Design and Study of Novel Antimicrobial Peptides with Proline Substitution

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This dissertation titled

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ABSTRACT

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Microorganism-related diseases and their resistance to conventional antibiotics are proliferating at an alarming rate and becoming a severe clinical problem. Therefore, it is urgent to develop novel approaches in antimicrobial therapy. Most living organisms produce and utilize at least some small peptides as part of their defensive system in combating infections by virulent pathogens. Research focusing on the structure and function of these antimicrobial peptides from diverse sources has gained a great number of interests in the past three decades. Generally, many naturally existing antimicrobial peptides are positively charged and have the potential to adopt either amphipathic $\alpha$-helix or $\beta$-sheet conformation. In this project, based on preliminary studies of $\beta$-sheet-forming peptides developed in our lab, analogs were designed to investigate the effects of introducing a single leucine-to-proline substitution on the structure and function of the peptides. The leucine-to-proline substitution was selected as a structural perturbation that could influence the antimicrobial and the cytolitic activity toward mammalian cells of these peptides. A series of experiments were performed in this project to investigate these potential changes, beginning with the determination of the antimicrobial activity and hemolytic activity of these peptides. Peptide conformation was determined by circular dichroism spectroscopy. Membrane permeability changes in both synthetic lipid bilayers and bacterial membranes were assessed by measuring peptide-induced calcein leakage.
from large unilamellar vesicles (LUV) and by peptide-induced entry of o-nitrophenyl-β-D-galactopyranoside into *E.coli* ML-35 cells. The ability of peptides to bind to lipid bilayers of defined composition was measured by tryptophan fluorescence enhancement, acrylamide quenching and 10-doxylnonadecane quenching. The activity of these peptides was further studied by measuring the planktonic bacterial cell killing (the live vs. dead bacterial viability), bacterial biofilm formation inhibition and inhibition of bacterial cells growth within established bacterial biofilms. The results show that the position of proline has a significant influence on the antimicrobial and hemolytic activity of these peptides. Some of these proline-containing analogs show high antimicrobial activity and good selectivity between bacterial vs. mammalian cells. In addition, the peptide binding studies suggest that at least some of the proline-containing peptides may kill bacteria by mechanisms other than simply inducing membrane leakage. In summary, amphipathic β-sheet-forming antimicrobial peptides with proline substitutions appear to be promising models for novel antimicrobial agents.

Approved: _____________________________________________________________

John F. Blazyk

Professor of Biochemistry
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CHAPTER 1: INTRODUCTION

A. History and Background

Throughout the modern era of medicine, the effort to search for natural, semi-synthetic or synthetic antimicrobial agents has never ceased. Researchers have always focused on finding chemicals that can kill or inhibit microorganisms effectively without introducing high toxicity to human cells. Among all the antimicrobial chemicals that have been discovered to date, antibiotics play a unique and significant role in clinical therapies and have provided a classic and effective means of defense against the invading microorganisms for almost 60 years (Hancock, 1997). However, since the application of antibiotics toward potential pathogens have been overused and improperly used, resistance or even multi-drug-resistance has emerged rapidly and seriously in many microorganism species. For instance, shortly after the discovery of penicillin in the 1940s, the bacterial strain *Staphylococcus aureus* rapidly developed strong resistance to this widely used antibiotic drug. Unfortunately, various bacterial strains that are resistant to most antibiotics have been reported (Monroe and Polk, 2000). Therefore, as conventional antibiotics are progressively demonstrating decreased efficacy, novel approaches will be required in the near future to maintain control of infectious diseases.

During the past three decades, there has been a constantly growing interest in host defense peptides because of their remarkable antimicrobial selectivity. Almost all living organisms including animals (both vertebrates and invertebrates), insects, plants, bacteria and fungi, produce an array of such small peptides as part of their innate defense systems (Reddy et al., 2004). A great number of such peptides have been isolated and
characterized from diverse sources. The following sections will review some distinctive representatives of these peptides that have been intensively investigated.

Magainin family is one of the most well-documented peptide families that possess strong antimicrobial activity toward a wide collection of bacteria, yeasts, fungi and viruses. These 23-amino acid-long peptides were initially isolated from the skin of African clawed frog *Xenopus laevis* (Zasloff, 1987). Approximately 500 peptides have been discovered from various amphibian skin glands and these peptides occupy a large portion of the total number of currently known natural antimicrobial peptides. Magainin family peptides have been extensively investigated, and their structure-function relationship and the mechanism of actions have been used as important references for many studies of other antimicrobial peptides. The first clinical antimicrobial peptide treatment was developed based on magainins although the outcome was eventually unsuccessful (Ge et al., 1999).

Cecropins are also a group of antibacterial peptides identified from natural sources. As a family, cecropins were first isolated from the silk moth *Hyalophora cecropia* (Hultmark et al., 1980); later their analogs have been found present in a variety of insect species and pigs (Hultmark et al., 1982; Maloy and Kari, 1995; Montville and Chen, 1998). These 31–39-residue-long peptides exhibit a broad-spectrum of activity against Gram-negative bacteria. However, it has been reported that for some Gram-positive bacteria such as *Staphylococcus aureus*, cecropins are relatively inactive (Andreus and Rivas, 1992; Wade et al., 1992).
Defensins, a family of peptides with up to 50 amino acid residues, also possess a broad spectrum of antimicrobial activity. Defensins are primarily found in epithelial cells and neutrophils in mammals. They are also found in insects and plants (Hancock and Chapple, 1999). Defensins can be categorized into three subfamilies, with each subfamily containing α-helix or β-sheet structure stabilized by disulfide crosslinks (Bastian and Schäfer, 2001; Hwang and Vogel, 1998; Jenssen et al., 2006).

A wide range of other collections of naturally occurring antimicrobial peptides have also been examined, suggesting that most of these peptides are gene-encoded, quickly synthesized at low metabolic cost, excreted shortly after microorganism infection, and act rapidly to kill or inhibit a broad range of microorganisms (Reddy et al., 2004). Despite their structural and functional diversity, these peptides share several common features. For instance, most of these peptides have an overall net positive charge (cationic) (Hancock, 1997). Furthermore, because most of the peptides kill bacteria by inducing target cell membrane permeabilization, they may have the potential to elude rapid emergence of resistance from various pathogenic microorganisms (Andreu and Rivas, 1999). All of these features make the antimicrobial peptides promising candidates for therapeutic drugs.

Although most naturally existing peptides have broad-spectrum antimicrobial activity and short response time toward pathogen invasion, their therapeutic application is limited due to the requirement of highly localized concentrations, and in some cases, severe toxicity toward host cells (Maloy and Kari, 1995). Compared to the extremely low concentration of conventional antibiotics that can acquire antimicrobial efficacy, the
peptides are relatively ineffective. In order to overcome this, a great deal of effort has been invested in increasing the antimicrobial potency and specificity between bacterial and mammalian cells of natural peptides or their derivatives, and deepening the understanding of the molecular mechanism by which these peptides achieve their antimicrobial function (Yeaman and Yount, 2003).

Generally speaking, the mechanisms of actions of antimicrobial peptides are not well established. Most antimicrobial peptides appear to target the lipid bilayers of the bacterial membrane. Since most of these peptides are cationic and there is a net charge difference in the membrane of host cells (the outer surface is relatively neutral) vs. bacterial cells (the outer surface is negatively charged), electrostatic interactions may contribute to the initial attraction of the membrane-targeting step and play a significant role in selective killing of bacteria (Hancock and Diamond, 2000). Most of these peptides can also form an amphipathic secondary structure upon binding to the membrane (Yeaman and Yount, 2003).

However, there could be other working mechanisms for some of the peptides. It is reported that a few peptides can bind to a specific receptor for cell targeting. For instance, a 34-amino acid-long peptide, nisin, exhibits extraordinarily strong binding affinity to Lipid II, which is essential for bacterial cell wall synthesis. As a result, nisin shows high antimicrobial activity against Gram-positive bacteria and good selective activity between bacterial and mammalian cells (Breukink and de Kruijff, 1999). In addition, some of these peptides may act on the bacterial DNA and interfere with the reproduction of
bacterial cells thus inhibiting/killing bacteria (He and Furmanski, 1995; Kanyshkova et al., 1999).

**B. Naturally Occurring Antimicrobial Peptides**

The diverse collection of peptides discovered in a wide range of species have exhibited a broad-spectrum of antimicrobial activity against various strains of bacteria, fungi, viruses and even tumor cells (Giuliani et al., 2008). Because of the fact that similar structural features are conserved in various peptides from widely different organisms, the early classification of antimicrobial peptides based on their origins has been found to be increasingly inadequate (Andreu and Rivas, 1999). Instead, categorization of these peptides based on structural homologies is more reasonable. The amino acid composition and the secondary structures of these peptides can be categorized into at least four major classes (Boman, 1995; Casteels et al., 1989; Hancock, 1997; Matsuzaki et al., 1997c; Schibli et al., 1999; Tossi et al., 2000):

1) Amphipathic α-helices

2) Amphipathic β-sheets (peptides with two or more disulfide bonds)

3) Loop structures (peptides with one disulfide bond)

4) Extended structures (peptides without cysteine but are rich in certain amino acids)

Table 1 shows some representative peptides in each category. Figure 1 shows structural schemes of each class. It is notable that for some antimicrobial peptides mixed structures exist (Figure 1 panel A) so that they possess features of more than one class.
### Table 1

