* studies.

## MEMBRANE ARCHITECTURE AND LIPID COMPOSITION

An enormous variety of lipid classes is found in nature, which exhibit a great diversity in their molecular and supramolecular structure. Nevertheless, the fundamental structural unit of biological membranes is mostly a highly dynamic, liquid-crystalline phospholipid bilayer [55] that acts as a fundamental permeability barrier. The concept of a characteristic lipid composition for a given cell membrane is well accepted, although changes in lipid composition may occur depending on environmental conditions. In the following paragraphs, we solely focus on the most

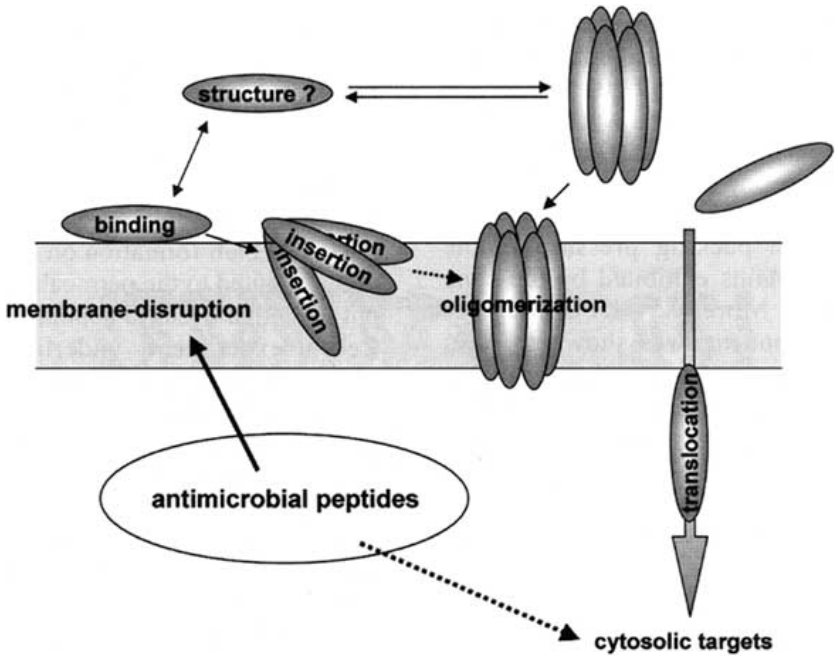


**Fig. (2)**. Simplified scheme of the different steps involved in the mode of action of AMPs. All these steps can be monitored by biophysical techniques. In order to disrupt the cytoplasmic membrane, AMPs have to bind to and translocate through the outer membrane and peptidoglycan layer, respectively (not shown for simplicity). In addition, some peptides internalize and bind to a cytoplasmic target, while other peptides (e.g. lantibiotics) dock to a specific lipid receptor. For details see text.

significant differences between bacterial and mammalian cell membranes, which are elaborated in more details elsewhere [52 and references therein].

Gram-positive bacteria have a simple lipid bilayer membrane, most likely reflecting an early evolutionary stage in which the genetic imperative for lipids is primarily the formation of a cell membrane [56]. This cytoplasmic membrane, which is protected by a teichoic acid layer, consists to a large extent of PG and its derivatives, mostly diphosphatidylglycerol (DPG or cardiolipin) and aminoacyl derivatives. Moreover, many Gram-positive species are characterized by a high content of branched-fatty acids, which are less common in Gram-negative bacteria. The cell envelope of Gram-negative bacteria is a more complex structure consisting of an inner membrane, a unique outer membrane layer and an intervening layer of peptidoglycan in the periplasmic space. The outer membrane has a distinctive, highly asymmetric composition. LPS are located exclusively in its outer leaflet, whereas phospholipids are confined to the inner leaflet of the outer membrane. Phosphatidyl-ethanolamine (PE) represents the major phospholipid class in both the outer and inner membrane [57]. In addition, negatively charged PG and DPG are considerable membrane constituents. Only limited insight exists regarding the distribution of phospholipids between the outer and inner leaflet of the cytoplasmic membrane of Gram-negative and Gram-positive bacteria [58]. The current knowledge of transbilayer movement of phospholipids in biogenic membranes was summarized recently leading to a model in which phospholipid translocation is mediated via membrane-spanning segments of a subset of proteins, characterized by a small number of transmembrane helices [59]. However, recent fluorescence studies incorporating pyrene-labelled PE and PG into *E. coli* and *B. subtilis* membranes indicated that PE and PG are segregated into distinct domains that differ in composition, proteo-lipid interaction and degree of order [60]. It was found that the proteo-lipid domains are enriched in PE. In this context it is of interest to note that membrane integration and functioning of transport proteins [61] as well as protein translocation [62] was severely impaired in *E. coli* mutants lacking PE. This implies a role of this non-lamellar phase preferring lipid in maintaining the functional structure of integral membrane proteins and was explained by the high lateral packing pressure in the hydrophobic core on these proteins exhibited by the cone shaped lipid molecule [63]. Moreover, DPG, another important bacterial lipid component, was shown to form large domains in the septal region and poles of *E. coli* [64]. In summary, these findings suggest the occurrence of a domain organization of bacterial membranes. Thus one may speculate that specific interaction of AMPs with these lipid domains or alterations of the physical state of the membrane by AMPs affecting the domain structures may interfere with normal membrane function.

The plasma membrane of erythrocytes serves as an archetype of mammalian cell membranes, and initial cytotoxicity tests of AMPs are usually performed by measuring their hemolytic activity on human erythrocytes. This membrane consists of about 60% of phospholipids and 25% of cholesterol [65]. An asymmetric distribution of phospholipids between the outer and inner lipid leaflet of the bilayer is well documented showing that the choline phosphatides (phosphatidylcholine (PC) and sphingomyelin (SM)), occur predominantly in the outer leaflet [66]. In many biological systems, these two choline phospholipids appear to occupy similar cellular compartments and their content is tightly regulated. In contrast, the aminophosphatides (phosphatidylserine (PS) and -ethanolamine (PE)) are almost exclusively located in the inner leaflet of the bilayer, which is regulated by an aminophospholipid translocase [59]. Owing to this asymmetric phospholipid distribution, uncharged, zwitterionic phospholipids are exposed to the outside of the cell, which represents the first site of interaction with AMPs, while the presence of large amounts of negatively charged lipids in the outer leaflet is a common feature of bacteria.

