Structure–function relationships and engineering of host–defense peptides

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Over the years, fatalities due to microbial infections have been reduced considerably due to the availability of a large number of potent antibiotics. The mechanism of action of these antibiotics involves either inhibition of bacterial cell wall synthesis, protein and nucleic acid biosynthesis or enzyme activities crucial to bacterial metabolism. Unfortunately, in recent years, microorganisms have developed resistance to a large number of therapeutically used antibiotics which were earlier very effective. Hence, the search for new molecules against which resistance may not develop easily is imperative. Species across the evolutionary scale from insects to mammals use peptides to combat bacteria. The endogenous antibacterial peptides exert their activity by permeabilizing the bacterial membranes. In this review, the approaches that have been employed in engineering endogenous antibacterial peptides to get molecules with improved activity, the biophysical properties that are responsible for activity and the possible therapeutic use of these class of peptides are discussed.

Species right across the evolutionary scale from insects to mammals are susceptible to microbial infections. Over the years, several antibiotics have been discovered and/or synthesized that are very effective in combating microbial infections in animals and humans. Therapeutically used antibiotics act by either inhibiting bacterial cell wall synthesis, protein and nucleic acid biosynthesis or inhibiting enzymes whose activities are crucial to bacterial metabolism. However, the endogenous molecular armaments that insects, amphibians and mammals use to counter micro-organisms are peptides which are composed of 20–50 amino acids. They are synthesized as precursors by the cells’ protein synthesis machinery which are processed to yield the endogenous antibacterial peptides. Hence, they do not contain L-amino acids or other unusual amino acids like the well-studied antibiotics gramicidin 

Primary, secondary structures and mechanism of action of endogenous antibacterial peptides

Information about primary and secondary structures of endogenous antibacterial peptides and their mechanism of action have been important inputs in the engineering and design of analogues, variant and model peptides. Hence, these aspects are briefly reviewed in this section.

The primary structures of a large number of endogenous antibacterial peptides have been determined from insects, amphibians and mammals. An examination of the various sequences indicates that they can be classified into two groups, i.e. (i) linear peptides composed of ~20–50 residues, (ii) peptides with one to three disulphide bridges. Since details of the primary structures are available in recent reviews, only representative sequences are shown in Table 1. A striking feature in peptides belonging to both groups is the preponderance of cationic and hydrophobic residues, although there is no primary structure homology. Most of the antibacterial peptides have been isolated from the haemolymph of...
insects, skin, gastrointestinal tract of amphibians and neutrophils, macrophages or small intestinal paneth cells in mammals. A few peptides having antibacterial activity and rich in cationic amino acids have also been characterized from the seminal fluid of insects\(^a\) and bovine species.\(^{31a,33} \) Peptide toxins like melittin (GIAGGLVATTGLPALISWIKRKRQKOQH) and pardaxin (GFFALIPKIISSLFNLGSQASGSSGQEOE) which are strongly haemolytic also possess potent antimicrobial activities.\(^{34-38} \) In addition to antibacterial peptides composed of 20 or more residues, there are few with only \(\sim 13\) residues like indolicidin\(^{37} \) and bactercin. The antimicrobial spectrum and the potencies are highly variable. Cecropins and magainins act only on bacterial cells and not eukaryotic cells whereas defensins exhibit both antimicrobial and cytolytic activities. The antibacterial peptides exhibit a broad spectrum of activity against gram-negative and gram-positive microorganisms. Cecropins and magainins are more active against gram-negative compared to gram-positive microorganisms. Since the assay methods in the different studies vary, comparison of the minimal inhibitory concentration (MIC) in different studies may not be valid. The MICs of these class of peptides are in the \(\mu\)M range.