*Antimicrobial Peptides (Andreus and Rivas, 1999)*

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence</th>
<th>Source</th>
<th>Structural Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecropin</td>
<td>RWKIFKKIEKMG</td>
<td>Silk moth</td>
<td>α-helix</td>
</tr>
<tr>
<td></td>
<td>NIRDGIVKAGPAIE</td>
<td><em>(Bombyx mori)</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIGSAKAI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magainin 1</td>
<td>GIGKFLHSAGKFGK</td>
<td>South African clawed frog</td>
<td>α-helix</td>
</tr>
<tr>
<td></td>
<td>AFVGEIMKS</td>
<td><em>(Xenopus laevis)</em></td>
<td></td>
</tr>
<tr>
<td>β-Defensin-1</td>
<td>DFASCHTNGGICLP</td>
<td>Ox</td>
<td>β-sheets with multiple</td>
</tr>
<tr>
<td></td>
<td>NRCPGHMIQIGCIF</td>
<td><em>(Bos taurus)</em></td>
<td>disulfide bonds</td>
</tr>
<tr>
<td></td>
<td>RPRVKCCRSW</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protegrin I</td>
<td>RGGRLCYCRRRFC</td>
<td>Pig</td>
<td>β-sheets with multiple</td>
</tr>
<tr>
<td></td>
<td>VCVGR</td>
<td><em>(Sus scrofa)</em></td>
<td>disulfide bonds</td>
</tr>
<tr>
<td>Brevinin-1</td>
<td>FLPVLAGIAAKVVP</td>
<td>Japanese frog</td>
<td>β-sheets with a single</td>
</tr>
<tr>
<td></td>
<td>ALFCKITKC</td>
<td><em>(Rana brevipoda porsa)</em></td>
<td>disulfide bonds</td>
</tr>
<tr>
<td>Drosocin</td>
<td>GKPGRPSYPSRPTb</td>
<td>Fruit fly</td>
<td>β-sheets with a single</td>
</tr>
<tr>
<td></td>
<td>SHPRPIRV</td>
<td><em>(Drosophila melanogaster)</em></td>
<td>disulfide bonds</td>
</tr>
<tr>
<td>Thanatin</td>
<td>GSKKPVPIYCNRR</td>
<td>Hemipteran</td>
<td>Extended structure</td>
</tr>
<tr>
<td></td>
<td>TGKCQRGM</td>
<td><em>(Podisus maculiventris)</em></td>
<td></td>
</tr>
<tr>
<td>Apidaecin IA</td>
<td>GNNRPVYIPQPRPP</td>
<td>Honeybee</td>
<td>Extended structure</td>
</tr>
<tr>
<td></td>
<td>HPRI</td>
<td><em>(Apis mellifera)</em></td>
<td></td>
</tr>
</tbody>
</table>
As outlined above, several conservations among antimicrobial peptides such as their overall net cationic charge and amphipathic structure have been observed across diverse families (Hancock, 2000). In order to highlight the compositional and structural features related to these conservations, the following sections will address the chemical and physical properties of antimicrobial peptides such as conformation, charge, amphipathicity and hydrophobicity.

*Figure 1.* Structural classes of antimicrobial peptides (Adapted from Jenssen et al., 2006). A: Mixed structure; B: Looped structure; C: β-sheet structure; D: α-helical structure; E: Extended structure.
1. Conformation

Several features in the secondary structure of peptides appear to be conservative despite their different sequences and sources, and peptides can be classified accordingly into several groups, as shown in Figure 1.

The first category is the $\alpha$-helix-forming peptide. Various naturally occurring cationic peptides have the ability to adopt an amphipathic $\alpha$-helical conformation upon binding to membrane or lipid bilayers and the formation of an $\alpha$-helix is believed to be an initial and critical step in the introduction of their antimicrobial activities (Jin et al., 2005). The $\alpha$-helix is a coiled structure stabilized by hydrogen bonding between the backbone carbonyl group of one amino acid and the imino group of the amino acid four residues away (Figure 2). In $\alpha$-helical structure, there are 3.6 amino acid residues per turn and each residue is connected to the next one by a rise of 1.5 Å, thus giving a pitch of 5.4 Å (Berg et al., 2002). Many antimicrobial peptides, such as magainins, PGLa and cecropins, can form this kind of $\alpha$-helix structure (Blondelle and Lohner, 2000; Shai 1999; Sitaram and Nagaraj, 1999; Tossi et al., 2000). These $\alpha$-helical peptides frequently have little or no apparent secondary structure in aqueous solution at neutral pH; but in the presence of lipid bilayers, bacterial membranes or nonpolar solvents such as trifluoroethanol (TFE), they essentially assume amphipathic $\alpha$-helical structure, a conformation that segregates the hydrophobic residues on one side of the helical axis and the hydrophilic residues on the other side. This highly organized amphipathic $\alpha$-helical characteristic can be illustrated by a helical wheel diagram, which is the top view of the helices with all the amino acid residues on the coils compacted into one circle (Figure 3).
The second structural class includes peptides such as defensins (Lehrer et al., 1993) and protegrins (Sokolov et al., 1999; Chen et al., 2000) with two or more cysteine-cysteine disulfide bonds that stabilize a conformation containing primarily \( \beta \)-sheets. This structure can also be stabilized by hydrogen bonds occurring between the carbonyl group and the imino group on the adjacent \( \beta \)-strands (Figure 4). Because the peptide chains are normally considered as directional, the \( \beta \)-sheet structures may consist of antiparallel or parallel \( \beta \)-sheets, or a mixture of both, based on the different/same proceeding directions of two adjacent \( \beta \)-strands (Berg et al., 2002). Antimicrobial peptides can also adopt amphipathic features by forming \( \beta \)-sheet structure, simply positioning polar and nonpolar side-chains of amino acid residues in two different directions to form the segregation between hydrophilic and the hydrophobic surface.

The third structural class includes antimicrobial peptides that possess only one disulfide bond as shown in Figure 1 panel B, such as thanatin (Mandard et al., 1998). They generally adopt loop structure.

The fourth class (and also the least common class) includes a only small portion of antimicrobial peptides that are rich in one or more amino acid residues, such as proline-rich (Otvos, 2002), arginine-rich (Wessolowski et al., 2004) and tryptophan-rich peptides (Junkes et al., 2008). They generally possess extended structure as shown in Figure 1 panel E.
Figure 2. Ribbon Diagram of α-helical structure of antimicrobial peptides (Adapted from Berg et al., 2002).

Figure 3. Helical wheel representation of amphipathic structure formed by α-helical antimicrobial peptides (Adapted from Blazyk et al., 2001; Matsuzaki, 1999).
2. Charge

Most of the naturally existing and synthetic antimicrobial peptides are cationic (Hancock and Diamond, 2000; Jin et al., 2003), and some of them contain highly ordered, positively charged domains such as cecropins (Sato and Feix, 2006). This cationic
characteristic is largely due to the presence of multiple lysine, arginine and histidine residues in the peptide sequence. The first step in the process of interaction between peptides and target membrane is the electrostatic interaction. The cationic antimicrobial peptides tend to interact with the bacterial cell membrane because the outer leaflet of the bacterial membrane is rich in negatively charged lipids. Moreover, lipopolysaccharides (LPS) of Gram-negative bacteria and teichoic or teichuronic acids of Gram-positive bacteria contribute additional negative charge to the outer surfaces of the respective organism (Yeaman and Yount, 2003).

In contrast to target bacterial membranes, eukaryotic cell membranes are overall much less negatively charged, especially on the outer surface. This feature would undoubtedly lead to less electrostatic interaction between eukaryotic cell membranes and antimicrobial peptides, and could account for at least part of the selective cytolytic activity of peptides between microorganism cells and host cells (Bessalle et al., 1992; Dathe et al., 1996; Matsuzaki et al., 1997a).

Therefore, increased positive charge of peptide may enhance the electrostatic interaction, causing an increase in the binding between bacterial membranes and antimicrobial peptides. Within a certain range, this correlation between peptide cationicity and antimicrobial activity has been demonstrated in a number of studies (Bassalle et al., 1992; Blondelle and Houghten, 1992; Oren et al., 1997). However, this relationship is neither entirely linear, nor unlimited. Beyond a certain point, increasing positive charge no longer leads to increased antimicrobial activity. Studies with magainin 2 derivatives showed that a net charge increase of +6 to +7 instead of +3 to +5 led to an
increased cytolytic activity toward human erythrocytes cells and a complete loss of activity against bacterial cells (Dathe et al., 2001). This decrease in antimicrobial activity may be a result of the combination of excessive positive charge of peptides and distribution of peptides inside the target cell membrane after initial binding. Increased cationicity can lead to strong electrostatic repulsion between bound peptides, prevent or disturb the translocation of the peptide into the inner domain of the target cell membrane and result in improper allocation of peptides, thus can possibly cause the loss of antimicrobial activity (Matsuzaki, 1997a; Yeaman and Yount, 2003).

3. Amphipathicity

Amphipathicity describes the degree of polarized arrangements of hydrophobic and hydrophilic domains within a peptide (Castano et al., 2000). Most antimicrobial peptides form amphipathic structures upon binding to artificial lipid bilayers or bacterial cell membranes. Amphipathic structure can be achieved via α-helix and β-sheet conformations. The α-helix- and/or β-sheet-forming peptides with a high amphipathicity would have most of their hydrophobic residues on one side of the structure and most of their hydrophilic residues on the opposite side, which can be visually displayed by a helical wheel and β-sheet-linear diagram (Figure 3, Figure 4). Amphipathic character allows peptides to bind to the lipid bilayer of the membrane and disrupt its structure. Fourier-transform infrared (FTIR), polarized attenuated total reflectance FTIR (ATR-FTIR), circular dichroism (CD), oriented CD (OCD) and Raman spectroscopy and nuclear magnetic resonance (NMR), have been used to determine the conformation of these peptides in solution and when bound to membranes (Sitaram and Nagaraj, 1999).
Similar to the results observed in correlation between peptide cationicity and activity, several studies demonstrated that an excessive high degree of amphipathicity with perfectly segregated hydrophilic and hydrophobic domain in peptide structure can lead to increased cytolytic activity toward human red blood cells (Dathe and Wieprecht, 1999; Kondejewski et al., 1999).

4. Hydrophobicity

The hydrophobicity of peptides can be used to estimate the overall polar/nonpolar features in the peptide structure (Eisenberg, 1984). Eisenberg’s consensus hydrophobicity scale is shown in Table 2 where each amino acid has been assigned a value according to the nature of its side chain.

Although a minimum level of hydrophobicity is required for antimicrobial peptide efficacy, excess levels of hydrophobicity are related to a loss of selectivity between bacterial vs. mammalian cells (Yeaman and Yount, 2003). Based on this finding, it is not surprising to note that a range of moderate hydrophobicity appears to be found in many naturally occurring antimicrobial peptides so that they can fully optimize activity against microorganism cell membranes (Wieprecht et al., 1997). Therefore, it is possible to enhance the selectivity of synthetic antimicrobial peptides by altering their hydrophobicity as long as the modification does not exceed a threshold value.

As discussed above, conformation, charge, amphipathicity and hydrophobicity, all contribute at different levels to the antimicrobial activity and the selective toxicity of peptides between bacterial vs. mammalian cells. Therefore, optimal antimicrobial peptide efficacy is a result of a delicately balanced coordination of all these compositional- and
structural-activity relationships. Moreover, all these parameters are cross linked together and modification of one of these properties often leads to collateral alterations in others.

