Thrombocidins, microbicidal proteins of human blood platelets

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Citation for published version (APA):

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Review

Mechanism and Design of Microbicidal Peptides

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Submitted for publication
Review

Chapter 7
INTRODUCTION

Peptide antibiotics form an integrated part of the defense system against invading microorganisms of most if not all species of life. Besides their widespread occurrence, they generally share a broad-range antimicrobial activity against Gram positive and Gram negative bacteria, as well as fungi. Because of these traits peptide antibiotics are often classified as belonging to the aspecific host defense system. Alternatively, the terms innate or first-line host defense are used to indicate that peptide antibiotics are readily available and need not be induced upon infection, as is the case with the components of the adaptive immune response.

Based on their structure and specific amino acid composition antimicrobial peptides have been categorized into four classes, β-sheeted peptides stabilized by one or more disulfide bonds, α-helical peptides, looped structures and extended coils (46). For a general overview of these classes and a fairly complete description of the peptides they encompass, the reader is referred to a number of comprehensive reviews (13,14,35,46,47,90).

Peptide antibiotics may have potential to complement conventional antibiotics, which are increasingly ineffective in the combat against microbial infections due to increased levels and rapid spreading of microbial resistance (22,26). Several reviews are available focusing on structures of peptide antibiotics (46,47,115), on specific classes of peptides (33,35,76,104,122), or on their clinical status (15,48). Although touched upon in several reviews (7,56,74,103) no comprehensive summary has been published regarding characteristics of peptide antibiotics required for antimicrobial activity. More confusing, studies on these characteristics have often been performed using membrane vesicles or planar lipid membrane systems. It is unclear how results from such experiments can be extrapolated to the actual killing of microorganisms. Therefore, the scope of this review is to evaluate the characteristics of peptides contributing to their antimicrobial activity, and to assess the value of findings in artificial membrane systems for the actual killing of microorganisms. Furthermore, directions will be discussed how this knowledge could be used to synthesize peptide antibiotics by rational design.

TESTING ACTIVITY OF MICROBICIDAL PEPTIDES

The rapid lysis of bacteria has been shown to be the basic mechanism of many microbicidal peptides. The concept that these peptides are membrane active agents forming pores and thereby causing leakage of nutrients or loss of membrane integrity is widely accepted. The method of choice to test peptide microbicidal activity is to expose selected microorganisms to a peptide and culture the surviving organisms. However, in order to obtain insight into the microbicidal mechanism other approaches are required. In this respect, particularly artificial (model) membranes have shown their usefulness.

Two types of membranes have been used most widely, planar bilayers and lipid vesicles. Planar bilayers are used to study ion permeability by measuring electrical conductance across the membrane as a function of the applied voltage. Normally lipid
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bilayers are impermeable for ions, but in the presence of microbicidal peptides ion-permeable channels can be formed.

Lipid vesicles (or liposomes) are aqueous compartments enclosed by a lipid bilayer. They can be formed by sonication of a suitable lipid (mixture) in an aqueous medium or by dispersion of the lipid and aqueous medium through a needle with small diameter. Liposomes offer a versatile system for studying several parameters, of which the following are the most common. Firstly, the lipid composition can be varied at choice. Bacterio-mimetic vesicles can be formed by using a mixture of zwitterionic (phosphatidyl choline (PC) or phosphatidyl ethanolamine (PE)) and anionic phospholipids (phosphatidyl glycerol (PG), diphosphatidyl glycerol (= cardiolipin, CL) or sphingomyelin). Subtle variation of membrane composition allows the study of requirements for peptide-lipid interaction and its consequences for membrane disintegration. Secondly, non-lipid compounds can be entrapped inside the vesicles to monitor their release after exposure to peptides. This method is used to study the size of pores formed, by measuring the (pace of) leakage of molecules of defined molecular weight. Finally, a membrane potential or pH gradient can be applied over the membrane. Both membrane potential and pH gradient may influence peptide activity, or may change upon peptide-lipid interaction.

An important prerequisite of effective peptide antibiotics is their selectivity for microbial membranes, i.e. eukaryotic membranes should be left undamaged. Eukaryotic membranes can be mimicked in liposomes by omission of the anionic phospholipids and by inclusion of cholesterol. In most studies however, erythrocytes are used to test cytotoxicity for eukaryotic membranes, since leakage of hemoglobin from these cells can be measured with high sensitivity.