Although the primary structures of a large number of antibacterial peptides are documented, detailed secondary structures of only a few have been determined by nuclear magnetic resonance (NMR) or X-ray methods. Detailed below are structures of peptides that have been proposed based on NMR or X-ray methods. In the presence of structure-promoting solvents, cecropin A is proposed to adopt helical conformation with a hydrophilic N-terminal region (residues 1–21) connected by a hinge region (residues 22–24) to a C-terminal hydrophobic \(\alpha\)-helical domain (residues 25–37).\(^{39} \) A continuous helical segment without the hinge has been proposed for cecropin P, a member of the cecropin family isolated from porcine small intestine.\(^{60} \) Magainin 2 also adopts helical conformation in aqueous TFE mixtures.\(^{41} \) However, recent studies in membranes employing Fourier transform infrared (FTIR) and solid-state NMR suggest two populations of the peptide, one in helical conformation and the other in \(\beta\)-sheet conformation.\(^{39} \) Human defensin HNP-3 adopts \(\beta\)-sheet structure in the solid state\(^{42} \) and in solution.\(^{22} \) The structures of several defensins have been determined in solution by NMR (the results have been summarized in ref. 22). All \(\alpha\)-defensins which have a conserved disulphide motif adopt \(\beta\)-sheet structure. \(\beta\)-Defensins though possessing disulphide linkages different from \(\alpha\)-defensins also adopt \(\beta\)-sheet structure. Insect defensins have regions of \(\beta\)-sheet and \(\alpha\)-helical structure. NMR studies have indicated that a \(\beta\)-hairpin region composed of 18-residues is less flexible than the rest of the molecule, suggesting that this region may be an important determinant of activity. The toxins melittin and pardaxin also adopt \(\alpha\)-helical conformation.\(^{34} \) The central proline residue introduces a kink in both the helices. The structures of antibacterial peptides as deduced from NMR and X-ray studies are shown in

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**Table 1. Representative structures of endogenous antibacterial peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Species</th>
<th>Sequence</th>
<th>MIC ((\mu)M)(^f)</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecropin(^a) A</td>
<td>H. cecropia</td>
<td>KWLFKKIEKKGQNIRGDIKAPRAVAVVEQATQIAK-CONH(_2)</td>
<td>0.2</td>
<td>&gt;200</td>
<td></td>
</tr>
<tr>
<td>Andropin(^a)</td>
<td>D. melanogaster</td>
<td>VFIIDILKVENA1NNAAOQVIIGFAPFPFKL1NPK</td>
<td>140</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Magainin-2(^a)</td>
<td>X. laevis</td>
<td>G1KFK1HSAFKQGAFQGEINS</td>
<td>1–7</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Bombinin(^a)</td>
<td>B. variegata</td>
<td>GI1G1S4A4G4LKL4GL4G4LAEM4F4A1-CONH(_2)</td>
<td>1.5</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Dermaseptin(^a)</td>
<td>P. savagii</td>
<td>AL1W1KINL1KK1GT1K1H1G1R1A1G1A4D1T1S1G1Q1TQ</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Seminalplasin</td>
<td>Bovine</td>
<td>SD1EK1S1PD1K1H1R1F1S1L1S1R1Y1K1L1N1R1L1N1F1K1L1LT1E1FL1K1W1G1D1R1G1N1R1S1V</td>
<td>10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Indolicidin</td>
<td>Bovine</td>
<td>I1L1P1W1K1W1P1W1R1W1R1-CONH(_2)</td>
<td>10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Defensin ((\alpha))</td>
<td>Human, HNP-1</td>
<td>ACYCR1PI1CA1T1AG1R1Y1G1T1C1V1Q1G1R1L1W1AF1C1C</td>
<td>10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Defensin ((\beta))</td>
<td>Bovine, BNBD-1</td>
<td>DF1A1S1C1T1N1G1G1I1C1L1P1N1R1C1P1G1H1N1Q1I1G1I1C1F1R1F1R1V1K1C1R1S1W1</td>
<td>act</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Defensin</td>
<td>P. terranovae</td>
<td>AT1C1O1L1S1G1T1S1G1I1N1H1S1A1C1H1A1C1R1L1R1N1R1G1Q1N1G1K1G1V1C1R1N1R1</td>
<td>na</td>
<td>act</td>
<td></td>
</tr>
<tr>
<td>Tachyplesin(^c)</td>
<td>T. tridentatus</td>
<td>KNC1F1R1V1C1Y1G1Q1Y1R1C1R1R1C1R1-CONH(_2)</td>
<td>1.5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Protegrin-1</td>
<td>Porcine</td>
<td>R1G1G1R1L1C1Y1C1R1R1R1F1C1R1V1C1G1R1</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Brevinin 1-E</td>
<td>R. esculenta</td>
<td>FL1P1L1A1G1L1A1A1N1FL1P1K1F1K1R1K1T1R1K1C</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) insects; \(^{a}\) amphibians; \(^f\) The MIC values are not directly comparable as the strains against which different peptides were tested are not the same; act, active; na, not active; S–S bonds are indicated.
Figure 1. Circular dichroism (CD), infrared and Raman spectroscopy have also been employed to characterize the conformation of some of these peptides. The acyclic peptides appear to favor helical conformation, especially in structure-promoting solvents and lipid vesicles.