Table 2

*Hydrophobicity of amino acids (Eisenberg, 1984)*

<table>
<thead>
<tr>
<th>Residue</th>
<th>Symbol</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>I</td>
<td>1.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>F</td>
<td>1.2</td>
</tr>
<tr>
<td>Valine</td>
<td>V</td>
<td>1.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>L</td>
<td>1.1</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>W</td>
<td>0.81</td>
</tr>
<tr>
<td>Methionine</td>
<td>M</td>
<td>0.64</td>
</tr>
<tr>
<td>Alanine</td>
<td>A</td>
<td>0.62</td>
</tr>
<tr>
<td>Glycine</td>
<td>G</td>
<td>0.48</td>
</tr>
<tr>
<td>Cysteine</td>
<td>C</td>
<td>0.29</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Y</td>
<td>0.26</td>
</tr>
<tr>
<td>Proline</td>
<td>P</td>
<td>0.12</td>
</tr>
<tr>
<td>Threonine</td>
<td>T</td>
<td>-0.05</td>
</tr>
<tr>
<td>Serine</td>
<td>S</td>
<td>-0.18</td>
</tr>
<tr>
<td>Histidine</td>
<td>H</td>
<td>-0.40</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>E</td>
<td>-0.74</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
<td>-0.78</td>
</tr>
</tbody>
</table>
C. Comparison of α-helix- and β-sheet-forming Antimicrobial Peptides and Their Analogs

Because of their ability to kill or inhibit a broad spectrum of microorganisms, hundreds of naturally existing antimicrobial peptides have been isolated from diverse sources and thousands of synthetic derivatives based on these peptides have been produced (Hancock, 2001). Conformational studies have revealed a wide range of structures, including amphipathic α-helices, β-sheets, loop and extended structures which are enriched in one or two amino acid residues (Jenssen et al., 2006). The majority of the research efforts focused on identification and characterization of small peptides with high antimicrobial activity and low toxicity toward host cells. In contrast, peptides with long amino acid sequences and high molecular weights have some limitations since it is highly possible that they may induce severe host immune reactions. Among the intensively investigated short antimicrobial peptides, the most abundant and widespread ones are α-helix and β-sheet-forming peptides and their analogs (Hancock and Scott, 2000).
1. α-Helix-forming Antimicrobial Peptides

Among the α-helix-forming peptides, magainins are a class of 23-amino-acid-containing antimicrobial peptides which have been extensively studied (Maloy and Kari, 1995). Magainins were isolated from the skin of *Xenopus laevis* (an African clawed frog) (Zasloff, 1987). Another group of peptides, PGLa, were discovered from the same source and have also been categorized into the magainin family (Hoffmann et al., 1983).

Various studies have been conducted in order to reveal the secondary structure of magainins, such as circular dichroism (CD) (Wieprecht et al., 1996; Matsuzaki et al., 1998a), vibrational (Hirsh et al., 1996; Jackson et al., 1992; Williams et al., 1990) and NMR spectroscopy (Bechinger et al., 1993; Hirsh et al., 1996). The results indicate that magainins (including PGLa) assume random coiled structure in neutral aqueous solution, whereas the peptides adopt α-helical structure upon binding to lipid bilayers or bacterial cell membranes. The orientation of the amphipathic magainin α-helix in relation to the plane of the cell membrane has been investigated by solid-state NMR (Bechinger et al., 1993; Bechinger et al., 1998) and fluorescence quenching experiments (Matsuzaki et al., 1994). The hydrophilic domain of the α-helix interacts with the polar head groups of the lipid bilayers or the cell membrane while the hydrophobic domain lies within the hydrophobic interior of the lipid bilayers or the membrane. Eventually, the α-helix can be accommodated into the cell membrane in a direction which is parallel to the plane of the membrane surface (Matsuzaki, 1999).

Magainins possess a wide range of antimicrobial activity against infectious microorganisms (Bessalle et al., 1990; Chen et al., 1988; Matsuzaki et al., 1997b; Zasloff,
The concentration of magainins to cause lysis of human red blood cells is 10~100-fold higher than the concentration that exhibits antimicrobial activity (Matsuzaki et al., 1995). Although magainins show promising selective cytolytic activity between microorganism and host cells, their antimicrobial potency needs to be enhanced. In some cases, a concentration more than 100 µg/ml is needed for magainins to achieve antimicrobial activity against some bacterial strains (Chen et al., 1988).

Therefore, a great number of efforts were addressed to increase the antimicrobial activity of the magainin peptides and their analogs. Initially, amino acid residue specific modifications with substitution or elimination of certain residues at different positions were applied to magainins. The results showed that a minimum length of peptide is necessary for antimicrobial activity of magainins (Zasloff et al., 1988). Several synthetic variants with single amino acid omissions in the C-terminal region of the magainin sequence (especially the ones with deletion of residues alanine-15 or glycine-18), showed higher activity against bacterial cells but also higher hemolytic activity toward human red blood cells compared to the original magainin parent peptide (Cuervo et al., 1988).

Increasing the positive charge of the magainins has proved to be another effective method to promote antimicrobial potency. Within a certain range, addition of extra positively charged residues, such as lysine or arginine, can increase antimicrobial activity (Besalle et al., 1992; Dathe et al., 2001). However, as outlined in the previous sections, this modification method has a limitation. Addition of too much positive charge can lead to excess strong electrostatic interaction and an excess high degree of amphipathicity, which cause magainin variants to completely lose their antimicrobial activity, as
exemplified in excess additions of lysine residues (Dathe et al., 2001; Jacob and Zasloff, 1994).

Furthermore, it is proposed that the increase of the degree of amphipathic magainin α-helix correlates to the increase of antimicrobial efficacy. Studies of a class of magainin analogs derived from the replacement of glycine or serine residues with alanine suggest that the degree of α-helix is highly related to the antimicrobial activities of magainin peptides (Chen et al., 1988). Alanine residues assume higher hydrophobicity than glycine and serine residues and thus can promote the content of α-helix. Substitution of alanine residues appears to dramatically increase the antimicrobial potency of magainin variants (Chen et al., 1988). All-D-isomer replacement of the peptide sequence is also an important method to improve the activity of magainin. In the early 1990s, potency studies of magainin derivatives with all-D-form and all-L-form amino acids indicated that the two exhibited almost identical antimicrobial potency and low cytolytic activity toward host cells (Bessalle et al., 1990). Since D-isomer-peptides possess higher tolerance to proteolysis compared to L-isomer-peptides, all-D-magainins can be more resistant to the cleavage of host enzymes and their antimicrobial potency is less likely to be dispelled by proteolytic reactions.

The mechanism of actions of magainins was investigated in numerous mechanistic studies. The results suggested magainin peptides use a pore- or hole-formation mechanism to achieve their antimicrobial efficacy (Epand and Vogel, 1999). The peptides assume α-helical structure, lie parallel to the surface of the lipid bilayer, then perpendicularly insert into the lipid bilayer or cell membranes, and induce
membrane perturbation by forming channel-like pores. (Matsuzaki, 1999; Matsuzaki, et al., 1998b). The pores seemed to be highly localized and only cause partial membrane disruption, unlike a complete membrane lysis caused by detergents (Yeaman and Yount, 2003).

Cecropins are another family of α-helix-forming peptides with sequences of 31-39 amino acids. They exist in random-coiled structure and assume α-helical configuration upon binding to the lipid bilayer or bacterial cell membranes. The α-helical structures of cecropins contain two distinct domains: the C-terminal region is a hydrophobic α-helix and the N-terminal domain is a nearly perfect amphipathic α-helix (Sato and Feix, 2006). These two domains are linked by a glycine- and/or proline- containing hinge. Cecropins exhibit promising antimicrobial activity against a wide variety of Gram-positive and some Gram-negative bacteria, but have low activity toward bacterial strains such as *Staphylococcus aureus* (Andreus et al., 1992; Boman et al., 1989). The effort made to modify and optimize cecropins is mainly focused on synthesis and characterization of cecropin-mellitin (CM) hybrid peptides.

The other fragment donor of CM hybrid peptides is mellitin, a 26-residue-long peptide first isolated from the honey bee *Apis mellifera*. Similar to the secondary structure of cecropins upon interaction with cell membranes, mellitin adopts a helix-hinge-helix conformation. But compared to cecropins, the order of the two distinct helices is reversed, e.g., the N-terminus becomes a hydrophobic α-helix and the C-terminus is an amphipathic α-helix (Piers and Hancock, 1994). While cecropin is minimally toxic toward host cells, mellitin is highly lytic toward mammalian cells.
However, mellitin acts effectively on *Staphylococcus aureus*, which can be utilized to overcome the inefficiency of cecropin to *Staphylococcus aureus* in CM hybrids.

Various studies have suggested that hybrids containing fragments from both cecropin and mellitin could retain or improve the antimicrobial activity of both peptides, but significantly lower the inherent toxicity from mellitin, or overcome cecropins’ disability to kill *Staphylococcus aureus* (Sato and Feix, 2006). The crucial step in the cecropin-mellitin hybrid design is to strategically determine the break point between the cecropin and mellitin segments. Judicious selections of each domain can yield peptides with potential binding and neutralizing abilities of teichoic acid of Gram-positive bacteria (Scott et al., 1999) and/or LPS of Gram-negative bacteria (Piers et al., 1994), thus to some extent can suppress severe immune responses induced by these bacterial membrane components.

Similar to their parent peptides, CM hybrids are amphipathic $\alpha$-helix-forming antimicrobial peptides. They only adopt their secondary structure in the presence of lipid bilayers or cell membranes, forming two $\alpha$-helices linked by a flexible hinge. This predominantly $\alpha$-helical structure has been observed by solution NMR (Oh et al., 1999; Sipos et al., 1991).

2. $\beta$-sheet-forming Antimicrobial Peptides

Some naturally existing antimicrobial peptides and their analogs are rich in the thiol(sulfhydryl) containing amino acid cysteine. Thus several intramolecular or intermolecular disulfide bonds can be formed between cysteines. As a result, these peptides can adopt a $\beta$-sheet structure which is stabilized by these disulfide bonds. The
most commonly known representatives of the β-sheet-forming peptide family include mammalian defensins (Bastian and Schäfer, 2001; Lehrer et al., 1993; Yasin et al., 2004), protegrins (Sokolov et al., 1999; Chen et al., 2000), thionins (García-Olmedo et al., 1998) and tachyplesin (Matsuzaki, 1999, Yasin, et al., 2000).