Leakage patterns of dyes from liposomes can be fitted in a model describing mode and time scale of pore formation by peptides, as well as the size of the pore. One of the archetypes of pore-forming antibacterial peptides is alamethicin, a 20-residue α-helical peptide isolated from the fungus Trichoderma viride (87). By insertion and oligomerization in lipid bilayers, alamethicin forms highly conductive transmembrane pores which are characterized by their high stability (31,40) (Fig. 1). Initially, pore formation by other antimicrobial peptides was postulated to proceed in a similar way, but detailed mechanistic studies necessitated rejection of this hypothesis. For the most extensively investigated antimicrobial peptides these studies will be described below.

In many cases the use of fluorescent techniques has proven highly useful to dissect mechanisms of pore formation. They not only enable monitoring of membrane leakage, but have also been used to investigate position and orientation of peptides in membranes. For a detailed description of such methodologies the interested reader is referred to a number of recent reviews (64,65).
Table 1. Sequences of representative antimicrobial peptides from different classes. Asterisks denote C-terminal amidation.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ß-sheeted</strong></td>
<td></td>
</tr>
<tr>
<td>HNP-1</td>
<td>ACYCRIPACIAGERRYGTCTIYGRLWAFCC</td>
</tr>
<tr>
<td>HNP-2</td>
<td>CYCRIPACIAGERRYGTCTIYGRLWAFCC</td>
</tr>
<tr>
<td>HNP-3</td>
<td>DYCRIPIACIAGERRYGTCTIYGRLWAFCC</td>
</tr>
<tr>
<td>NP-1</td>
<td>VVCACRRALCLPRERRAGFCRIRGRHPLCCR</td>
</tr>
<tr>
<td>NP-2</td>
<td>VVCACRRALCLPRERRAGFCRIRGRHPLCCR</td>
</tr>
<tr>
<td>Protegrin 1</td>
<td>RGGRLCYCRRRFCCVGR*</td>
</tr>
<tr>
<td>Protegrin 3</td>
<td>RGGGLCYCRRRFCCVGR*</td>
</tr>
<tr>
<td><strong>α-helical</strong></td>
<td></td>
</tr>
<tr>
<td>Cecropin A</td>
<td>KWFKKKIEKMGRNIRDGIVKAGPAIEVIGSAKAI</td>
</tr>
<tr>
<td>Cecropin P1</td>
<td>SWLSTAKKLESAXKAGKRISEGIAIAIQGPR</td>
</tr>
<tr>
<td>Magainin 1</td>
<td>GIGKFLHSAKGKAFVGEIMKS</td>
</tr>
<tr>
<td>Magainin 2</td>
<td>GIGKFLHSAKGKAFVGEIMNS</td>
</tr>
</tbody>
</table>

MICROBICIDAL MECHANISM OF ß-SHEETED PEPTIDES

ß-Sheeted cysteine-containing peptides: 1. Defensins Human polymorphonuclear neutrophils (PMN) contain a range of antimicrobial peptides (23,71,108). Most abundant are the defensins, human neutrophil peptides (HNP) 1 to 4 (Table 1), constituting 5 to 7% of the total cellular protein content of PMN (70). The structure of HNP-3 was elucidated first (52), and appeared to be representative for structures of HNPs to be resolved later (96). A HNP monomer is characterized by three antiparallel ß-sheets, and the molecule is given a compact conformation by three intramolecular disulphide bridges. In the crystallized HNP two monomers form a stable basket-like configuration with an apolar base and a polar top, making it a structure with amphiphilic characteristics (52). Unlike α-helices, which are preferentially formed at a lipid interface (see below), the rigid structure of defensins in aqueous solution remains intact upon interaction with lipid (32). This rigidity and the stability of the dimer imply that this is also the biologically active conformation of HNPs (52).

Defensins are active against a broad range of microorganisms, including gram positive and gram negative bacteria (34), fungi (69) and enveloped viruses (23). Antibacterial and antifungal activity depend on metabolic activity of target organisms (68,69). Defensins were shown to subsequently permeabilize the outer and inner membrane of E. coli causing loss of vital functions (68). In conjunction with the observation that defensin (HNP-1) can form voltage-dependent channels in lipid membranes (58), the
bacterial cytoplasmic membrane has since been considered to be the primary target of these peptides.

In order to discern the cidal mechanism the ability of defensin to induce fusion and lysis of lipid vesicles of various composition has been studied. Since HNP-2 specifically interacted with anionic, and not with zwitterionic lipids in liposomes (72) and loss of lipid organization was observed only in vesicles composed of anionic lipids (32,128), initial defensin-lipid interaction is most likely governed by electrostatic interactions. Human defensins were shown to be able to fuse negatively charged vesicles (32), thereby causing lipid mixing of the outer leaflet without mixing of the vesicle content (128). Rather than to this hemi-fusion of cells, the underlying mechanism of HNP-mediated microbial killing has been related to its ability to cause membrane leakage. Entrapped fluorescent probes could be released from vesicles quantitatively, and dextran-derivatives of up to 19 kDa could be liberated at least partly (128). This indicated the presence of pores with a diameter of 25 Å, estimated to be formed by oligomers of 6-8 defensin molecules (128).