The amphipathic nature of structures and the presence of cationic residues strongly suggest that interaction of endogenous antibacterial peptides with membranes mediates their biological activity. Both acyclic and cyclic peptides like the defensins bind to lipid vesicles and permeabilize them, resulting in the release of entrapped solutes. Electrical conductance of bilayer membranes doped with these peptides shows characteristic features of single channel conductance. Direct interaction and permeabilization of bacterial membranes has been demonstrated for peptides like defensins, tachyplesins, magainins and seminalplasmin. The recent observations that D-enantiomers of cecropins, magainins and melittin show antibacterial activity comparable to the L-enantiomers indicate that no chiral recognition is involved in their mechanism of action.

While it is evident that the mechanism of action of endogenous antibacterial peptides involves membrane permeabilization, the molecular architecture of the channels or pores formed in membranes is still debated upon. Magainins are presumed to bind to the membrane surface, which results in deformation and bending in a toroidal fashion, resulting in the formation of pores lined with polar head groups of the lipids rather than trans-membrane channels formed by peptide aggregates like in alamethicin. Cecropin P is presumed to bind to the membrane surface and destabilizes the phospholipid packing, leading to permeabilization. However, defensins are presumed to form transmembrane channels with the conducting unit being a dimer.

**Antibacterial activities of peptides that correspond to segments of endogenous antibacterial peptides**

Table 1 indicates that several acyclic peptides are composed of more than 25 residues which may not be necessary for interaction with membranes. Attempts to delineate short segments in these peptides that can bind...
to lipid vesicles and exhibit antibacterial activity have been successful. In SPLN (Table 1), hydrophobicity analysis has indicated a 27-residue stretch more hydrophobic than rest of the peptide. Synthetic peptides corresponding to the 27-residue segment and the 13-residue segment (PKLLETFLSKWIG) exhibited antimicrobial activity\(^{59,60}\) though with less potency than SPLN. The short peptides bound to lipid vesicles and permeabilized bacterial membranes like SPLN\(^{49}\). Interestingly both peptides exhibited haemolytic activity not observed in the parent peptide.

Pardaxin is another molecule that has been investigated extensively\(^{58,62,63}\). The N-terminal 1–18 residues are essential for activity. Pardaxin and a peptide without the C-terminal seven amino acids exhibited potent antibacterial and haemolytic activities\(^{35,36}\). However, a peptide corresponding to the 1–18 segment showed selective antimicrobial activity with haemolytic activity even at high concentrations\(^{64}\). Peptides corresponding to the N-terminal segment composed of 14 or 15 residues, though could interact with model membranes did not possess antibacterial or haemolytic activities\(^{65,66}\).

A synthetic peptide corresponding to N-terminal 18-residue segment of dermaspin showed antimicrobial properties comparable to the parent 37-residue peptide\(^{67}\). Short peptides with deletions of amino acids from N or C terminal regions corresponding to cecropins\(^{68–74}\) and magainins\(^{5,75,76}\) have not yielded bioactive molecules. However, hybrid peptides composed of hydrophobic segments from cecropins and melittins\(^{18,70,77,78}\) have been shown to possess antimicrobial activity. Thus, by judicious choice of hydrophobic or amphiphilic segments from antibacterial peptides, it is possible to generate endogenous bioactive peptides.

Engineering of ‘short’ bioactive peptides to improve potency and selectivity

Although peptides ~18 amino acids derived from longer peptides and some naturally occurring peptides like indolicidin ~13 residues long do have antibacterial activity, some of them also lyse erythrocytes and other eukaryotic cells. This property is not desirable if these peptides are to be used as drugs. Hence, there have been attempts to engineer such peptides to get desirable biological activities.