Therefore, in order to gain insight into the specificity towards particular lipid components exhibited by AMPs, which would trigger membrane target specificity and disruption, it is of interest to study their interaction with model membranes consisting of mixtures of the various lipid components that mimic bacterial and mammalian cell membranes. A large number of biophysical techniques exist that allow the detailed investigation of these processes.

## BACTERIAL KILLING BY MEMBRANE DISRUPTION - MORE THAN ONE MECHANISM

The biological activity of AMPs is thought to stem primarily from their ability to disrupt bacterial cell membranes, although other mechanism(s) of bacterial killing exist (see below). The first evidence that AMPs act on bacterial membranes was demonstrated upon studying the interaction of human defensins with *E. coli* [67]. In this initial study, permeabilization of membranes was tested by using a membrane impermeable substrate, *o*-nitrophenyl galactoside, which was hydrolized by L-galactosidase upon its release from the cytoplasm in case of permeabilization. These experiments indicated that the antibacterial activity of the peptide is associated with the permeabilization of both the outer and inner membrane of *E. coli*, whereby permeabilization of the inner membrane appeared to be the lethal event. The same conclusion was derived from electron microscopy studies on *E. coli* cells exposed to magainin [51]. The bleb formation on the surface of the bacterial cell was attributed to the permeabilization of the outer membrane but the lethal event to depolarization of the inner membrane. Recent experiments underline these results. Thereby the effects of the frog skin antimicrobial peptide PGLa on live *E. coli* in culture medium were analysed using atomic force microscopy [68]. Perturbation of the outer membrane was observed in a first stage followed by rupture of the inner membrane associated with loss of cytoplasmic material. Further evidence that for most peptides the site of action is the lipid bilayer and that their activity is not related to interaction with a specific membrane protein receptor comes from studies using all D-magainin, cecropins and melittin. All of these D-peptides exhibited similar antibacterial and hemolytic activity than the naturally occurring L forms [69-71]. In addition, both the natural L- and the D-enantiomer of protegrin-1 showed the same effect on membrane model systems, suggesting that the antimicrobial activity does not depend on an interaction with chiral centers on bacterial

membranes, but is rather due to perturbation of the lipid matrix of the target membrane [72]. However, other AMPs such as the proline-arginine-rich peptide, PR-39, or cecropin P were found to exhibit different antimicrobial activity in their L- and D-form which in addition appeared to depend strongly on the bacteria species [73].

## Pore Formation and Carpet Mechanism - Classical Models

Two distinct mechanisms regarding membrane permeation and disruption have particularly been presented and discussed. One model suggests a membrane disruption via the so-called "carpet" mechanism [74], while the second model proposes that AMPs act by perturbing the barrier function of membranes through transmembrane pore formation [75]. In brief, the essential characteristics of the carpet model are (1) the adsorption of AMPs at the membrane surface until a certain threshold concentration is reached and (2) the subsequent insertion of the AMPs into the hydrophobic core resulting in permeation/disruption of the membrane. The detailed mechanism of membrane permeation or disruption is less defined. It could be disintegration in a detergent-like manner [49] or formation of pores [76, 77] and channel aggregates [78]. In contrast to the carpet mechanism no covering of the membrane surface by peptides is theoretically necessary in the case of pore formation, as peptide oligomerization and transmembrane pore formation may occur below the threshold concentration. For example, pore formation of magainin was observed at lipid-to-peptide molar ratios well below 100:1 [79].

A number of studies have been devoted to the elucidation of the structure of these peptide pores and process of pore formation, mainly by the group of Matsuzaki and of Huang. A pore structure that involves lipids and peptides was first proposed by Matsuzaki *et al.* [80] based on the observation that magainin 2 induces rapid lipid flip-flop coupled with pore formation. Thereby this fluorescence study showed that flip and flop rate were identical and independent of lipid species. Huang succeeded by neutron scattering to solve the structure of the phospholipid-magainin pore complex [77]. In this complex - named toroidal or wormhole pore - the phospholipids bend from the membrane interface towards the hydrophobic interior and are intercalated between the long axis of the peptide molecules which are oriented perpendicular to the bilayer plane "Fig. (**3**)". Subsequent studies revealed that other peptides such as protegrin-1 [81] or the lytic bee venom peptide, melittin [82] arrange in such a pore. A pore of the barrel-stave type was described only for alamethicin [83]. In addition, circular dichroism studies on oriented phospholipid multilayers demonstrated that these AMPs have two binding states in a bilayer. At low concentrations they adopt a surface state, S, and transform upon a certain threshold concentration into a pore-forming state, I. This process exhibits some analogy to the carpet model, where insertion of AMPs also occurs only above a certain threshold concentration. The transition from the S state to the I state has a sigmoidal dependence indicating cooperativity in the peptide-bilayer interaction. Connected with this process is a thinning of the membrane. Early X-ray studies showed that the bilayer thickness decreased almost linearly with increasing magainin 2 concentration [84]. The membrane thinning effect of AMPs was further studied in more details by X-ray diffraction indicating that this effect is a plausible mechanism for the peptide-induced pore formation [85]. A recent analysis of the pore formation by