Defensins are a predominant class of β-sheet-forming antimicrobial peptides that are widely distributed in animals, plants and insects (Cole et al., 2002; Hristova et al., 1997; Schroder, 1999). Mammalian defensins can be categorized into three subfamilies, α-, β- (Eisenhauser et al., 1992; Jones and Bevins, 1993) and θ-defensins (Tang et al., 1999).

Although the origin and sequence of α- and β-defensins are quite different, they all contain six cysteine residues and thus three intermolecular disulfide bonds (Sinha et al., 2003). This structural homology of cysteine-cysteine pairings is generally consistent in α- and β-defensins across various species and their β-sheet structures are largely stabilized by these intramolecular disulfide bonds (Bastian and Schäfer, 2001; Jones and Bevins, 1993; Lehrer et al., 1993).

While the α- and β-defensins are diversely found in many organisms, θ-defensins have a very limited source. They have only been isolated from monkeys Rhesus macaques (Tang et al., 1999). Although θ-defensins also contain six cysteine residues, they tend to form intramolecular disulfide bonds rather than intermolecular disulfide bonds which are common in α- and β-defensins. This structural feature enables θ-defensins to adopt a cyclic backbone consisting of two antiparallel β-sheets, which are stabilized by the three intermolecular disulfide bonds. It is interesting to note that θ-
defensins and their synthetic analogs may have anti-viral activities. Several reports suggest that these peptides can protect host cells from various viral infections induced by HIV-1, Herpes Simplex Virus (HSV) type 1 and 2, cytomegalovirus, vesicular stomatitis virus and influenza virus (Cole, 2003; Daher et al., 1986; Yasin et al., 2000; Yasin et al., 2004).

Plant defensins uniformly contain two more cysteine residues than mammalian defensins, thus can form four intramolecular disulfide bonds. The structure of plant defensins are also predominantly β-sheets, although there is an additional α-helix existing. Insect defensins, very similar to plant defensins, also contain four disulfide bonds and a triple stranded β-sheet (García-Olmedo et al., 1998).

Since this diverse collection of defensins significantly differ in activities against various species of microorganisms, it is not surprising to find that they can use different action mechanisms. For instance, according to an in vitro study based on planar bilayer membranes, defensins can induce leakage of K⁺ and form ion-permeable channels depending on voltage change (Kagan et al., 1990). This is apparently different from the simple pore-formation mechanism used by many β-sheet-forming peptides.

Protegrins are 16-18 amino acid residue long peptides, initially found in pig neutrophils (Kokryakov et al., 1993). They are highly cationic as they are rich in positively charged amino acid residues (4-6 arginine residues). Protegrins exhibit potent antimicrobial activity for many bacterial species (Chen et al., 2000). So far five structurally categorized protegrin subfamilies have been identified. Native protegrin-1, protegrin-2 and protegrin-3 were the first three classes that were isolated from pigs (Chen
et al., 2000). Protegrin-4 and protegrin-5 were subsequently discovered based on a cDNA clone (Zhao et al., 1994) and a genomic clone (Zhao et al., 1995). As a matter of fact, protegrins belong to a large and diverse group of antimicrobial peptides, the cathelicidins family (Zanetti et al., 1995).

The characteristic feature of protegrins secondary configuration is the formation of β-sheet structure. Protegrins contain two antiparallel β-strands that are linked by two intramolecular disulfide bonds. Instead of forming a cyclic peptide like θ-defensins, the amphipathic conformation of protegrins shows a β-sheet structure with only one turn in the center domain of the peptide sequence (Chen et al., 2000).

It is proposed that the presence of β-sheet is crucial to the antimicrobial activity of protegrins. Structural and activity studies of a group of protegrin-1 variants without stable β-sheet structure shows a reduced antimicrobial activity. These protegrin-1 derivatives are synthesized based on amino acid residue specific substitutions and include linear analogs (without any disulfide bond or a β-turn) and analogs with only one disulfide bond across the β-sheet (Chen et al., 2000). Furthermore, it is remarkable to find that the ratio of charged to uncharged residues in the peptides applies a profound effect on the antimicrobial activity. For this consideration, even though analogs with reduced positive charge tend to be less active, the overall cationic charge of peptides is not as important as the charged/uncharged residue ratio, due to the reason that the latter one can further impact the amphipathicity of the β-sheet (Chen et al., 2000). Stereochemically designed protegrin analogs with all-D-amino acids retain comparable antimicrobial potency as the
all-L-amino acids peptide, implying that the interaction between protegrins and target bacterial cell membranes may be non-specific electrostatic attraction (Chen et al., 2000).

Mechanistic study using electron microscopy reveals that protegrins acquire their antimicrobial activity via membrane disruption, similar to many other β-sheet-forming peptides (Gennaro and Zanetti, 2000). The net charge on the outer surface of the bacterial cell membrane and an energy-dependent efflux pump in bacteria may interfere with protegrins and thus affect their activity (Gennaro and Zanetti, 2000). Moreover, the interaction between protegrins and lipopolysaccharide (LPS) of Gram-negative bacteria may work as a recognition step in the initial process of bacteria killing (Chen et al., 2000).

Thionins are plant-originated antimicrobial peptides, which were initially found in wheat seeds (García-Olmedo et al., 1989). Generally speaking, thionins contain 45–47 amino acids and can be categorized into five classes. Although these subfamilies of thionins differ significantly in the peptide sequence, they share similar secondary structural characteristics, often referred to as a Γ-shaped structure. This three dimensional structure is a mixture of α-helix and β-sheet, and composed of a long arm (two antiparallel α-helices) and a short arm (two antiparallel β-sheets). Disulfide bonds are formed between the two α-helices and this Γ-shaped structure is highly amphipathic (García-Olmedo et al., 1998). Thionins also cause cell membrane perturbation of bacteria, which is supported by the peptide induced leakage experiment in negatively charged synthetic lipid vesicles (García-Olmedo et al., 1989). However, some divalent cations can reverse this leakage-inducing effect (García-Olmedo et al., 1998). Toxicity of thionins toward host cells have been reported for different thionin types, which were supported by
various *in vitro* studies based on cultured mammalian cells (Carrasco et al., 1981; Vernon et al., 1985). This toxic effect is also a result of the ability of thionins to induce cytoplasmic membrane leakage, but on the membrane of mammalian cells (García-Olmedo et al., 1998).

Tachyplesins, including polyphemusins, are a family of peptides containing 17-18 amino acids, which were first isolated from the horseshoe crab *Tachypleus tridentatus* (Nakamura et al., 1988). Subsequently, three sub types of tachyplesins and two sub types of polyphemusins from other species were discovered (Miyata et al., 1989; Muta et al., 1990; Nakamura et al., 1988). Tachyplesins show potent antimicrobial activity and considerable hemolytic activity toward human red blood cells (Matsuzaki et al., 1997). These peptides are rich in cysteine and arginine residues. Thus, similar to the intramolecular disulfide bridges formed in θ-defensins, the secondary structure of tachyplesins is a cyclic β-sheet stabilized by two disulfide bonds (Matsuzaki, 1999). FTIR spectroscopy revealed that tachyplesins adopted β-sheet structure in both the aqueous solution and lipid bilayer/membrane environment (Matsuzaki et al., 1993). NMR studies further indicated that this β-sheet configuration is an imperfect amphipathic structure (Tamamura et al., 1993). However, despite tachyplesins assuming β-sheet structure even in water solution, the orientations of the peptides appear to be slightly different in the presence of lipid bilayers or cell membranes (Oishi et al., 1997). Some tachyplesins and their synthetic analogs are reported to display of anti-HIV capabilities (Tamamura et al., 1993; Tamamura et al., 1996).
D. Proposed Mechanisms of Peptide Antimicrobial Action

The mechanisms employed by the peptides to achieve their antimicrobial activity and selective toxicity are not entirely clear. Although abundant evidence favors the concept that the peptides interact with and permeabilize target cell membranes to perform their antimicrobial actions, the mechanisms used by peptides to kill or inhibit the growth of microorganism cells appear to vary for different peptide species (Giuliani et al., 2008).

Rather than a mammalian cell membrane with a relatively neutral outer surface, most cationic antimicrobial peptides preferentially bind to bacterial cell membranes with a mainly negatively charged outer surface. After this initial electrostatic interaction or other peptide-target bindings, several activity determining events subsequently take place to allow peptides to further interact with bacterial cell membranes and finally acquire their antimicrobial activity (Zhang et al., 2000).

After the initial membrane binding, peptides accumulate on the surface of target cell membranes and reach a crucial concentration, generally known as the threshold concentration (Breukink and de Kruijff, 1999; Yang et al., 2000). Beyond this point, an overwhelming peptide-to-lipid ratio would lead to further changes in the structure and conformation of peptides.

Another critical event closely related to peptide activity is the structural and conformational transition of peptides after initial membrane binding. This transition has been investigated in detail by numerous studies. Many antimicrobial peptides have extended or random coil conformations in aqueous environments but can adopt highly
ordered amphipathic α-helical or β-sheet conformations upon binding to lipid bilayers or membranes (Dathe and Wieprecht, 1999; Giuliani et al., 2008, Jenssen et al., 2006).

However, as outlined in the previous section, the secondary conformation of some β-sheet-forming peptides such as defensins (Yasin et al., 2004) and tachyplesins (Matsuzaki, 1999) appear to remain unchanged during this stage of peptide-target interaction. These peptides naturally assume highly ordered β-sheet structure, which are constrained by disulfide bonds thus even in aqueous solution they are likely to assume the same configuration.

Additionally, this discriminated conformation change may prevent peptides from assuming proper amphipathic structure during the interaction with the mammalian cell membrane and play an important role in the process of antimicrobial peptides reducing cytolytic activity toward host cells. For example, increasing the amount of negatively charged phospholipids within the artificial liposomes can increase the extent of α-helix of some cecropin analogs in vitro. In comparison, the liposomes mimicking the phospholipid composition of mammalian cell membranes significantly decrease the extent of α-helix of peptides, which necessarily leads to a low lytic activity of the corresponding cecropin derivatives toward host cells (Wang et al., 1998).