Rabbit defensins (NPs) (Table 1) are closely related to human defensins (96) but form monomers in solution (54). NPs are able to cause leakage of entrapped probes from vesicles and, like with HNPs, this process was highly dependent on the presence of negatively charged lipids in the membrane, especially cardiolipin (55). Dextrans of up to 50 kD were released, which would require pores of 60 Å in diameter, consisting of an estimated 50 defensin monomers (55). Since formation of such large pores is very unlikely, a model was proposed in which release was explained by assuming transient membrane disturbance in a diffusion-limited process (55).

By release of fluorescent probes (65) the mechanisms of vesicle leakage due to HNP and NP were shown to be different. The first mechanism was characterized by an all-or-none release of the probe (128), possibly reflecting pore formation by oligomerization of HNP in the membrane. The mechanism observed for NPs showed a graded leakage of the probe from vesicles composed of PG (54), suggested to be caused by transient membrane disturbance. However, a much faster release was observed from vesicles composed of E. coli-extracted lipids (55), indicating that the mechanism of membrane permeabilization depended on lipid composition. The difference in the mechanism of leakage from PG vesicles due to HNP and NP is suggested to be caused by the presence of HNP as dimers, as opposed to NPs which are predominantly monomeric (54).

Detailed structure-function studies of defensins have not been performed yet due to difficulties in synthesizing peptides with three intramolecular disulfide bridges. However, studies using natural defensins on model membranes, as described above, indicate several crucial elements required for defensin activity. These include membrane interaction promoted by the positive charge of the peptides, and possibly peptide multimerization in the membrane. However, the exact relevance of liposome-leakage models for microbicidal activity remains unclear. For instance, although linearized defensins had no antibacterial activity, they were more effective in causing vesicle leakage than the folded forms (32,128). Furthermore, the voltage-dependence of pore-forming activity initially observed in planar bilayers (58) has not become apparent from the work using liposomes.
ß-Sheeted cysteine-containing peptides: 2. Protegrins. Protegrins are a group of small (16-18 residues) ß-sheeted peptides containing two intramolecular disulphide bridges, imposing a hairpin-like structure to these molecules (6,28). Protegrins were isolated from porcine leukocytes (60,133), where they are stored in inactive form. They are activated upon excretion by the elastase-mediated removal of an N-terminal domain (95,111).

Although less is known about the detailed microbicidal mechanism of protegrins compared to defensins, these are promising compounds for possible application as antiinfective agents for a number of reasons. In the first place, protegrins are active against a broad range of microorganisms, including Gram-positive and Gram-negative bacteria and fungi (20,30,88,99,117). Secondly, as opposed to defensins, activity of protegrins is retained in the presence of salt (20,49) and moderate amounts of serum (111,130). Probably owing to these characteristics protegrins significantly reduced mortality due to S. aureus, P. aeruginosa and VRE infections in an in vivo infection model (117). Therefore, protegrin variants are presently under investigation in clinical trials (47).

Structure-function studies of protegrins revealed that the presence of at least one cysteine bridge is needed for antimicrobial activity, although full activity requires completely folded protegrin. Removal of both disulfide bridges almost entirely abolished activity (20,49,131). This implies that the secondary structure of protegrins is an important determinant for microbicidal activity. In addition, residues in the central part of protegrins appear to be required for full activity since modest C-terminal truncation is tolerated, while truncation of >4 N-terminal residues led to considerable loss of activity (131).

Only few studies are available on the antimicrobial mechanism of protegrins at the molecular level. It has been observed that protegrins, like other ß-sheeted peptides (see above), can form ion-permeable channels in membranes (75). Since the all-D enantiomer of protegrin-1 was as active as the natural L-form, the involvement of specific receptors is unlikely, and the microbial lipid bilayer is thought to be the target of protegrins (20,131).

Protegrin-1 is present as a monomer in solution (6). In the presence of zwitterionic micelles, however, dimers are formed which possibly associate to higher-order clusters, in analogy to defensins (102). A more detailed study of protegrin-1 in anionic bilayers revealed that the peptide could be oriented into the membrane in multiple states, of which the extremes were called S and I (51). Orientation in either state depended on relative humidity of the system and peptide concentration. Although no conclusive evidence could be given about the exact orientation in S or I state, it was suggested that the S state, at low peptide concentration, corresponded to surface absorbed peptide. The I state, at high peptide concentration, most likely represents a membrane spanning orientation (51). Transition from one state to the other was observed, but how this effect relates to microbicidal activity remains to be resolved.