The 13-residue seminalplasmin-related peptide PKLLETFLSKWIG (SPF) had antibacterial and haemolytic activities at comparable concentrations\(^{59}\). A single amino change of glutamic acid to lysine resulted in considerable increase in antibacterial activity over haemolytic activity\(^{79}\). The activity profiles of several other analogues related to SPF\(^{80}\) indicate that the aromatic residues were not important for antimicrobial activity. However, haemolytic activity was reduced considerably when the aromatic residues F and W were replaced by L. Likewise transposing KT and SK residues also favoured antibacterial over haemolytic activities. Replacement of K by E resulted in complete loss of biological activities.

Peptides having antibacterial and/or erythrocyte lytic activities could also be generated from the inactive N-terminal 16-residue segment of pardaxin, GFFALIPKIISPLFK\(^{41}\). The analogue GFFKLIPKIISPLFK–CONH\(_2\) showed selective antimicrobial activity whereas the analogues GFFKLIAKIISPLFK–CONH\(_2\) and GFFARIKIIKISPLFK–CONH\(_2\) exhibited both antimicrobial and erythrocyte lytic activities. In peptide amides corresponding to the 18-residue segment, GFFALIPKIISPLFKTL\(^{64}\), replacement of P (7) by A imparted haemolytic activity in addition to antibacterial activity. Antimicrobial activity but not haemolytic activity was abolished when K residues were replaced by Q.

Indolicidin is a peptide isolated from bovine neutrophils having an unusual amino acid composition\(^{72}\). Five of the 13 residues are W. There are also 3 P residues. The trp residues are essential for both the biological activities of indolicidin as replacement of W by F results in specific antibacterial activity\(^{82}\). The proline residues do not appear to be essential for activity as their replacement by A is tolerated. A similar study on the haemolytic peptide crabrolin (FLPLIRKIVTAL–CONH\(_2\)) has resulted in the identification of an analogue having specific antibacterial activity\(^{83}\).

By making judicious change of amino acids, it is possible to convert an inactive peptide to an active one. For example, peptide corresponding to the ordered \(\alpha\)-helical segment of the haemolytic toxin \(\delta\)-haemolysin, IISTIGDLVKWIIDTV does not have erythrocyte lytic activity\(^{84}\). Analogues where D have been replaced with K showed potent antibacterial and haemolytic activities. Replacement of one or two D residues resulted in varying biological activities.

Several 15-residue peptides which are hybrids of melittin and cecropin have also been obtained possessing antimicrobial activities\(^{70,78}\). The segments from cecropin and melittin correspond to various hydrophobic regions in the parent peptides. There have been attempts to generate analogue with improved activity by replacement of amino acids in the full length cecropin, magainins\(^{69–78}\) (summarized in ref. 18) and pardaxin\(^{63}\). Analogues of magainins have been obtained with considerably improved activity over the parent molecule\(^{76}\). However, in some analogues, improved antibacterial activity also resulted in increased haemolytic activities. In pardaxin, introduction of \(\delta\)-amino acids resulted in selective antibacterial activity over haemolytic activity\(^{85}\). Selective replacement of amino acids by \(\delta\)-isomers in melittin also resulted in selective lysis of bacteria but not mammalian cells\(^{86}\).
Apart from efforts to engineer endogenous peptides by a rational approach to get short peptides with improved selective activity, there have been attempts to generate bioactive peptides by combinatorial methods. Hexapeptides with good antibacterial activity, especially against *S. aureus* have been reported. However, the combinatorial method is restrictive and cannot be easily used for the synthesis of peptides composed of even 10 residues due to the large number of combinations involved.

**Biophysical principles that govern the biological activities of antibacterial and haemolytic peptides**

The membrane activity of several 'engineered' peptides described in the previous section has been investigated in detail. The peptides bound to lipid vesicles and permeabilized them so as to cause the release of entrapped hydrophilic molecules like calcein or carboxyfluorescein. Interestingly, peptides that possessed both antibacterial and haemolytic activities were more effective in permeabilizing model membranes compared to peptides possessing only antibacterial activity. Now erythrocytes and other eukaryotic cells have efficient protein 'pumps' in the plasma membrane as a result of which small osmotic imbalance can be tolerated. Hence effective permeabilization of the plasma membrane is required for lysis in the case of erythrocytes. However, the bacterial plasma membrane is the site of respiration and even a small perturbation of the membrane could affect respiration. Thus, a peptide which is not an effective membrane perturbant could have selective antimicrobial activity. In many peptides the biological activities correlate with model membrane permeabilization. However, the molecular structure of the lesions formed by these peptides in membranes is yet to be established unequivocally.