Many α-helix and β-sheet antimicrobial peptides are well documented for self-association or multimerization in the process of peptide-membrane interactions. This assembling of peptides can lead to the formation of peptide-peptide or peptide-lipid complexes and facilitate subsequent peptide actions (Matsuzaki, 1999; Yeaman and Yount, 2003).
Although many critical events in the process of peptide-membrane interaction have been comprehensively understood, the orientation of bound peptide to the lipid bilayer in the target cell membrane structure is not clearly defined (Matsuzaki, 1999). At moderate peptide-to-lipid ratios, the antimicrobial peptides essentially assume a parallel orientation with respect to the plane of the membrane surface. Numerous structural studies using solid-state NMR (Bechinger et al., 1998; Hirsh et al., 1996), Raman-FTIR spectroscopy (Hirsh et al., 1996; Williams et al., 1990) and fluorescence quenching experiments (Matsuzaki et al., 1994) have supported this theory.

But in order to induce pore-formations, the peptides need to change their orientation and adopt a transmembrane position at a certain point in the process of the peptide-membrane interaction. When the peptide-lipid-ratio increases beyond a threshold value, peptides appear to penetrate the cell membrane. In this case, if the peptides maintain the previous parallel orientation, they may encounter unfavorable exposure of their hydrophilic domain to the nonpolar hydrophobic core of lipid bilayers. Therefore, it is possible for the peptides to change their orientation and penetrate perpendicularly in the membrane surface (Ludtke et al., 1994). A considerable amount of experimental data has shown that both $\alpha$-helical and $\beta$-sheet peptides can transfer from a parallel to perpendicular orientation with respect to the plane of lipid bilayers (Heller et al., 2000; Ludtke et al., 1994). This orientation change is closely related to peptide induced membrane perturbation (Heller et al., 1997 and 1998).

An impressive amount of effort has been invested in searching for uniform models for peptide actions but to date there has been little conclusive evidence to support
any universal concepts. As microorganism cell membrane composition and structure are widely variable, many peptides may act via one or more mechanisms in specific target membrane environments. (Giuliani et al., 2008; Yeaman and Yount, 2003)

Three generally accepted models for membrane disruption induced by amphipathic peptides have been proposed:

(1) The “barrel-stave” mechanism (Breukink and Kruijff, 1999; Ehrenstein and Lecar, 1977);

(2) The “toroidal pore” (or “wormhole”) mechanism (Ludtke et al., 1996; Matsuzaki, 1999);

(3) The “carpet” mechanism (Shai and Oren, 2001).

1. The Barrel-Stave Model

In this model, peptide monomers first bind to the outer surface of target cell membranes. At this point, the peptides rapidly adopt amphipathic structure and lie in an orientation parallel to the plane of lipid bilayers in the membrane.

These monomers then continue with further assembling, undergo self-association and/or multimerization. After exceeding the threshold concentration, peptides appear to adopt a transmembrane orientation and insert perpendicularly into the inner leaflet of the membrane, creating a localized expansion in the cell membrane (Ehrenstein and Lecar, 1977). Upon initiation of pore formation, more peptide molecules can be transported into the inner membrane leaflet via a self-promoted uptake mechanism driven by hydrophobic interactions, resulting in an increased extent of membrane lipid relaxation. Finally, a certain amount of membrane lipids is pushed aside and the formation of barrel-like cyclic
aqueous pores are completed with multiple stave-like transmembrane peptides
penetrating through the cell membrane vertically. The interior surface of the pore consists
of hydrophilic domains of peptides, which are segregated from the hydrophobic region of
the cell membrane. The hydrophobic domains of peptides face outward and interact with
the hydrocarbon chain of membrane phospholipids (Figure 5, left panel).

Many peptides mainly employ the barrel-stave mechanism, such as pardaxin
(Rapaport and Shai, 1991 and 1992) and alamethicin (Beven et al., 1999; He et al., 1996;
Yang et al., 2001).

2. The Toroidal Pore (Wormhole) Model

Although the toroidal pore mechanism also involves transmembrane pore-
formation, some cell membrane lipids in this model can translocate into the pore, thus
significantly differing from the lipid alignment in the barrel-stave mechanism (Matsuzaki,
1999).

In this model, the binding and subsequent assembling of peptide monomers is
very similar to those of the barrel-stave model. Peptides also assume amphipathic
secondary structure and then undergo the parallel-to-perpendicular orientation change.
NMR, fluorescence quenching, CD and X-ray studies (Hara et al., 2001; Ludtke et al.,
1995) have confirmed this two state transition theory of peptide orientation.

The unique feature of the toroidal pore model lies in the process of peptide
penetration. While peptides insert perpendicularly into the cell membrane, they can apply
curvature stress to the membrane lipids and bend the lipid polar head groups toward the
center of the transmembrane pore (Ludtke et al., 1996). As a result, the membranes are
progressively spanned and deformed with a considerable amount of membrane lipids intercalating between peptides. Eventually the integrity of the cell membrane is irreversibly disrupted and the transmembrane pores/channels are formed with membrane lipid polar head groups introduced in the aqueous phase of the pore, interacting with the hydrophilic domains of peptides (Figure 5, center panel).

Furthermore, the toroidal pore model can offer access to potential intracellular targets for antimicrobial peptides (Matsuzaki, 1999). During the process of membrane lipid bending under curvature stress, some peptide can be transferred to the inner leaflet of the membrane and finally enter the cytoplasmic space of bacterial cells via the transmembrane channel (Uematsu and Matsuzaki, 2000). Magainins appear to follow the toroidal pore mechanism, suggested by an impressive amount of experimental data (Matsuzaki, 1999).

3. The Carpet Model

The carpet mechanism reflects the diffuse action employed by some peptides to achieve their antimicrobial activity. It is notable that the membrane perturbation effect in the carpet model is not like a uniform collapse of the entire target cell membrane structure induced by detergents (Giuliani et al., 2008).

In the carpet model, during initial steps in the process of peptide-membrane interactions, cationic antimicrobial peptide monomers bind to and accumulate on the target cell membrane surface, similar to other models. The continuous peptide self-association can condense a high quantity of peptide carpeting the surface area of the cell membrane and lead to crucial changes in membrane properties.
With the peptide density increasing beyond the threshold value, micelle-like complexes consisting of peptides and membrane lipids are formed in the carpet mechanism instead of the formation of a transmembrane channel or pore. The hydrophobic domains of peptides face toward the hydrophobic core of the membrane while a large amount of membrane phospholipids are wrapped with peptides inside the micelle structures. The formation of micelles creates transient gaps or breaches in the lipid bilayers, so that the membrane is subject to stress and eventually loses its integrity (Figure 5, right panel).

Due to the formation of transient holes in lipid bilayers, the carpet model can also facilitate the invasion of peptides into the cytoplasmic leaflet of the target cell membrane and help the interaction between the peptides and intracellular target molecules, (Falla et al., 1996; Oren et al., 1999). The orientation of peptide in relation to the plane of the cell membrane is parallel during the initial binding and accumulating stage. But subsequently the peptides change orientation with membrane lipids re-orienting accordingly to form micelle-like structures (Oren and Shai, 1998). It is proposed by some researchers that the actions of magainins may follow the carpet model instead of the toroidal pore model, supported by differential scanning calorimetry (Matsuzaki et al., 1991) and the fluorescence energy transfer (Schümann et al., 1997) studies.
4. Other Possible Action Mechanisms

Although many antimicrobial peptides follow the action model of target membrane permeabilization, the effort to search for a universal action mechanism of peptide has met limited success. Therefore, it is feasible for peptides to use additional mechanisms of action besides membrane perturbation.
Cecropins are normally known as peptides with an action site on the cell membrane. However, a cecropin subtype peptide, cecropin A, is believed to be able to dissipate the transmembrane electrochemical gradient of both artificial lipid vesicles and bacterial cell membranes (Silvestro et al., 1997 and 2000). This dissipation effect is dependent on peptide-concentration but not directly related to bacterial cell membrane lysis action of cecropin A. In the Gram-negative bacteria *Escherichia coli*, cecropin A dissipated ion gradients and showed potent activity at low peptide concentrations without inducing the leakage of bacterial cytoplasmic components (Silvestro et al., 2000).

Some antimicrobial peptides are able to penetrate the bacterial cell envelope, translocate across the cell membrane and bind to intramolecular target molecules to achieve their antimicrobial activity. Several arginine- and proline-rich antimicrobial peptides such as apidaecin, drosocin and pyrrhocoricin as well as some cathelicidin family derivatives may acquire their antimicrobial potency via interaction with outer membrane or intramolecular polymers and inhibition of bacterial cellular protein synthesis and protein folding (Otvos, 2002). In stereochemical studies, all-D-amino-acid analogs of these peptides dramatically lost all antimicrobial activity, indicating that the action mechanism of these peptides might follow specific site/receptor recognition and docking but not include non-specific pore-formation (Casteels and Tempst, 1994). The initial recognition begins with binding of peptides to the outer membrane component LPS of bacteria, the final receptors are cellular proteins GroEL and DnaK, suggested by isotope incorporation experiments (Boman et al., 1993; Castle, et al., 1999). These peptides utilize an energy driven, permease/transporter-mediated mechanism to be
transported into the bacterial cell cytoplasm and kill bacteria by interfering with their metabolic process (Castle, et al., 1999).

Similar to these proline-rich peptides, a 21-amino-acid long peptide buforin II also adopts a specific-binding mechanism without inducing membrane permeabilization. Buforin II contains a single proline at the middle position of the sequence and this residue is crucial for its ability to translocate the cell membrane, supported by a proline-to-alanine substitution study (Park et al., 2000). This peptide finally binds to nucleic acids (both DNA and RNA) to kill bacteria (Park et al., 1998 and 2000).

This membrane penetrating ability has also been found in tryptophan- and arginine-rich peptides. Lactoferricin, a 25 amino acid long peptide with a predominance of tryptophan and arginine residues, is believed to be able to spontaneously penetrate the outer and inner cell membrane (Vogel et al., 2002). The prevalence of highly cationic arginine and aromatic tryptophan residues are not only important to amphipathicity of lactoferricin, but also critical for the translocation of the peptide across the membrane (Wessolowski et al., 2004). Human milk lactoferricin shows two high affinity binding sites in its N-terminus for specific regions of bacterial DNA thus can regulate the transcription processes and lead to the killing of bacteria (Kanyshkova et al., 1999).