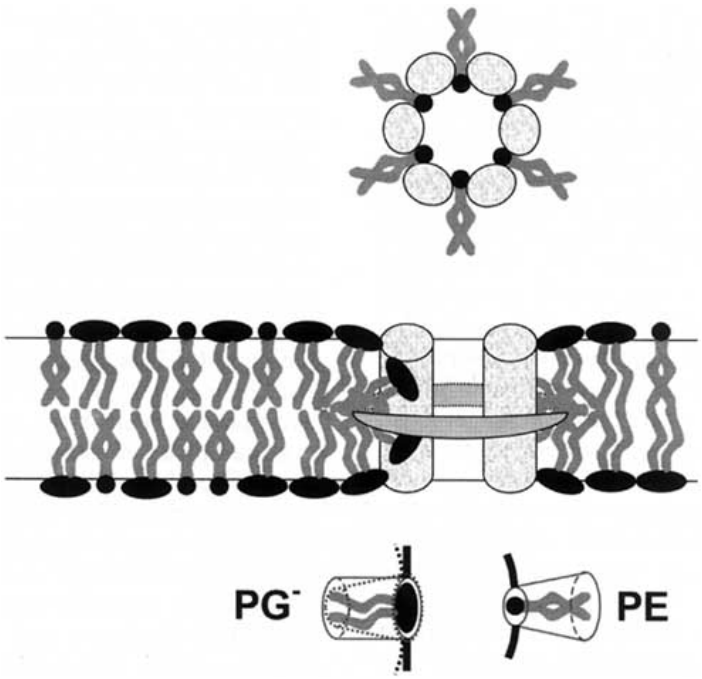


**Fig. (3)**. Geometrical requirements related to the packing of the phospholipids forming a toroidal pore; positive curvature if viewed normal to the bilayer plane and negative curvature parallel to the bilayer plane. The latter is most pronounced in the methyl-region of the hydrocarbon chains (shaded area and topview). In addition, the molecular shape of PG and PE, major phospholipid components of cytoplasmic bacterial membranes, is represented.

alamethicin (barrel-stave) and melittin (toroidal) showed that assuming an internal membrane tension associated with the membrane thinning can explain the observed behaviour by X.ray diffraction and oriented CD spectroscopy [86] which is a linear decrease of the bilayer and parallel orientation of the peptides until the threshold concentration is reached.

The geometrical requirements related to the packing of the phospholipids forming the toroidal pore are twofold: a positive curvature normal to the bilayer plane and a negative curvature parallel to the bilayer plane "Fig. (**3**)". The extent of negative curvature however depends on the polar angle of the peptide that determines the diameter of the pore [87]. If the diameter exceeds about 1.1 nm, positive curvature facilitates pore formation [79]. In addition, PE was shown to inhibit the formation of such pores, which may be explained by its molecular shape. Thus, PE can be described as an inverse cone-shaped molecule that preferentially adopts structures of negative curvature [88]. This contrasts with the requirements for a pore-forming lipid that has to pack predominantly in a positively curved structure as can be more easily adopted by PGs. Hence one could speculate that pore forming peptides would preferentially interact with negatively charged PGs. Consequently, one can further assume that this mechanism would be rather relevant for bacteria with cytoplasmic membranes of high PG content as seen for many Gram-positive bacteria, while such pore formations would be inhibited in bacterial membranes rich in PE such as the Gram-negative bacteria *E. coli*, *P. aeruginosa* etc. In support of these premises, magainin was found to kill more effectively bacteria containing inner membrane with higher amounts of PG [51].

There has been an ongoing debate whether pore formation or carpet mechanism is the correct model. A number of arguments against pore formation are based on the lack of preferred stoichiometries for the pores as demonstrated by the wide variability in conductance increases induced by peptides in model membranes [89] and on the fact that once a stable pore is formed, it should result in a lethal effect [90]. This in turn should be reflected in MICs below the micro-molar concentration range experimentally observed. However, membrane permeabilization by pore formation may be also compensated by pumps in bacterial membranes, as the membrane organization is intact [75]. In addition, pore formation is a cooperative process and hence reduction in the peptide density in the outer leaflet due to translocation into the inner leaflet would significantly decelerate subsequent pore formation, and, in turn, the system would reach a quasi-equilibrium state, where pores no longer exist. This could also reflect a way to translocate peptides from the outer to the inner leaflet of the bilayer [75]. Moreover, it was also argued that these mechanisms may represent gradual or specific steps of membrane perturbation which strongly depend on the nature of the peptides, lipids and environmental conditions [6, 91, 92]. Studies on the interaction of magainin with liposomes composed of different negatively charged lipids further underline this suggestion [79]. As mentioned above magainin effectively formed pores in PG model membranes well below lipid-to-peptide molar ratios of 100:1. However, pore formation in liposomes composed of DPG, phosphatidylserine (PS), or phospatidic acid (PA) was only observed at much higher peptide concentration (lipid-to-peptide molar ratios 50:1 to 10:1). In addition, this was accompanied by some morphological changes of the liposomes. The different behavior of these acidic phospholipids was attributed to their ability to form non-lamellar structures under conditions of reduced headgroup repulsion such as low pH or high ionic strength. Also binding of cationic AMPs will locally neutralize the bilayer charge and hence affect membrane curvature which seemingly is one parameter that determines besides of electrostatic interactions the kind and efficacy of interaction of AMPs with lipid membranes [79]. These observations emphasize that membrane lipid composition plays an important role by which mechanism AMPs permeabilize or disrupt membranes.

**Domain Formation and Defect Structures - Impairment of Membrane Function**

It is evident from the discussion above that the molecular mechanism of bacterial killing obviously depends on a number of parameters such as the nature of the peptides, the membrane lipid composition, lipid-to-peptide molar ratio and environmental conditions such as ionic strength or pH, and that there is no “one” mechanism of membrane permeation/disruption. Another parameter less considered is the initial mass imbalance between the outer and the inner leaflet upon binding of AMPs to the membrane, although there were early suggestions that this acts as a driving force for pore formation and translocation of peptides across the membrane [93]. Upon peptide insertion we may observe two extreme situations: perpendicular and parallel orientation of the peptide to the membrane plane, whereby the latter situation is of particular interest "Fig. (**4**)". Insertion and accumulation of peptides mean that the lipid bilayer has to accommodate the peptides and hence has to balance the mass increase in the outer leaflet of the bilayer. One may argue that this can be easily compensated by flipping of phospholipids into the inner leaflet of the bilayer. However, the half-time of translocation in biogenic bacterial cytoplasmic membranes is on the order of minutes [59 and references therein] and therefore, packing constraints will exist in the outer leaflet resulting in compression of this lipid monolayer and/or increased curvature strain. In addition, voids in the hydrophobic core of the membrane are created below the peptides, if they lie parallel to the bilayer plane. The extent of this perturbation depends on the depth of penetration which is governed by the hydrophobicity of the peptide. This is energetically an unfavorable situation and hence the system will rearrange in a way that hydrophobic interactions are maximized. One can envision that the lipids with a high degree of flexibility in the liquid-crystalline phase will fill this void by e.g. increased trans-gauche isomerization of the neighboring lipids and/or by moving the interior leaflet towards the peptides "Fig. (**5**)". As a result, membrane domains may be formed that differ significantly in their local properties (curvature strain, membrane thickness and fluidity) as compared to the membrane bulk phase. Because of the differences in the physicochemical properties between the bulk phase and AMP-enriched domains packing defects may arise at their border lines resulting in increased membrane permeability. In addition, exclusion of certain lipids, e.g. segregation of PG, from areas of the cell membrane due to their preferential interaction with AMPs may occur. Re-ordering of the native