The secondary structures of the various analogues and variants have been studied almost exclusively by CD spectroscopy. The spectra suggest predominantly helical conformation for the peptides in trifluoroethanol and membrane environment. In water, almost all peptides show spectra which are characteristic of unfolded conformation. Helical content has been estimated from $\theta_{222}$ values. Since there are inherent limitations in quantitating secondary structure from CD data, the molecular structures of the peptides cannot be commented upon. However, as large number of related peptides with varying activities have been studied, their results do permit some correlation of structures with activity. A striking observation is that several peptides that have only antibacterial activity have less propensity for helical conformation than peptides which are strongly haemolytic in addition to possessing antibacterial activity. In fact, several analogues of magainins that have enhanced antibacterial activity are also haemolytic and are more helical than the parent peptide. In melittin and pardinax, introduction of $\alpha$-amino acids, which result in decrease in helical content, show selective antimicrobial activity. However, there are exceptions to the above mentioned observations. For example, dermaseptin and its N-terminal 18-residue peptide, though exhibit only antibacterial activity show high helical content in trifluoroethanol. However, it is evident that the acyclic peptides adopt predominantly helical structure in hydrophobic environment and are largely unordered in aqueous medium. Lower helical content also correlates with lower membrane-perturbing ability.

In summary, lower helical content and consequently less membrane-perturbing ability favours specific antibacterial activity. For haemolytic activity, high helical content appears to be necessary. The presence of cationic residues is essential for antibacterial activity but not for haemolytic activity. Studies on peptides containing disulphide bridges have largely been confined to the naturally isolated ones. These peptides adopt $\beta$-sheet structures and breaking of the disulphide bridges leads to unfolding and loss of biological activities.

**Do endogenous antibacterial peptides or their 'engineered' variants have potential as therapeutic agents?**

The 'endogenous' host-defense peptides and the 'engineered' synthetic peptides have broad spectrum antibacterial activities against a variety of gram-negative, gram-positive microorganisms and fungi. Anti-viral activity has also been observed in some peptides. Clinically isolated strains that are resistant to conventionally used drugs are also susceptible to these peptides. Magainins and their analogues have been shown to have antitumour activities against murine and human cell lines. *In vivo* activity has been demonstrated in a murine model. By suitable engineering based on biophysical principles, it is possible to eliminate cytolytic activity against eukaryotic cells and also reduce the length of the peptides substantially compared to endogenous peptides. The problem of inactivation of the all L-peptides by proteases can also be overcome by selective incorporation of $\alpha$-amino acids or non-coded amino acids. As they are part of the endogenous host-defense mechanism, toxicity is unlikely to be a serious problem. In the case of indolicidin which has cytotoxic activity in addition to its antibacterial activity, entrapment in liposomes reduced cytotoxicity considerably and was effective in treating animals systemically infected with *Aspergillus fumigatus*. By appropriate formulation, it should be thus possible to reduce toxicity when present and render the peptides suitable for systemic use. Hence,
these class of antibacterial agents have all the features requisite for use as therapeutic agents. To-date, no reports of hormone action or undesirable immune reaction related to these class of peptides have been reported. Yet, to-date either endogenous or engineered antibacterial peptides have not found extensive use as antibacterial drugs for systemic or even topical applications. The one factor that could come in the way of their use as drugs is the costs involved in bringing an antibiotic drug to market. It appears that costs have to be less than US $10 per gram to be competitive with other drugs in clinical use. However, the dictates of economy should not come in the way of using peptides as drugs considering the serious problem of resistance to currently used antibiotics, especially when the immune system is compromised like in AIDS. Defensins have also been shown to act against non-tuberculosis mycobacteria and hence could be active even against M. tuberculosis. Since resistance to several currently used drugs by M. tuberculosis is also being recognized as a serious health problem, there is an urgent need to try novel approaches to combat bacterial infections. Even the fluoroquinolone class of antibiotics are not refractory to the development of resistance. Hence, antibacterial peptides that act by permeabilizing membranes are promising alternatives to currently used drugs. Economics should not overrule an obvious scientific advantage.

SPECIAL SECTION: CHEMISTRY–BIOLOGY INTERFACE


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