A series of other alternative action mechanisms of antimicrobial peptides have also been proposed, such as LPS binding and an endotoxin neutralizing mechanism used by indolicidin (Staubitz et al., 2001). A considerable amount of debate exits about the exact mechanisms used by particular peptides even in some groups of peptides which have been intensively studied.
E. Biological Membranes

Since the antimicrobial potency of nearly all peptides is believed to inherently involve peptide-membrane interactions, it is necessary to outline the compositional and structural characters of the biological membrane, and to make comparisons between bacterial versus mammalian cell membranes in various aspects.

A biological membrane (or so called biomembrane) is an amphipathic layer that acts as a selectively-permeable barrier within or around a cell. The typical function of biological membranes are to allow some molecules with particular size, charge, and other physical/chemical properties to cross it, while retaining the majority of organically generated molecules inside the cell. Biological membranes also have mechanical characteristic or elastic properties (Verkleij and Post, 2000).

Although the biomembranes are diverse in structure and function, they have a number of important inherent characteristics in common. The most commonly accepted model of the biomembrane structure is the fluid mosaic bilayer model (Singer and Nicolson, 1972). According to this concept, developed in the early 1970s, all biological membranes are primarily composed of a fluid mosaic of proteins and lipids (Figure 6). Membrane proteins are embedded in the biomembranes and mediate various functions, serving as channels, receptors, pumps, enzymes and energy transducers (Dumas et al., 1999; Killian, 1998). Membrane lipids are relatively small molecules with hydrophobic head groups and hydrophilic tails. They form a bilayer frame with hydrophobic and hydrophilic domains (Berg et al., 2002).
Besides these different domains within the lipid bilayer of the biological membranes, a different distribution of membrane lipids exist in the outer and inner membrane leaflets, resulting in membrane asymmetry (Deveaux, 1992; Op den Kamp, 1979; Roelofsen et al., 1981; Roelofsen and Op den Kamp, 1994). Additionally, biological membranes are not rigid or static structures. The fluid mosaic organization of membrane proteins and lipid allow these molecules to diffuse readily in the plane of the

*Figure 6. Diagram of biological cell membrane (Adapted from Berg et al., 2002).*
biomembranes, unless they are constrained by some other anchor mechanism; in contrast, membrane proteins and lipids are not allowed to rotate across the membrane freely (Zachowski, 1993).

It has been well accepted that there are several important compositional and structural characteristics that differ between bacterial and mammalian cell membranes. It is this group of distinctive structural features of bacterial cell membranes that allows antimicrobial peptides with amphipathic features to distinguish and interact with their target cell membranes.

1. Composition of Biological Membranes

It is generally acknowledged that one of the essential and elementary components of all biological membranes is the lipid bilayer. There are three common types of membrane lipids: phospholipids, glycolipids and cholesterol.

Phospholipids include phosphoglycerides, sphingomyelin, and other lipids that contain a more complex alcohol backbone. Most phospholipids are phosphoglycerides, composed of a glycerol backbone attached to two hydrophobic fatty acid chains and a hydrophilic phosphate head group. The fatty acid tails usually contain 14 to 24 carbons, and there is usually one saturated and one unsaturated chain. The phosphate group is attached to an alcohol by the formation of an ester bond and several common phospholipids are derived from different phosphate-alcohol head groups, including phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG). The structures of these phospholipids are shown in Figure 7.
Sphingomyelin (SM) is another type of membrane phospholipid. Instead of glycerol, the backbone in SM is sphingosine, an amino alcohol that contains a long, unsaturated hydrocarbon chain (Figure 8).

Among these phospholipids, PC, PE and SM are neutral while PG, DPG, PS and PI are negatively charged (anionic).
Figure 7. Some common phospholipids found in biological membranes (Adapted from Berg et al., 2002).

\[
R_1, R_2, R_3, R_4 = \text{Fatty Acids}
\]

R =

- **Phosphatidylserine**
- **Phosphatidylethanolamine**
- **Phosphatidylcholine**
- **Phosphatidylglycerol**
- **Diphosphatidylglycerol**
Glycolipids are sugar-containing lipids, which are also derived from glycerol or sphingosine residues (Figure 9). The backbone of glycolipids can be bound to a variety of sugar units like an acylglycerol (common in many bacteria) (Ratledge and Wilkinson, 1988). Glycolipids can act as recognition receptors for specific biomolecules, facilitate the stability of the membrane and attach cells together to form tissue. Their orientation in biomembranes is parallel to the orientation of phospholipids within the lipid bilayer, with their sugar residues extending from the lipid bilayer into the extracellular environment (Pralle et al., 2000).

*Figure 8. Structure of sphingomyelin. (Adapted from Berg et al., 2002).*
Cholesterol is a class of membrane lipids containing a hydroxyl head and a hydrocarbon tail, with each of the two groups attached to one of the two ends of a steroid nucleus (Figure 10). Cholesterol is present in all animal biological membranes but is absent from most bacterial cells. It is an important component of the hydrophobic core of the membrane and helps to assemble phospholipids in the cell (Berg et al., 2002). Sterols, such as cholesterol and ergosterol, are generally neutral.

Membrane proteins are the second group of essential constituents in biological cell membranes. For unclear reasons, the majority of membrane proteins which have been identified to date are glycoproteins. A diverse collection of distinct categories of glycoproteins have been discovered, some membrane proteins constantly protrude their hydrophilic domains into the extracellular region, while other proteins intercalate and span the membrane. (Branton et al., 1981).
2. Difference between Bacterial and Mammalian Cell Membranes

The membrane lipid composition and distribution significantly differ between bacterial and mammalian cells. These fundamental differences may provide potential selective targets for antimicrobial peptides.

Unlike mammalian cells, bacteria possess a cell wall structure in addition to the cytoplasmic membrane. The bacterial cell wall is located outside of the cytoplasmic membrane and peptidoglycan is present as a major component. As the name implies, the peptidoglycan molecule includes two parts: a peptide portion and a glycan (sugar) backbone. The peptide portion is composed of multiple connected amino acids, and the glycan portion is composed of alternately repeating units of N-acetylglucosamine and N-acetylmuramic acid linked to each other by β-1-4 glycosidic bonds. Peptidoglycan is
responsible for the structural rigidity and the shape of bacterial cells (Demchick and Koch, 1996; Gitai, 2005; van Heijenoort, 2001).

Not all bacterial cell walls have the same overall characteristics although the elementary component peptidoglycan is universally present in all bacterial cell walls. There are two major types of bacterial cell walls, Gram-positive and Gram-negative, which are differentiated by whether or not they retain crystal violet dye during a Gram staining procedure (Shih and Rothfield, 2006). In Gram-negative bacteria, the cell wall, also known as the cell envelope, consists of an outer and an inner membrane (cytoplasmic membrane). Compared to Gram-negative strains, Gram-positive bacteria have a much thicker peptidoglycan layer and the outer membrane is absent (Schaffer and Messner, 2005; Weidenmaier and Peschel, 2008). Most of Gram-positive strains contain a group of polymers known as teichoic acids (TA), which are not present in Gram-negative strains. Teichoic acids are covalently linked to the peptidoglycan layer (Figure 11, upper panel).

Compared to the Gram-positive bacteria, the cell wall structure of Gram-negative bacteria is far more complex. The cell envelope extends outward from the surface of the cytoplasmic membrane to form an outer membrane (Figure 11, lower panel). The inner and outer membranes are separated by space known as periplasm. Peptidoglycans are present as the major structural component of the periplasm. The periplasmic space of Gram-negative bacteria may also contain periplasmic β-glucans (MDO). The outer membrane contains mostly lipopolysaccharide (LPS) in the outer leaflet (Wasiluk et al., 2004).
Figure 11. Diagrams of bacterial cell wall (Adapted from Gennis, 1989; Ghuysen and Hackenbeck, 1994). Upper panel: Gram-positive bacteria; lower panel: Gram-negative bacteria.
Besides this lack of a peptidoglycan cell wall, mammalian cell membranes exhibit other important differences compared to bacterial cell membranes. It is generally recognized that bacterial cell membranes are much more negatively charged on the outer surface compared to that of mammalian cell membranes. This difference is greatly but not solely based on the lipid compositional difference between the bacterial and mammalian cell membranes. Moreover, it can also partly be a result from the asymmetric distribution of membrane lipids, e.g., the membrane lipids are not randomly or evenly dispersed over the two membrane halves in either of these two kinds of cell membranes (Verkleij and Post, 2000).

As for membrane lipid composition, many bacterial cell membranes are composed of a considerable to a predominant amount of negatively charged phospholipids such as phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) (Tyler et al., 1995). Plus, the glycolipids are also anionic. However, there are significant differences in lipid classes and contents between different strains of Gram-positive and Gram-negative bacteria.

In the extensively studied Gram-positive strain, *Staphylococcus aureus*, phospholipids account for around 80% of total membrane lipids, and glycolipids constitute 10-20%. PG, DPG and lysylPG (lysyl ester form of PG) are the predominant classes (more than 90%) of phospholipids (Hayami et al., 1979), and the presence of other classes such as PE, PC and PS have also been reported (Brice et al., 1979). For another Gram-positive strain, *Staphylococcus epidermidis*, although the percentage of
phospholipids in the total lipid (around 75%) is close to that of Staphylococcus aureus, PG appears to constitute almost all the phospholipids (Ratledge and Wilkinson, 1988).

As for the Gram-negative organism, Escherichia coli, a well investigated bacterial strain, contains different proportions of phospholipids in the membrane compared to Gram-positive bacteria. The percentages of each class in the total amount of phospholipids are: PE 75-85%, PG 10-20%, DPG 5-15% (Raetz, 1978). In Pseudomonas aeruginosa, another common Gram-negative strain, 90% or more of the total membrane lipid is phospholipid. The phospholipid composition is 70% of PE and 20% of PG/DPG (Hancock and Meadow, 1969). It is notable that much lower percentages of PG/DPG are present in Escherichia coli and Pseudomonas aeruginosa cell membranes compared to those of Staphylococcus aureus and Staphylococcus epidermidis.