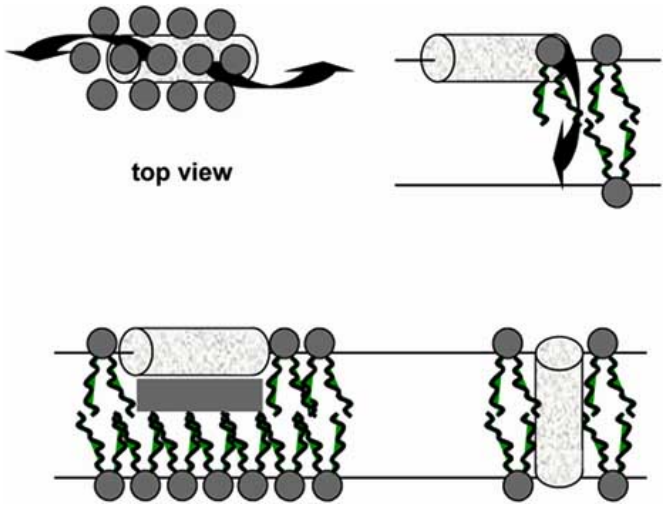


**Fig. (4)**. Peptide insertion into a bilayer. Peptides oriented parallel to the bilayer plane may create voids in the hydrophobic core (shaded area), an energetically unfavorable situation. In addition, lipids in the outer leaflet of the bilayer have to move upon insertion of the peptides within the plane or across the bilayer as indicated by arrows. Peptides are represented as a cylinder (α-helix of about 25 residues to scale with phospholipids).

membranes in this manner may have important implications on membrane structure and integrity and influence the function of membrane proteins.

In support of these premises differential scanning calorimetry and X-ray diffraction indicated that PGLa induces lipid segregation in PG liposomes, most likely resulting in peptide-depleted and peptide-rich domains as deduced from the occurrence of an additional phase transition observed at temperatures above the chain-melting transition of PG [94]. Such additional transitions were also observed for human neutrophil peptide (HNP-2) [95] and the cyclic peptide rhesus theta defensin (RTD-1) [96]. In contrast to these AMPs protegrin-1 induced an additional phase transition below the chain-melting transition of PG liposomes [52]. There is also evidence that other biologically active amphipathic molecules, such as cardiotoxin [98] and synthetic peptides ([99] and references therein) interact with model membranes inducing lateral separation of phospholipids into co-existing domains. Phase separation is not restricted to PG liposomes, but was also detected in mixtures of PE/PG. Such liposomes represent a simple, but adequate model system to mimic bacterial cell membranes [100]. Using this model system microcalorimetric studies revealed that in the presence of HNP-2 [95] and PGLa [92] additional phase transitions appeared below the chain melting transition of such mixtures. An analogous behavior was also observed for LL-37 "Fig. (**6**)". Already at a lipid-to-peptide molar ratio of 100:1 the major endothermic peak, which is related to the chain melting of the lipid mixture, is shifted towards higher temperature in comparison to the dipalmitoyl-PG/dipalmitoyl-PE equimolar mixture. This shift indicates a depletion of PG molecules from the lipid mixture (PE is the higher melting component) and consequently the formation of peptide-enriched PG domains, which melt at lower temperature. Again this emphasizes a preferential interaction with negatively charged lipids as expected because of the cationic nature of the peptide. This behavior is typical for a large number of AMPs, such as for example the α-helical peptides magainin 2 [51] and buforin II [102], the β-sheet peptides tachyplesin [13] and protegrin-1 [103], the cyclic peptide gramicidin S [104] as well as nisin Z [105] to name a few.

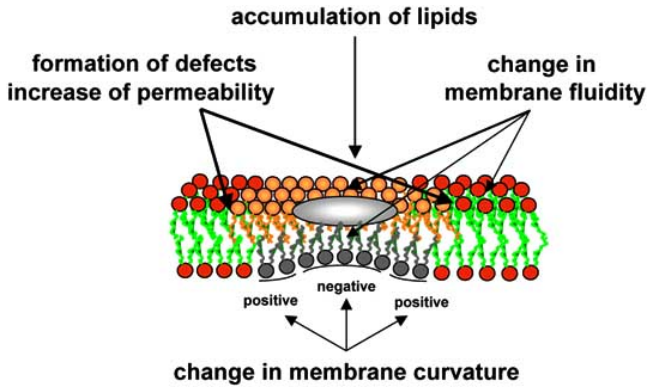


**Fig. (5)**. Scheme of a lipid-peptide domain indicating possible changes in membrane fluidity as well as curvature and formation of defect lines.

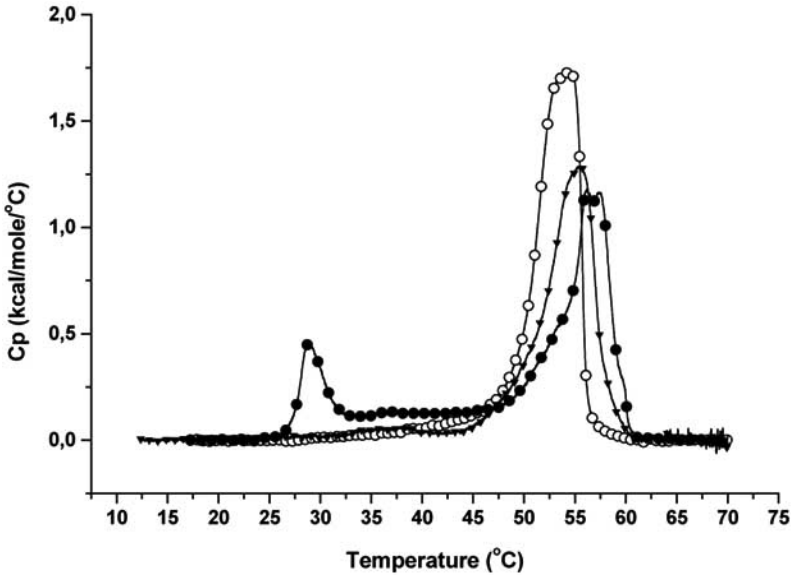


**Fig. (6)**. Excess heat capacity curves of dipalmitoyl-PG/dipalmitoyl-PE (1:1 mol/mol) (❍) and in the presence of LL-37 at a lipid-to-peptide molar ratio of 100:1 (▼) and 25:1 (●), recorded at 30°C/hr in 10 mM Na-phosphate buffer pH 7.4, 130 mM NaCl.