Tachyplesin is an amphiphilic, cyclic 17-residue peptide with an antiparallel ß-sheet structure, analogous to protegrins. The two disulfide bridges in tachyplesin have been shown to be of crucial importance for antibacterial activity (85). When linearized, tachyplesin was still capable of destabilizing bilayer organization, but antimicrobial activity was much weaker. The antimicrobial effect of tachyplesin most likely is based on its ability to span vesicle and bacterial membranes, and to traverse these membranes. Linearized tachyplesin had lost this property (85).
MICROBICIDAL MECHANISM OF \( \alpha \)-HELICAL PEPTIDES

\( \alpha \)-Helical peptides: 1. Magainins. Of the many antimicrobial peptides presently known in frog skin (10), the magainins were the first to be isolated (132) and have since become the model representatives of antimicrobial peptides in numerous studies. Magainins 1 and 2 are closely related 23-residue peptides (Table 1) isolated from granular glands of Xenopus laevis skin (132) and have broad spectrum microbicidal activity (39,132). Although magainins have no defined structure in solution, they readily adopt an amphipathic \( \alpha \)-helical conformation upon interaction with lipids (77). Magainin dissipated electrical membrane potential across various energy transducing membranes by the formation of weakly anion-selective pores (27,123). Uncoupling of controlled respiration by interference in ATP synthesis seems to be part of the killing mechanism of magainins (123).

Further studies on peptide-membrane interactions revealed that magainins are highly selective for acidic membranes (127). Magainins do not bind liposomes composed of zwitterionic lipids, and magainins with increased charge more potently disturbed lipid organization (127) indicating that their initial contact with membranes is governed by electrostatic interactions. Interaction with lipids leads to a peptide orientation which is parallel to the membrane surface, embedding the hydrophobic face of the helix in the acyl-chain region (8,80). In this process, no stable peptide aggregation is observed (105), but peptides seem to be in a monomer-dimer equilibrium (80). However, calcein release from liposomes suggested that pore formation occurs, and from the initial rate constant of magainin-induced calcein leakage it was deduced that pores were formed by 4-6 monomers, which had a transient character judging from their short life span (73,80). These pores were formed by a supramolecular structure consisting of peptide and lipid (73,78) in contrast to the pores of peptide alone as in the barrel-stave model of alamethicin (31).

Before the equilibrium state was reached, pores acted as ion channels dissipating membrane potential, and as molecular sieves releasing 600 Da fluorophores and retaining larger molecules. Disintegration and thus closure of the pore (equilibrium state) caused redistribution of magainins, leaving a number of the peptides on the outside of the membrane, but also translocating 20-30% peptides across the bilayer to the inner leaflet (78) (Fig 1). Furthermore, peptide redistribution was concomitant with exchange of lipids from the outer to the inner leaflet and vice versa, leading to lipid asymmetry (79).

Although this model was valid for PG liposomes, in PS bilayers no pore formation occured, probably owing to the accumulation of peptide on the liposome surface (83). On PS-membranes, magainin is more likely to act by a carpet mechanism characterized by random deposition of peptide on the membrane, thereby destabilizing membrane integrity (83).

Membrane permeabilization is not only influenced by lipid charge, but also by charge of the peptide. Magainin derivatives with increased positive charge effectively killed bacteria (82), but did not show maximal permeabilizing activity in a liposome model (81). This was due to pore destabilization, most likely to occur by repulsion of the helices (81).
In contrast to the permeabilization of strongly negatively charged liposomes composed of PG, which was crucially dependent on electrostatic interactions, permeabilization of weakly negative liposomes (PG/PC mixture) depended on hydrophobic interactions (127). Increase of peptide hydrophobicity led to an increased liposome leakage as well as an increased bactericidal effect, but also to increased hemolytic activity (124). Thus, peptide charge should be sufficiently high and hydrophobicity sufficiently low to retain a selective antibacterial effect (124).

**α-Helical peptides: 2. Cecropins.** Cecropins are α-helical peptides initially identified in the hemolymph of the *Cecropia* moth, and have also been found in other insects (13). Although cidal for both Gram-positive and Gram-negative bacteria, they are more active against the latter (39,89). A mammalian cecropin, cecropin P, has been isolated from porcine intestine (66).

Insect cecropins consist of two helical segments, separated by a β-turn hinge (4). The N-terminal segment has an amphipathic character, while the C-terminal segment is more hydrophobic. Cecropin P maintains a more rigid structure since it does not contain the hinge (38,114).