Therefore, in both Gram-positive and Gram-negative bacteria, though negatively charged phospholipids are not always the predominant membrane lipids, they can still confer the total anionic feature of bacterial cytoplasmic membranes. In contrast, the outer leaflet of eukaryotic cell membranes contains mainly zwitterionic phospholipids such as PC, PE and SM, and a neutral sterol component, cholesterol. For example, in human erythrocytes, the approximate membrane lipid composition percentages from several studies are as follow: zwitterionic phospholipids PC 30%, PE 25%, SM 25%, negatively charged phospholipids PS 15%, PI 2% and neutral cholesterol 3% (Dodge and Phillips, 1967; Nelson, 1967; Ways and Hanahan, 1964). Apparently the portion of zwitterionic phospholipids is much higher than the portion of negatively charged phospholipids.
As outlined above, although a considerable amount of anionic phospholipid PS is present in human erythrocytes, the potential negative charge is shielded by the asymmetric distribution of lipids in the cytoplasmic cell membrane. In the mammalian cytoplasmic cell membrane, such as human erythrocytes (Figure 12), anionic PS is completely distributed in the inner leaflet of the membrane, while most of the neutral phospholipid PE is largely dispersed in the inner leaflet. Although PC and SM are mainly located in the outer leaflet of the membrane, they are also neutral phospholipids thus the outer surface of human erythrocytes cytoplasmic cell membrane is relatively neutral compared to bacterial cytoplasmic membrane.

Figure 12. Asymmetric phospholipid distribution in the cytoplasmic membrane of human erythrocytes (Adapted from Gennis, 1989).
On the contrary, the anionic PG is mostly located in the outer leaflet of the cytoplasmic membrane of the Gram-negative strain *Escherichia coli* (Figure 13). Similar asymmetric lipid distribution was also observed for *Staphylococcus aureus* (White and Frerman, 1967; Hayami et al., 1979; Nahaie et al., 1984) and many other bacterial strains (Ratledge and Wilkinson, 1988).

*Figure 13. Asymmetric phospholipid distribution in the cytoplasmic membrane of *Escherichia coli* (Gennis, 1989).*

Moreover, Gram-negative bacteria contain negatively charged LPS in the outer membrane (Jeralal and Porro, 2004) and Gram-positive bacteria contain negatively
charged acidic polysaccharides such as teichoic acids (Weidenmaier and Peschel, 2008). They may contribute additional anionic properties to the bacterial cell membrane.

All of these attributes tend to create a negatively charged cell membrane outer surface for bacterial cells, but a relatively neutral outer leaflet for mammalian cell membranes. Due to the cationicity of most antimicrobial peptides, it is reasonable to interpret at least part of the selective activity of peptides as a result of target specific electro-interactions.

F. Endotoxin and Biofilm of Bacteria

Endotoxin and biofilm are main virulent factors involved in bacterial infections. Some antimicrobial peptides can neutralize endotoxin and/or inhibit biofilm formation, or kill bacterial cells inside established biofilm. Therefore, it is important to highlight the properties of typical bacterial endotoxin and biofilm.

The lipopolysaccharide (LPS) is a major component of the gram-negative cell envelope and a main virulent endotoxin in bacterial infections (Hancock and Rozek, 2000). LPS (Figure 14) is composed of 3 distinct components: the O-polysaccharide chain (O-antigen), the core region and lipid A (Bhor et al., 2005; Raetz et al., 2007). The core regions are generally conserved for most strains of Gram-negative bacteria (Magalhães et al., 2007).

During the process of infection, LPS can be released from bacteria (Holzheimer, 2001; Morrison, 1998) and induce the generation of a series of inflammatory mediator molecules, which finally leads to severe physiological damage (Blagbrough et al., 2000; Ulevitch, 2000).
Therefore, besides a fundamental understanding of the unique structure of LPS, it is urgent to develop antimicrobial agents that can neutralize the virulent effects of LPS (Jerala and Porro, 2004). Many antimicrobial peptides are able to neutralize LPS but antimicrobial activity is not necessary to yield endotoxin neutralization (Zorko et al., 2005). Polymyxin B is a well known LPS neutralizing agent. Although polymyxin B
exhibits efficient endotoxin neutralizing ability, the serious toxicity toward host cells has limited the therapeutic applications of polymyxin B (Bruch et al., 1999).

Besides endotoxins, bacterial biofilms account for various virulent effects in many bacterial infections, especially in chronic infections (Olson et al., 2002). The biofilm network structure can be produced by virtually all the bacterial strains. Bacterial resistance to conventional antibiotics can be significantly increased by biofilms. The virulence of biofilms may be a result of their interference with the host innate immune system (Sutherland, 2001).

Bacterial cells are able to adhere to smooth surfaces both in vitro and in vivo (Singh et al., 2002; Itoh et al., 2005), excrete extracellular polysaccharide material (EPS, known as “slime” in the past), and form a matrix like capsular structure known as biofilms (Vuong and Otto, 2002). EPS appears to be a key component of the biofilm structure. A striking feature of charge diversity of EPS has been observed. Most known EPS molecules to date consist of either uronic acids or ketal-linked pyruvates and are anionic (Otto, 2006). Some EPS are neutral, while the other few are cationic such as the adhesins isolated from Gram-positive strains Staphylococcus epidermidis (Mack et al., 1996). Interspecies biofilms were also reported by various researchers (James et al., 1995; Skillman et al.; Tomlin et al., 2001). Not all the species in these mixed biofilms are able to produce EPS but stability of all bacterial strains involved in the matrix can be enhanced.

EPS molecules originating from different biofilm sources display distinct features related to the bacterial cells established inside the biofilm and the surrounding
environment. Thus no universal consensus has been reached to support any unifying structural models of biofilm-specific EPS (Sutherland, 2001). However, the general lifecycle of bacterial biofilm has been intensively investigated by various studies, as shown in Figure 15.

![Figure 15. Model of biofilm formation and lifecycle (Vuong and Otto, 2002).](image)

It is generally recognized that planktonic forms of bacteria are much easier to treat whereas the same strains established inside biofilms have a decreased susceptibility to antibiotics (O’Toole and Kolter, 1998; Takahashi et al. 2007; Vuong and Otto, 2002). This resistance of biofilm-grown microorganisms may result from profound physiologic changes introduced by the bacterial biofilm.
First of all, the bacterial cells contained inside the biofilm possess a slow growth and metabolic rate. Since most conventional antibiotics are targeting certain points in the process of the growth and metabolism of bacteria, the status of low metabolic activity may act as a basis for biofilm resistance (Otto, 2006).

Numerous studies have also demonstrated that EPS plays an important role in the resistance of biofilm-forming bacteria toward antibacterial agents (Sutherland, 2001). For example, polysaccharide intercellular adhesin (PIA), an adhesive EPS of *Staphylococcus epidermidis* and *Staphylococcus aureus*, protects bacteria inside the biofilm from several antimicrobial peptides from human skin. This bacterial resistance may result from the electrostatic repulsion between the cationic antimicrobial peptides and the similarly positively charged of PIA (Mack et al., 1996; Vuong et al., 2004). In biofilm formed by *Pseudomonas aeruginosa* strains, two anionic EPS components appear to be able to remove antimicrobial peptides from the cytoplasmic membrane of bacterial cells (Chan et al., 2004). Additionally, an EPS protective layer, the poly-γ-glutamic acid (PGA) capsule generated by many strains such as *Bacillus anthracis*, *Staphylococcus epidermidis* and *Escherichia coli* also appear to increase the biofilm resistance (Hornef et al., 2002), but the mechanism of action is unclear.

The treatment toward medical implant or medical device related biofilm infections has been limited by the resistance of bacterial biofilms to antibiotic therapy (Otto, 2006). Thus, several different techniques have been used to study the physicochemical characters of various biofilms and develop potential biofilm-inhibitory agents.
In the 1980s, the smooth surface of polystyrene test tubes and 96-well tissue culture plates was utilized to study the adherence of staphylococci biofilm to medical device and a quantitative model by determination of absorbance of crystal violet stained biofilm matrix was also established (Christensen et al., 1982 and 1985). In the following decade, researchers developed the modified Robbin’s device (MRD) to study the potential antibiotic susceptibility of biofilms (Ceri et al., 1999; Morck et al., 1994).

Recently, a new technology, the Calgary Biofilm Device (CBD) has been used in various biofilm studies (Ceri et al., 2001, Harrison et al., 2005 and 2006; Spandj et al., 2003). This device consists of a 96-well-plate base and a polystyrene lid with 96 identical pegs. These pegs offer a defined average surface area for biofilm formation thus it is accurate and efficient to conduct quantitative investigations of biofilms. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) are also used to determine morphological features of biofilms (Kaplan, 2003; Harrison et al., 2006).

G. Synthetic Antimicrobial Peptides Designed to Form $\alpha$-helix or $\beta$-sheet

As described in detail previously, various approaches have been applied to alter naturally existing peptides to increase their effectiveness against virulent microorganisms (Yeaman and Yount, 2003; Giuliani et al., 2008). However, it is difficult to assess the effects of peptide modifications based only on derivatives from natural sources. Additionally, the delicate and complex correlation between peptide length, composition and structure frequently result in the peptide alterations that yield undesired outcomes. In order to simplify the peptide design and relevant problems, synthetic peptides with
simplified sequences have been used as model templates in various studies. These peptides have been designed with the ability to form amphipathic \( \alpha \)-helix or \( \beta \)-sheet structures when bound to target membranes. Amphipathic structure is a central feature in the design of synthetic antimicrobial peptides, since this property, coupled with the electrostatic interactions between peptides and cell membranes, is a critical factor in promoting the interactions between peptides and bacterial cell membranes. Unlike naturally occurring \( \beta \)-sheet peptides, where secondary structure is dictated by intramolecular disulfide bonds, synthetic linear peptides designed to adopt \( \beta \)-sheet secondary structure possess this capability solely based on the amino acid sequence, as described below.

For the design of synthetic peptides that have the potential to form an amphipathic \( \alpha \)-helix, the goal is to have two segregated domains with the hydrophobic residues on one side of the helical axis and the hydrophilic residues on the other side. Since there are 3.6 amino acid residues per turn in an \( \alpha \)-helical structure, the polar and nonpolar amino acid residues have to be placed at different positions in a manner that leads to a reasonable segregation. For example, in our lab, a PGLa peptide analog (KIAGKIA)_3-NH_2 was designed with the potential to form a highly amphipathic \( \alpha \)-helical structure (Figure 16, A) with the six polar lysine residues, along with three glycine residues clustered on one side of the helical face and the nonpolar isoleucines and alanines on the opposite face (Blazyk et al., 2001).
For the design of synthetic peptides that have the potential to form an amphipathic β-sheet, a similar goal needs to be reached but the distribution of amino acid residues must differ significantly from that of α-helix-forming peptide, since the β-sheet is a linear, strand-like structure. The polar and nonpolar residues need to be placed at alternating positions in the peptide sequence so their side chains can extend in opposite directions to form segregated hydrophobic and hydrophilic leaflets. For example, in our

*Figure 16. Helical wheel diagram of (KIAGKIA)_3-NH₂ (panel A) and β-strand diagram of (KIGAKI)_3-NH₂ (panel B) (Blazyk et al., 2001). “+”: lysine; white: glycine; gray: alanine; black: leucine or isoleucine.*
lab, (KIGAKI)$_3$-NH$_2$ was designed with the potential to form a highly amphipathic $\beta$-sheet structure (Figure 16, B) with the side chains of all the polar residues (lysine and glycine) placed at one leaflet of the peptide strand, while the side chains of all the nonpolar residues (alanine and isoleucine) are pointing in the other direction (Blazyk et al., 2001).