Using nonperturbing solid state magic angle spinning $^{31}P$ NMR and $^{2}H$ wide-line NMR nisin was also shown to induce phase separation in PC/PG model membranes [106]. Again the negatively charged PG partitions preferentially into the nisin-associated environment which was quantified from $^{2}H$ NMR measurements of selectively headgroup-deuterated PC. Deuterium NMR of the selectively headgroup-labelled PG provided further evidence of a strong interaction between the charged lipid component and the peptide. It was suggested that the observed lipid partitioning in the presence of nisin is driven, at least initially, by electrostatic interactions confirmed by isothermal titration calorimetry [107]. From the latter experiments an increase of peptide concentration near the membrane surface by about 2-3 orders of magnitude compared to its bulk concentration was estimated. This high surface concentration was suggested to be the driving force for the peptide transition from the lipid-water interface into the membrane. Further, the small intrinsic partition coefficient ($K = 1.8\ M^{-1}$), indicated a correspondingly small hydrophobic energy contribution to the binding process. This electrostatic model was confirmed with nisin mutants in which valine-32 was replaced with either lysine or glutamate increasing or decreasing the electric charge by 1 unit. The contribution of electrostatic and hydrophobic free energy to non-specific binding of peptides to charged (PG) and neutral (PC) membrane interfaces were systematically investigated using indolicidin analogs which covered a wide range of hydrophobic free energy [108]. These studies showed that these components are not always simply additive and that the effective valences of the peptides are smaller than the formal ones, which may lead to an underestimation of the hydrophobic interactions. The principal issues for peptide-lipid interactions [109] concern the structural complexity of the membrane interface, the thermodynamic contributions of the membrane bilayer itself as well as conformational and solvation changes of the peptides upon interaction. Therefore, the authors argue that integration of these components will require atomic level descriptions such as taken into consideration by the Honig and McLaughlin groups [110-113].

**Membrane curvature strain and membrane disruption**

Microorganisms such as e.g. *Escherichia coli* or *Acholeplasma laidlawii* have been shown to precisely regulate the amounts of lamellar and non-lamellar phase preferring lipids [114]. The importance of the proper balance between these types of lipids has been widely discussed [115, 116]. Their lipid composition is in a narrow window close to a lamellar to non-lamellar phase boundary, thereby conferring upon these membranes a degree of non-lamellar structure forming propensity. The presence of non-lamellar phase forming lipids such as PE, DPG or MGDG led to an increase in the lateral pressure in the center of the bilayer owing to the cone-shaped molecular geometry of these lipids. This results in a negative curvature stress or in other words in a lamellar bilayer state where the desire for monolayer curvature is physically frustrated. This is in contrast to lamellar phase forming lipids such as PG or choline phospholipids. These phospholipid molecules exhibit a cylindrical molecular shape and therefore exhibit a uniform lateral pressure throughout the hydrocarbon chain region of the bilayer. The different packing properties may have also implications for membrane function. For example, as mentioned earlier it has been suggested that the high lateral hydrocarbon chain pressure, exhibited by such lipids, controls the conformation of integral membrane proteins. In accordance with this assumption are observations that non-lamellar lipids are often required for functional reconstitution of membrane proteins (64 and references therein) and that PE is found in protein rich membrane domains [60]. Therefore, one may speculate that AMPs that reduce the lateral hydrocarbon chain pressure upon insertion may lead to conformational changes of integral membrane proteins and hence to impairment of membrane function.

It is also tempting to speculate that AMPs are able to induce membrane rupture by lowering the lamellar to non-lamellar phase boundary as schematically shown in "Fig. (**7**)". Such a mechanism may be proposed for bacteria with membranes of high content of non-lamellar phase forming lipids. This suggestion is supported by X-ray measurements on liposomes composed of lipid extracts from *E. coli* and *A. laidlawii*, respectively [117]. In this study it was demonstrated that the cyclic peptide gramicidin S of *Bacillus brevis* has considerable potential for disrupting the structural integrity of lipid bilayer membranes by markedly decreasing the energetic barriers against the formation of non-lamellar lipid phases. It was proposed that the formation of the bicontinous cubic lipid phases is due to the limited flexibility of the β-turn of gramicidin S as well as to the clustered location of the ornithine side chains. This conformation facilitates an accommodation of the peptide in the lipid membrane that strongly promotes negative curvature which leads to disruption of the lamellar structure. A significant increase in monolayer curvature stress is likely to be of major importance for the formation of non-lamellar structures and was suggested to be key to the membrane-disruptive properties of some AMPs [92, 99].