Cecropins, like magainins, can form voltage-dependent ion channels in planar bilayers (21). The positively charged residues near the N-terminus are thought to initiate contact with lipid, while the hydrophobic C-terminal helix is assumed to insert into the membrane. Dissipation of the electrochemical membrane potential was proposed to be the main physiological effect, since the peptide with the most efficient depolarizing ability also had the most potent bactericidal activity (21). In addition to depolarizing the membrane, cecropins can also induce formation of larger pores as evidenced by the release of fluorescent probes (113,118) and even the large marker β-galactosidase from vesicles (113). Pore formation appears to be dependent on peptide concentration and only occurs at high peptide/lipid ratios (113). At lower ratios, only membrane depolarization occurs, and since cecropins kill bacteria at this lower concentration, the bactericidal effect is most likely due to this membrane dissipation (113).

Initial interaction between cecropin and lipid bilayers results in the deposition of peptide in monomeric form on the vesicle surface (37). A barrel-stave-like insertion as proposed for alamethicin is unlikely, judged from the relatively large number of peptides per monomer (>100) that are needed to cause initial ion leakage (37). Instead, peptides are thought to diffuse to the target cytoplasmic membrane, since no peptide aggregation occurred in the outer membrane (37). Cecropin P is thought to act by a similar mechanism, since in PE/PG lipid bilayers peptide is mainly found oriented parallel to the surface in a monolayer without formation of stable pores (38). Killing would be achieved by the disintegration of lipid packing and by formation of transient pores (36).
The need for a balance in charge and hydrophobicity of peptides for pore formation in liposomes was shown in a study using two cecropin variants consisting either of two amphiphilic or two hydrophobic helices (121). Compared to the hydrophobic variant, the amphiphilic variant bound more strongly to membranes composed of acidic...
Design of microbicidal peptides

lipids, but caused less vesicle leakage. This implies that initial binding of the peptide is
driven by electrostatic interactions. When these interactions are too strong, peptide
aggregation or retention of the peptide at the membrane surface will avoid final leakage to
occur. As evidenced in this study, the critical step in pore formation is based on
hydrophobic interactions (121).

Cecropins are unable to bind to erythrocytes and lyse these cells (118). Initially it
was proposed that this would be caused by the presence of cholesterol in erythrocyte
membranes. However, incorporation of cholesterol in lipid vesicles did not hamper
cecropin binding, nor cecropin-induced lysis (113,118). Apparently other factors like sialic
acid, abundantly present on erythrocytes, are responsible for the protection against
cecropins.

CHARACTERISTICS OF SYNTHETIC PEPTIDES INFLUENCING
MICROBICIDAL ACTIVITY

From the mechanistic studies detailed above, charge and hydrophobicity of a
microbicidal peptide have emerged as determinants for its cidal activity. Parameters like
peptide helicity (in the case of α-helices), length, and amphipathicity are additional entities
which may influence peptide microbicidal activity. These parameters have been studied
using synthetic peptides with two goals: improving microbicidal activity and improving
target selectivity, i.e. reducing cytotoxicity. In most studies helical peptides have been
investigated since their properties can predictably be modulated by modification of amino
acid composition, and because techniques are available to study conformation and
orientation of these peptides in various environments. For β-sheeted peptides this is far
more difficult, but some data are available.

Helical peptides

Helicity. A great number of antibacterial peptides with a helical character have been
identified, suggesting that helicity may be a major determinant for microbicidal activity.
Indeed, studies in which peptide helicity was disturbed by the replacement of (natural) L-
amino acids by their D-enantiomers, while preserving other parameters, showed decreased
bilayer-disturbing potency of these peptides (24). In other studies, the helical character of
peptides appeared only of limited importance: diastereomers consisting of D- and L-lysines
and leucines without α-helical character were found to have full antibacterial activity (92),
while stabilization of an α-helix as such not necessarily improved such activity (53).
Furthermore, the design of optimally helical amphipathic helices may be countereffective,
since these peptides are highly hemolytic (19). Although helical conformations are
abundantly present in antimicrobial peptides, other properties, most prominently the ability
to accomplish electrostatic and hydrophobic interactions with the target membrane, may be
of more importance for their biological activity (24,97).
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Length. Helical peptides generally vary in length from 16 to 35 amino acids. Assuming that pore formation in a membrane is a requirement of microbicidal activity, a peptide of approximately 20 residues would be required. Indeed, in a series of amphipathic helices varying from 16 to 24 residues, only the longest one could form a stable conducting pore (1). The shorter peptides only produced occasional membrane currents, and were not bactericidal (1). In another study of model peptides consisting of lysines and leucines, peptides of 14-15 residues had the most potent microbicidal activity (12). Longer peptides were not only less active, but also had higher hemolytic activity (12). As is apparent from these contradictory results, and as underlined in yet another study (92), peptide length does not seem to be decisive for microbicidal activity, or at least effects of variation in length are overruled by other peptide characteristics.

Charge. In numerous studies the influence of charge on microbicidal and membranolytic activity has been demonstrated. Initial peptide-lipid contact has especially been shown to be mediated by electrostatic interactions, which are stronger at high peptide charge. Introduction of additional lysines in leucine/lysine model peptides resulted in increased bactericidal activity (2) showing that peptide charge should be sufficiently high in order to obtain optimal bactericidal activity. Increase of charge generally leads to decreased hemolytic activity (12,92). However, when the peptide is very cationic, permeation of even only weakly anionic liposomes is reduced, and antibacterial activity is also low (92). This is probably due to retention of the peptide on the outside of the (bacterial) membrane. Lowering peptide charge resulted in a more potent antibacterial activity (92). In summary, a charge optimum exists for peptide microbicidal activity.

Hydrophobicity and amphipathicity. Two closely linked properties readily recognized as major determinants for effective bactericidal activity are hydrophobicity, and the distribution of hydrophobic regions over the peptide, designated as the hydrophobic moment or amphipathicity (67). Amphipathicity is defined as the degree of segregation of polar and non-polar residues along the axis of the α-helix to either of the faces of the helix, and is an important property for peptide interaction with lipids in general (107). Amphipathicity not only determines antibacterial but also hemolytic activity. Therefore, the impact of changes in amphipathicity on these activities could provide insight for the design of peptides with selective antibacterial activity.

Hemolytic (11) and antibacterial activity (24,67,126) of peptides both depend on hydrophobic properties, although in different ways. Hydrophobicity of melittin-derived (11) as well as model peptides (12) was directly related to hemolytic activity, while an inverse correlation was found for antibacterial activity. Large numbers of contiguous hydrophobic residues appear to enhance hemolytic activity, and thus need to be avoided in selective bactericidal peptides (12).

In a liposome model system, the degree of membrane permeation not only depends on peptide hydrophobicity, but also on membrane composition. Vesicles composed of strongly negatively charged phospholipids are more effectively lysed by peptides with increased overall hydrophobicity (121), despite the lower level of binding of these peptide to the membrane. In other words, in this example a larger fraction of the bound peptides is
actively involved in causing membrane leakage than in the case of a membrane composed of weakly anionic lipids (24). Peptides with increased hydrophobic moment caused enhanced leakage from vesicles composed of zwitterionic lipids, and had increased antibacterial as well as hemolytic activity, and thus a decreased membrane selectivity (126). On vesicles with anionic lipids, which are considered to mimic microbial membranes, increased peptide hydrophobic moment did not alter permeabilizing activity (126).

The hydrophobic moment of an α-helix can be expressed as the angle formed by the hydrophobic residues in an α-helix when looking along the helix axis. In a series of model peptides varying in hydrophobic angles, peptides with angles >180° had considerable hemolytic activity, while reducing the angle also reduced hemolysis (59). However, none of the synthesized peptides had any antibacterial effect at 100 µl/ml (59). In another study, the angle formed by the positive charged residues was varied, keeping other properties (hydrophobicity and hydrophobic moment) constant (125). A large angle (140-180°) increased the permeation of zwitterionic as well as weakly anionic vesicles, and increased antibacterial as well as hemolytic activity. Lower angles reduced all these activities.

Taken these studies on helical peptides together, peptides have highest antimicrobial activity at high hydrophobicity, high hydrophobic moment and a high angle formed by the positive charge (25). Overall peptide charge needs to be sufficiently high, but not higher than an optimum, while peptide length is of minor importance. However, to obtain a peptide selectively active against microorganisms and not lysing erythrocytes, moderate hydrophobicity, reduced hydrophobic moment and a small angle of the positive charge, in combination with a moderate overall positive charge may be favored (25). In a multiple regression analysis studying the individual contribution of α-helicity, mean hydrophobicity, and mean hydrophobic moment (amphipathicity), the latter was identified as the major contributor to antibacterial activity against E. coli (97).

**β-Sheeted peptides**

Bactericidal peptides with β-sheeted structures have been investigated less systematically than their α-helical counterparts. Although β-sheeted structures can be designed and synthesized (94), the requirement of cysteines and the need of a folding step to induce disulfide bridge formation is a major drawback. Therefore, no data are available e.g. on systematically modified defensins to discern influence of alterations in charge or hydrophobicity as described for helical peptides in the previous section. However, some studies have been done on smaller, less complicated β-pleated peptides.