Furthermore, several AMPs were shown to promote the formation of non-lamellar lipid structures in PE model systems. For example, alamethicin induces a cubic phase, when incorporated in small amounts in dielaidoyl PE [118]. Moreover, a phase sequence of $H_{II}$ – bicontinous cubic of various space groups - $H_{II}$ was observed in dioleoyl PE liposomes with increasing concentration of alamethicin [119]. It was suggested that alamethicin induces such lipid structures by changing the thickness and/or flexibility of the lipid bilayer. This is supported by the observation that binding of alamethicin to diphytanoyl PC, exhibiting also a cone shaped molecular geometry, causes membrane thinning. Concomitantly an increase in chain disorder over a large area was observed [83, 120]. Huang and coworkers proposed that this decrease in membrane thickness is compensated by an increase of the hydrophobic cross sectional area of the lipid acyl chains. In case of PE this lateral expansion would further enhance the existing mismatch between the cross-sectional areas of the headgroup and hydrocarbon side chains, inducing the lipid monolayer to curl. A different mechanism of induction of negative membrane curvature strain was suggested for nisin which lowered the lamellar to inverse-hexagonal phase transition of dioleoyl- and palmitoyloleoyl-PE, respectively [121]. It was proposed in analogy to hydrophobic molecules such as squalene [122] and fluarizin, a drug used in cardiovascular pharmacology [123] that insertion of the large hydrophobic section of nisin (segment 1-19) will lead to an increase of the hydrophobic volume in the bilayer interior. This will again promote negative curvature and hence formation of inverted non-lamellar structures. Formation of such structures owing to reduced headgroup hydration in the presence of nisin was ruled out by IR-measurements. The impact of insertion of a large hydrophobic volume into the bilayer can also be deduced from a recent study on a custom-designed cecropin B analog [124]. While the natural cecropin B has one amphipathic and one hydrophobic α-helix, the synthetic peptide (CB3) was composed of two hydrophobic α-helices. Both peptides differed markedly in their interaction with model membranes. While for cecropin B pore formation was proposed, CB3 induced rapid formation of irregular shaped, non-lamellar structures. Microscopy on giant PE/PA vesicles showed that these structures rapidly disintegrated into twisted, microtubule-containing debris before being completely destroyed. Finally, the impact of penetration depth was recently demonstrated by studies on a 17 β-amino acid oligomer (beta-17). Formation of $H_{II}$-structures were facilitated by expanding the regions of the bilayer below the pivotal plane more than regions close to the interface resulting again in increased negative curvature strain [125]. This synthetic peptide exhibiting similar antimicrobial and hemolytic activity than magainin 2 also showed like other AMPs specificity for negatively charged phospholipids.

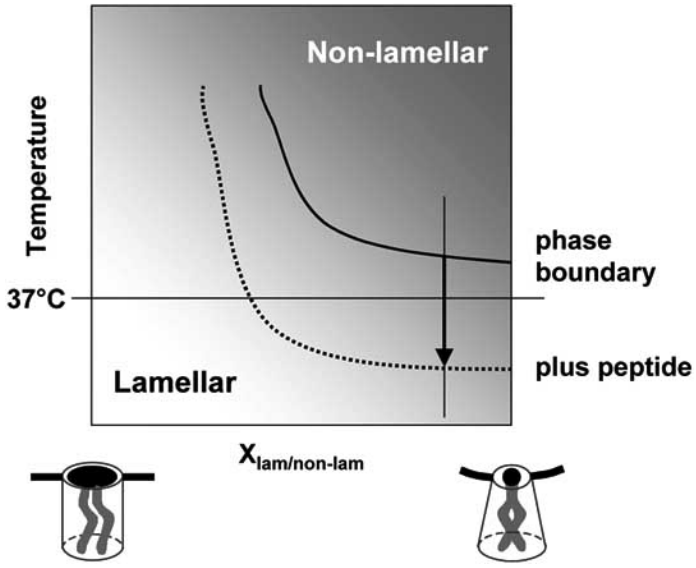


**Fig. (7)**. Schematic phase diagram and phase boundary of lipid mixtures composed of lamellar and non-lamellar phase preferring lipids. At high amounts of non-lamellar phase preferring lipids the phase boundary can be close to physiological temperature, which can be lowered dramatically by addition of antimicrobial peptides [117].

However, beta-17 did not discriminate between PG and PS liposomes like magainin 2 indicating different mechanism of membrane perturbation for these peptides. Antimicrobial activity has been reported recently for a number of β-peptides [126-129] which have the advantage of being resistant to proteolytic degradation [129] and hence makes them interesting candidates for novel antibiotics. Obviously the membrane-disrupting capacity of several AMPs is related to the non-lamellar phase-forming propensity of the target cell membrane. This has some interesting implications for specific targeting and peptide design. Moreover, it suggests a role for lipidomics, i.e. mapping of the lipid molecular species and using physicochemical methods for characterizing and modeling lipid-peptide interactions, especially in lipid microdomains of biological membranes [130], in the research area of antimicrobial peptides.

## BACTERIAL KILLING BY RECEPTOR-LINKED PROCESSES

Results from recent studies on model systems utilizing mainly fluorescence techniques and measuring the depolarization of planar lipid membranes have intensified discussions that AMPs may exert their action by binding to cytosolic targets. While these aspects are still a matter of debate [5, 78], certain lantibiotics were shown to bind to a specific membrane lipid precursor. Therefore, this chapter is devoted to receptor-linked processes that may lead to bacterial killing.

### Membrane Translocation of AMPs - Binding to Intracellular Targets

Experiments on the interaction of different classes of AMPs with planar bilayers and with the inner membrane of *E. coli* showed that there was no clear correlation between membrane depolarization and the minimal inhibitory concentration of the peptides [89]. Furthermore, systematic studies on model membranes using a series of natural and synthetic AMPs from various structural classes covering practically all structures known (α-helical, β-sheet, cyclic and extended) showed that while all peptides interacted with the phospholipid bilayer, the impact on these membranes was quite heterogeneous [131]. It was therefore proposed that the known abilities of AMPs to act on lipid membranes rather reflect the peptides ability to cross membranes and that their target(s) are located in the intracellular space. Effective internalization without significantly permeabilizing the membranes was also suggested earlier for buforin II, a histone H2A-derived AMP of 21-residues and +6 net charge from the Asian toad Bufo bufo gargarizans [132]. Circular dichroism measurements revealed an amphipathic helix distorted around Pro(11) with a flexible N-terminal region [102]. Thus, it was suggested that proline may play a role in the translocation process, supported by the observation that Ala substitution of Pro(11) resulted in structural and membrane perturbing properties similar to magainin 2. The latter is considered to represent an archetype for membrane-disrupting AMPs. Furthermore, buforin II was more efficiently translocated across lipid bilayers but unlike magainin 2 no lipid flip-flop was associated with the translocation indicating that the mechanism of membrane translocation of both AMPs differs from each other.