Gramicidin S is a cyclic, 10-residue β-pleated peptide with hemolytic activity and with antibacterial activity against Gram-positive bacteria. When peptide length and positive charge were increased in order to form peptides with pronounced amphipathic properties, activity was extended to Gram-negative bacteria and hemolytic activity was lost (3). This suggests that, like for helical peptides, appropriate hydrophobicity and proper orientation of hydrophylic and hydrophobic groups determine antibacterial activity and membrane selectivity (3).
In another study, replacement by D-amino acids in an α-helical peptide led to loss of helical character and the induction of a (linear) β-sheet structure (93). These diastereomers disrupted anionic and zwitterionic bilayers and were bactericidal, but had lost the hemolytic activity of the parent peptide (93). In a more general approach, the presence of a hydrophobic face of both amphipathic α-helical and β-sheeted peptides controlled interactions with hydrophobic surfaces, indicating that these physical parameters were more important than secondary structure (116).

RATIONAL DESIGN OF MICROBICIDAL PEPTIDES

Having identified the major determinants for microbicidal activity, ideally peptides could be designed and synthesized with optimized microbicidal activity. Several strategies are employed to achieve this goal, varying from modification of natural peptides to de novo design of synthetic peptides. Taking into consideration general guidelines as deduced from mechanistic studies described above, several attempts have been done to develop such peptides.

Zhong et al (134) performed a database search for amphipathic structures in proteins as a selection criterion for the design of antimicrobial peptides. These amphipathic regions were synthesized as peptides after adaptation of cationicity and amphiphilicity to the putative needs of a peptide antibiotic, i.e. low hydrophobicity and high hydrophobic moment. Unfortunately, antimicrobial activities of these peptides was very low (134). Tossi et al (119) were more successful by extracting the most common features from over 80 antimicrobial peptides. They synthesized a number of α-helical peptides, 18 to 22 residues in length, intermediate in positive charge, balanced in hydrophobic and hydrophilic sectors and with high amphipathicity. MIC values for a number of Gram positive and Gram negative bacteria ranged from 1 to 8 μM, which is in the range of activity of several natural peptides (119).

A number of hybrid peptides consisting of the amphipathic part of cecropin and the hydrophobic part of melittin (CEME) have received considerable attention (17). The most active hybrid was 26 residues in length, was 100-fold more active against S. aureus than cecropin A (2 vs. >200 μM) and had only limited hemolytic activity (17). This hybrid could be further downsized to 15 residues without loss of activity (5).

The observation that α-helical nature of peptides is not required for their antibacterial activity, while it is needed for hemolytic activity, was exploited by a number of researchers synthesizing D-enantiomers based on helical peptides characterized previously (9,61,110,120,127). Such L/D or all-D enantiomers have the additional advantage that they are more resistant to proteolytic cleavage and thus presumably have a longer half-life (9,86). By modulating charge and hydrophobicity of L/D enantiomers, synthetic peptides could be constructed with selective and potent antibacterial activity (92).

Thus, based on general rules derived from studies in which peptide properties are systematically modified, it is possible to design antimicrobial peptides with potential for clinical application, but the number of publications reporting successful rational design of such peptides is still limited at present.
FUTURE DEVELOPMENT OF MICROBICIDAL PEPTIDES: IMPACT OF THE MODEL SYSTEM

Research aiming to elucidate the microbicidal mechanism of peptide antibiotics has profited from the use of liposomes as a model system. Liposomes have allowed the identification of many peptide as well as membrane characteristics crucial for microbicidal activity. However, several examples exist of discrepancies between the effects of peptides on liposomes and on microorganisms. For instance, linearization of defensins led to the loss of antibacterial activity (109), but still caused membrane leakage of liposomes (32,128). Cecropins could dissipate membrane potential in membranes, and induce pore formation. It remains unclear, however, which of these two mechanisms causes the microbicidal effect. Similarly, antimicrobial peptides of various sources caused dissipation of membrane potential in vesicles, but failed to do so on cytoplasmic membranes of E. coli at MIC concentrations (129). Other peptides fully dissipated E. coli membrane potential at concentrations below MIC (129). From these results two things may be concluded. First, effects of peptides on liposome membranes may not always be representative for effects on bacterial membranes, and thus the results obtained with liposomes as a model system should be interpreted carefully. Second, the cytoplasmic membrane may not be the target for (several) microbicidal peptides at all (129). As proposed by Matsuzaki (78) peptides may be transferred over the membrane and kill bacteria by intracellular processes, for instance by inhibition of DNA or protein synthesis (16).