Internalization was also reported for human neutrophil peptide-1 (HNP-1) into *Mycobacterium tuberculosis* [133] and pyrrhocoricin, a proline-rich insect peptide for which a specific enzymatic target has been identified [101, 134]. Thus, pyrrhocoricin binds to the *E. coli* heat shock protein DnaK diminishing its ATPase activity, while the inactive D-pyrrhocoricin analogue and both membrane-active AMPs cecropin A and magainin 2 failed to inhibit the DnaK-mediated phosphate release from ATP. Studies were also performed to gain information on the other important biological function of DnaK, the refolding of misfolded proteins, by assaying the alkaline phosphatase and beta-galactosidase activity of live bacteria. Again only pyrrhocoricin reduced the activity of these enzymes. Experiments with labelled pyrrhocoricin analogues suggested that binding of the peptide prevents the frequent opening and closing of the multi-helical lid over the peptide-binding pocket of DnaK, permanently closing the cavity, and thereby inhibiting chaperone-assisted protein folding. Remarkably, pyrrhocoricin binding was not observed to the homologous DnaK fragment of *S. aureus*, a pyrrhocoricin non-responsive strain. Hence, owing to the prominent sequence variations of prokaryotic and eukaryotic DnaK molecules in the multi-helical lid region, it was proposed that finding this new target paves the road for the design of strain-specific antibacterial peptides and/or peptidomimetics.

In summary, these observations suggest that for a number of AMPs the ultimate target is not the cytoplasmic membrane but intracellular. One can then envision multiple intracellular targets in bacteria such as DNA and RNA as well as proteins as shown e.g. for pyrrhocoricin. Nevertheless, in order to gain access to these targets the peptides have to overcome the membrane barrier, which again emphasizes a possible role of the membrane lipids regarding the specificity and/or efficacy of such AMPs. So far, little is known about the molecular mechanism of peptide translocation across lipid bilayers.

Remarkably, many of the AMPs that are believed to act on cytoplasmic targets are rich in arginine and/or proline. Therefore, one may speculate that these peptides are moved across the bilayer by a mechanism similar to arginine-rich peptides derived from e.g. HIV-1 proteins, flock house virus coat proteins or cancer related proteins [135]. These peptides have attracted attention as potential intracellular delivery vehicles of macromolecules. The wide structural variety of such membrane permeable peptides and findings on the translocation of these peptides through eukaryotic cell membranes were recently reviewed [136]. Systematic studies on synthetic linear and branched-chain arginine peptides revealed a considerable degree of flexibility in the spatial disposition of the positive charges in these peptides showing that a linear structure was not indispensable for their translocation [137]. Moreover, typical endocytosis and metabolic inhibitors had little effect on the translocation of the peptides suggesting a common internalization mechanism ubiquitous to arginine-rich peptides, which is not explained by a typical endocytosis [138].

Peptide translocation through a eukaryotic cell membrane may however differ from translocation through a bacterial membrane owing to the differences in lipid composition of the two membranes. Evidence for a role of lipids in

translocation of molecules comes from studies on *E. coli* mutants deficient in PE synthesis. These mutants exhibited a significantly reduced ability to transport proteins across the cytoplasmic membrane, which was restored upon addition of diunsaturated PE [62]. Interestingly, such lipids have a strong tendency to form inverse-hexagonal ($H_{II}$) lipid aggregate structures, when dispersed in water [88]. The presence of such transient inverted lipid structures in protein translocation or membrane fusion has been a long-standing issue in membrane biophysics [139]. Having in mind that in particular PEs are particularly prone to undergo such structural changes and that these lipids are major components of Gram-negative bacterial membranes, one can envision that PEs may also play an essential role in the translocation process of AMPs.

Participation of lipids in peptide translocation from the outer to the inner monolayer of a bilayer was outlined by Matsuzaki [75]. As described above translocation of magainin 2 was coupled to a transient pore formation and phospholipid flip-flop. Thereby, the peptide translocates stochastically into the inner leaflet upon disintegration of the pore. This process is not specific for magainin 2, but was also shown for the α-helical frog skin peptide PGLa [87], the β-sheet peptide tachyplesin I from horse shoe crab [140], mastoparan X [141] and the α-helical bee venom peptide melittin [142]. Notably, none of these peptides belongs to the class of arginine rich AMPs. The formation of the toroidal pore and translocation of the peptide lasts seconds to minutes depending on the peptide-lipid ratio, whereby an increase of the positive charge of the peptide would reduce the stability of the pore as a result of enhanced electrostatic repulsion between the side chains [143]. Hence, one could speculate that rapid pore formation and disintegration facilitate the internalization of a peptide without significantly perturbing the barrier properties of the membrane.

### Lipids as Receptors for AMPs – the Specific Case of Lantibiotics

Lantibiotic peptides secreted by a wide range of Gram-positive bacteria exhibit activity toward Gram-positive organisms and are usually several orders of magnitude more potent than traditional antibiotics such as penicillin [144]. Many of the lantibiotic peptides have been shown to use a particular lipid component of bacterial membranes, lipid II, as a specific docking molecule [reviewed in 155]. Lipid II is the prenyl chain-linked donor of the peptidoglycan building blocks and hence represents a target for many antibiotics. Binding of such antibiotics lead to inhibition of peptidoglycan biosynthesis, thus preventing the formation of the peptidoglycan layers surrounding the bacterial membrane, which is essential for bacterial cell survival. Lantibiotics contain a number of unique structural features including dehydroamino acids and the thioether amino acids lanthionine and 3-methyllanthionine resulting in the formation of characteristic, intra-chain ring structures. Two groups of lantibiotics have been reported: the elongated amphiphilic, screw-shaped peptides of the type A-lantibiotics and the globular type B-lantibiotics. The latter can be further subdivided based on mode of action. Thereby, peptides of the cinnamycin-subtype inhibit phospholipases and other enzyme functions by binding to PE, while mersacidin and actagardine inhibit the bacterial cell wall biosynthesis by blocking the membrane-bound peptidoglycan precursor lipid II [145]. In particular, the hinge region (Ala-12 and Abu-13) of mersacidin allows an effective alteration of the overall backbone depending on the environment [146]. Thus a compact structure of mersacidin has been observed by NMR measurements in the presence of dodecylphosphatidylcholine (DPC) micelles comparable to its structure described in water/methanol [147] and crystalline state [148], while upon binding to lipid II exposure of the charged groups was observed. This suggests that electrostatic interactions most likely govern the binding mechanism between mersacidin and its receptor molecule. Similar structural changes (flattening of the peptide structure upon binding to a lipid II analog [149]) was also observed for ramoplanin, a lipoglycodepsipeptide antibiotic considered as a promising clinical candidate for treatment of Gram-positive bacterial infections that are resistant to antibiotics such as glycopeptides, macrolides, and penicillins [150]. Although ramoplanin does not exhibit sequence homology to mersacidin and actagardine, it is topologically similar. Based on these studies, it is now assumed that conformational changes are a common feature of peptides that bind to lipid II [146].

Binding of the type-A lantibiotics, nisin and epidermin, to lipid II also results in inhibition of peptidoglycan biosynthesis, and in addition induces the formation of pores [145]. Nisin was the first example of such a targeted membrane-permeabilizing peptide antibiotic [54]. A dual role was suggested for lipid II, namely to function as a receptor for nisin, thereby increasing its specificity for bacterial cells (MIC in nanomolar range), and to be involved in the pore formation. This has now been elucidated in detail using mass spectrometry and fluorescence spectroscopy [151]. Using pyrene-labelled lipid II a model for the nisin-lipid II pore complex was proposed: a uniform structure composed of an identical number (5-8) of nisin and of lipid II. The latter is separated by about 1.8 nm. Seemingly no restriction to the length of the hydrophic anchor was found, although a minimal chain length is required to obtain a stable pore complex. This led to the assumption that lipid II is located at the outer boundaries of the pore complex. The specific interaction however occurs at the pyrophosphate-MurNAc(pentapeptide)-GlcNAc headgroup, which is mediated by the N-terminus of nisin [152-154].

## CONCLUDING REMARKS - STRUCTURE ACTIVITY RELATIONSHIP STUDIES

As outlined at the beginning of the review biophysical studies have contributed strongly to the elucidation of the structure of AMPs in solution and membrane bound/inserted state, their aggregational state and finally, their interaction on a molecular level with membranes (mostly model systems) and hence the molecular mechanism of action of AMPs. Together with biological activity data this knowledge forms the basis of structure-activity-relationship analysis. Less attention was paid in this review to the former issues, i.e. peptide structures and aggregational state, because this would exceed the size of this review. Moreover, an excellent review appeared very recently on the relationship

between peptide structure and antibacterial activity [5]. The authors also focused only on one representative peptide from each structural class in respect of SAR studies which basically reveal two main requirements for antimicrobial activity: a positive charge and an induced amphipathic conformation. Significant contributions to an understanding of amphipathicity of α-helical peptides were made by the groups of Dathe [156 and references therein] and of Shai [49 and references therein]. The former group performed systematic studies on synthetic α-helical peptides varying the angle subtended by charged residues, the hydrophobic moment and total hydrophobicity, while Shai has demonstrated that breaking the α-helix by incorporation of D-amino acids results in loss of hemolytic activity but retained antimicrobial activity. This is in agreement with earlier studies on combinatorial α-helical 18-mer peptides composed of leucine and lysine residues, termed YLK [41]. Peptides with a high amphipathic structure were hemolytic, while peptides showing the greatest antimicrobial activity have a low estimated amphipathicity. A very recent surface plasmon resonance study showed that diastereomeric peptides bound similarly to both PC/cholesterol bilayers and monolayers, while the L-amino acid peptides bound 10-25-fold stronger to bilayers as compared to the monolayer [157]. The different behavior was explained by a two-state reaction model indicating insertion of the L-amino acid peptides into the PC/cholesterol bilayers and a surface localization for the diastereomers. In contrast, using PE/PG model membranes only a 2-fold difference was found. Moreover, the diastereomers bound approximately 100-fold better to PE/PG than to PC/cholesterol membranes, whereas the L-amino acid peptides bound similarly to both lipid matrices suggesting that selectivity is determined in the first binding step. Diastereomeric peptides were also used to test the role of pre-assembly on their ability to discriminate among bacteria, erythrocytes, and fungal cells by using monomeric peptides with variable lengths and their covalently linked pentameric bundles [158]. All the bundles expressed similar potent hemolytic and antifungal activity as well as high antimicrobial activity. In contrast, all the monomers showed length-dependent antimicrobial activity, were less active toward bacteria and fungi, and were devoid of hemolytic activity. Attenuated total reflectance Fourier-transform infrared spectroscopy revealed that peptide assembly affects the structure of the peptides as observed by an increased α-helical and β-sheet content in membranes. This also points out the need of a careful characterization of the structure of AMPs which frequently differs between the solution state and membrane bound/inserted state. It will not be sufficient to perform these studies routinely with CD spectroscopy but to determine a detailed high resolution three-dimensional conformation [5]. For example, even small peptides such as a hexapeptide that originated from a synthetic combinatorial library [41] exhibited a marked amphipathic structure in SDS with its hydrophobic residues on one side of the structure as deduced from 2D-NMR spectra [159]. Applying the same technique a fragment of the human lactoferrin, LF11, that exhibits no structure in aqueous environment was shown to form a well-defined hydrophobic core, when bound to LPS with Trp-3 at the interface between polar and nonpolar groups of the lipid A, and to adopt a loop structure in anionic micelles [160]. These observations emphasize that a number of peptides may adopt different structures depending on the lipid counterpart. This was early recognized in lipid-peptide interaction studies, where α-helical structure was only induced in the presence of negatively charged lipids, which was proposed to be one way of discrimination between bacterial and mammalian membranes that differ markedly in their lipid composition [52]. As outlined throughout the review the mechanisms of bacterial killing also depend on the lipid matrix, e.g. pore formation may rather occur in PG-rich membranes, while disruption of the membrane will be rather observed in PE-rich membranes. Considering the diversity of lipids in bacterial membranes it may not be too surprising that different mechanism of action have evolved in nature. In respect of understanding the mode of action of AMPs and to get a profound knowledge for the rationale design of novel antibiotic peptides it will be necessary to study the interactions between antimicrobial peptides and membrane lipids with accurate model systems. Only then we shall be able to deduce the proper mechanism of action.

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