Liposomes may be an oversimplification as a model system for bacteria also because of their limited complexity. For instance, it has been shown repeatedly that electrostatic interactions between cationic peptides and negatively charged phospholipids in liposomes are crucial for ultimate membrane leakage. In Gram negative bacteria, however, it has been estimated that 90% of the outer surface negative charge arises from LPS (100), which is absent in the liposomes used. Based on our own observations, the contribution of negatively charged phospholipids of the inner membrane of bacteria in the entrapment of cationic peptides is questionable. E. coli HDL11, a strain with inducible synthesis of negatively charged phospholipids (63), was found to be equally susceptible to recombinant platelet thrombocidins in presence or absence of negatively charged phospholipids (unpublished results).

LPS has been shown to interact with antibacterial peptides indeed, and it has been implicated that LPS plays a role in bacterial susceptibility for peptide antibiotics. For instance, magainin 2 binds to and disorders fatty acyl chains of Salmonella LPS (100,101), and a good correlation was found between the susceptibility for magainin and the sugar chain length in LPS of this organism (100).

Besides the considerations concerning the elucidation of the exact mechanism of peptide antibiotics, more clinically relevant observations would argue against the use of liposomes in the development of designer peptides. In recent studies modifications in LPS of several bacteria, including Salmonella spp, were found to be associated with (intrinsic) resistance for peptide antibiotics (41). In Salmonella, this resistance is induced upon invasion of macrophages, and is most likely needed to withstand activity of the abundantly present intracellular antimicrobial peptides (29,42). The induced resistance was caused by
substitutions of phosphate groups (L-4-aminoarabinose or ethanolamine) or derivatization of acyl chains (palmitate or 2-hydroxymyristate) in the lipid A moiety of LPS. This adaptive response was orchestrated by the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems (43-45). Although studied most extensively in *Salmonellae*, aminoarabinose and/or ethanolamine substitutions have been found in other species resistant to antimicrobial peptides, including *Proteus* (112), *Klebsiella* (50) and *Escherichia* (91). In *E. coli*, lipid A-derivatization by palmitate, L-4-aminoarabinose and ethanolamine was induced by NH$_4$VO$_3$ (57), while acylation with palmitate was induced by cold-shock (18). It has not been investigated whether these substitutions confer resistance to peptide antibiotics, but this is to be expected. Thus, enzymatic pathways exist in several Gram negative bacteria capable of inducing resistance to antibacterial peptides. This poses serious concerns regarding the possible development of resistance in other bacteria. Gram positive bacteria do not possess LPS, but it has been shown that *Staphylococcus aureus* sensitivity to cationic antibacterial peptides was associated with the presence of negatively charged cell wall components (98).

These data indicate that LPS (or a negative surface charge in general) is an important component mediating susceptibility to antimicrobial peptides. Electrostatic interactions between LPS and peptide may be of decisive importance for ultimate microbicidal activity, as suggested in a recent study of synthetic model peptides. A correlation was found between LPS binding and antimicrobial activity, while no correlation was found between peptide antimicrobial activity and peptide charge, length or hydrophobicity (106).

Thus, the development of effective microbicidal agents calls for a model system in which LPS is included, like liposomes with incorporated LPS (84), hybrid vesicles containing defined phospholipids combined with cell membrane components isolated from bacteria, or entire bacteria. LPS-containing liposomes would be the easier model system, but the latter two systems will provide more direct biological relevance. The use of defined mutants in LPS, cell wall or membrane composition or of (isogenic) strains differing in membrane potential (62) could shed more light on the cidal mechanism of antimicrobial peptides and aid in the development of clinically applicable microbicidal peptides.

**CONCLUSION**

Antimicrobial peptides have received considerable interest in studies on the nature of innate immunity. The increase in microbial resistance for conventional antibiotics has further stimulated the interest in these compounds (115). It is well imaginable that the role of conventional antibiotics in antiinfective treatment will decrease, and thus alternatives need to be sought. Since peptide antibiotics are abundantly present in nature and have been successful for millions of years, they potentially provide an attractive source of alternative antimicrobial agents. Although the exact mechanism of action has not been elucidated yet, the insight in requirements for peptide microbicidal activity is growing. As a result of past and current research efforts, several antimicrobial peptides are currently being evaluated in various clinical trials (46-48). Further detailed studies on structure-function relationships of
Design of microbicidal peptides, using model systems representative for the actual microbial target, should allow the design of increasing numbers of aiding in the combat against microbial infections.

REFERENCES