1. Comparison of (KIAGKIA)$_3$-NH$_2$ and (KIGAKI)$_3$-NH$_2$ Peptides

Because most currently known natural linear antimicrobial peptides are capable of forming $\alpha$-helix structure with at least a considerable extent of amphipathicity (Epand and Vogel, 1999; Sitaram and Nagaraj, 1999), preliminary studies in our laboratory began with an investigation of whether the $\alpha$-helix is necessary for potent antimicrobial effects. The analogs (KIAGKIA)$_3$-NH$_2$ and (KIGAKI)$_3$-NH$_2$ were designed to compare different amphipathic secondary structures in peptides with the same overall charge and hydrophobicity (Blazyk et al., 2001). The 21-residue peptide containing KIAGKIA repeats was designed to form amphipathic $\alpha$-helical structure (Figure 16, A), while the 18-residue peptide containing KIGAKI repeats was designed to form an amphipathic $\beta$-sheet (Figure 16, B). These peptides both possess an equal positive charge and hydrophobicity. Using CD and FTIR spectroscopy, (KIAGKIA)$_3$-NH$_2$ was shown to adopt a highly ordered $\alpha$-helical structure upon binding to the lipid bilayers. In contrast, (KIGAKI)$_3$-NH$_2$ assumed highly amphipathic $\beta$-sheet structure (Blazyk et al., 2001).

The antimicrobial potency and hemolytic activity of these peptide analogs were measured. (KIAGKIA)$_3$-NH$_2$ and (KIGAKI)$_3$-NH$_2$ displayed an equivalent antimicrobial potency, which is higher than that of PGLa. More significantly, (KIGAKI)$_3$-NH$_2$ showed
an enhanced capability compared to (KIAGKIA)3-NH2 in inducing leakage in synthetic lipid vesicles composed of POPE/POPG mixtures compared to POPC/POPG mixtures. Since PE is a neutral phospholipid that is present in many bacterial plasma membranes while PC is also neutral but mainly present in mammalian plasma membranes such as *E. coli*, (KIGAKI)3-NH2 displayed better selectivity that discriminates bacterial vs. mammalian cell membranes than (KIAGKIA)3-NH2. The hemolytic activity of (KIGAKI)3-NH2 was also lower than that of the (KIAGKIA)3-NH2 peptide (Blazyk et al., 2001). These experimental results suggest that β-sheet-forming peptides such as (KIGAKI)3-NH2 could be promising candidates for further modifications, which may yield a good combination of antimicrobial potency and selective cytolytic activity between bacterial and host cells.

Based on these initial studies, two families of synthetic peptide were synthesized and analyzed in our laboratory to further study the functional significance of amphipathic secondary structures (Jin et al., 2005). The first group consisted of three hexamer repeats, each containing two lysines, two isoleucines, a glycine and an alanine. The second group consisted of three heptamer repeats, each consisting of two lysines, two leucines, two alanines and a glycine. Each peptide possessed the same positive charge and hydrophobicity, but with varying propensities to form amphipathic β-sheet or α-helix structures.

The hemolytic activities of the hexamer family peptides were uniformly negligible, whereas those of the heptamer family peptides varied considerably. Significant differences were observed among these peptides in antimicrobial potency,
ability to induce leakage in different vesicles with varying lipid compositions or in \textit{E.coli} ML-35 cells, and in their secondary structures in the presence of lipid bilayers (Jin et al., 2005). Overall high antimicrobial potency was only observed in those peptides that can form a highly amphiphatic structure (either $\beta$-sheet or $\alpha$-helix), which once again strongly supported the relationship between amphiphatic structure and antimicrobial activity of peptides. The amphiphatic $\beta$-sheet-forming peptides in the hexamer family exhibited less hemolytic activity than the amphiphatic $\alpha$-helical peptides in the heptamer family. Thus, these results further confirmed that antimicrobial peptides that can form amphiphatic $\beta$-sheet secondary structures might offer a selective advantage in targeting bacterial cells.

Despite many years of attempts in numerous laboratories, limited success has been gained in the effort to design or discover new amphiphatic $\alpha$-helical peptides with a satisfactory toxic discrimination between microbial and host cells. Therefore, instead of the $\alpha$-helix-forming peptides, our laboratory has focused on $\beta$-sheet-forming peptides and their variants in an attempt to optimize antimicrobial potency and selectivity.

2. Synthetic KL-repeat Containing Peptides and Tryptophan Incorporation

The design of (KIGAKI)$_3$-NH$_2$ peptide and its derivatives provided an excellent starting point to search for optimal antimicrobial agents. However, the (KIGAKI)$_3$-NH$_2$ peptides did not possess sufficient antimicrobial potency and with 18 amino acids, they were judged to be too large to be viable for successful clinical development. In order to achieve greater antimicrobial potency and reduce peptide size, the design of amphiphatic $\beta$-sheet-forming peptides was extended to a new direction.
Theoretically, an increase in amphipathic character of β-sheet structure might lead to an increase in antimicrobial potency of peptides. The β-sheet structures of the (KIGAKI)$_3$-NH$_2$ family peptides were only moderately amphipathic. In order to create a more highly ordered amphipathic structure, our laboratory simplified the sequence, using only two amino acids, one with a positive charge (lysine) and the other one with a hydrophobic side chain (leucine). A perfect amphipathic β-sheet structure can be created by placing these two amino acid residues at alternating positions, so that the lysine and leucine residues extend their side chains in two opposite directions with respect to the plane of the β-sheet (Figure 17).

![Figure 17](image.png)

*Figure 17.* β-strand diagram of KL-repeat containing peptides showing the distribution of lysine and leucine side chains. White: lysine; black: leucine.

A series of such amphipathic β-sheet peptides with a sequence containing alternating lysine (K) and leucine (L) repeats was synthesized and investigated earlier (Castano et al., 2000). Peptides containing 9-15 amino acid residues were shown to form
antiparallel $\beta$-sheet structures in the presence of lipid bilayers using FTIR and polarization-modulated IRRAS spectroscopy (Castano et al., 2000). The antimicrobial activity of these peptides was not investigated in this study; however, the peptides were highly hemolytic, demonstrating their ability to induce leakage in human red blood cells.

A major problem with KL-repeat containing peptides is their high cytolytic activity toward mammalian cells. We focused our effort on how to achieve maximal antimicrobial potency of peptides while maintaining a selective advantage by targeting only bacterial cells. Our goal was to identify peptides with MIC values as low as 1 µg/ml with little or no hemolytic activity at 100x MIC.

In our laboratory, we began by examining longer (up to 18 residues) KL-repeats. Although they possessed good antimicrobial activity, these peptides were very hemolytic. In addition, they were not very soluble in aqueous solution. The fact that these peptides showed $\beta$-sheet structure in the absence of lipids suggested that they aggregate in water, leading to precipitation. By shortening the peptide to 11 residues, the solubility problems were eliminated. KL-11 possessed good antimicrobial activity, although it showed little selectivity based upon its high hemolytic activity.

In earlier work with KIGAKI peptides (Jin et al., 2003), we replaced a nonpolar isoleucine by a tryptophan (W) in order to introduce an intrinsic fluorescent probe to assess the interactions of the peptides with lipid bilayers. Our group found that the position of W in the sequence influenced the functional activity and ability of the peptide to induce leakage in lipid vesicles. Introduction of W at position 2 or 10 resulted in enhanced antimicrobial and hemolytic activity (Jin and Blazyk, 2002). Next, we modified
the most active peptide, W2-KL-11, by substituting single leucine residues with either alanine or glycine in an attempt to reduce hemolytic activity while retaining antimicrobial activity (Blazyk et al., 2003; Blazyk et al., 2004). Alanine substitutions lowered both antimicrobial and hemolytic activity. Glycine substitution at position 4 or 6 completely eliminated hemolytic activity, while retaining antimicrobial activity for *E. coli* but not for *S. aureus* or *P. aeruginosa*.

Our laboratory also tested whether shorter KL-repeat peptides might be effective. Good antimicrobial activity was observed for peptides containing 9 or 10 residues. Shorter peptides were insufficiently active for further study. W4-KL-10, where leucine was replaced by tryptophan at position 4, was nearly as potent antimicrobially as the 11-residue peptides but less hemolytic. Similarly, W2-KL-9, where leucine was replaced by tryptophan at position 2, showed good antimicrobial activity and only moderate hemolytic activity. These two peptides were selected as the parent compounds for further modifications.

The strategy employed with the alanine and glycine replacements was to reduce the hydrophobicity of the nonpolar face of the β-sheet. In the work presented here, we are using a new approach involving the introduction of local structural distortions along the peptide backbone to modulate peptide conformation.

3. Synthetic KL-repeat Containing Peptides with Leucine-to-Proline Substitutions

In this project, based on two model templates W4-KL-10 and W2-KL-9, a novel approach of peptide sequence modification to modulate the geometric property of the peptides by leucine-to-proline (P) substitutions was conducted and intensively studied.
Unlike the other 19 natural amino acids, proline (Figure 18) possesses a unique pyrrolidine ring and its $\alpha$-nitrogen atom is constrained within this rigid ring structure (Allen et al., 2004). This special structure can cause a conformational hindrance in the formation of the $\alpha$-helix or $\beta$-sheet which cannot be provided by the other amino acids.

The amino acid residues in peptides are connected together by peptide bonds and in each amino acid residue the two bonds of alpha carbon, $C\alpha$ – N (nitrogen in the amino group) and $C\alpha$ – C (carbon in the carboxyl group) can rotate to accommodate the normal geometry of the $\alpha$-helix or $\beta$-sheet structure. The rotation angles of these two bonds are referred as the phi ($\phi$) and the psi ($\psi$) angles (Figure 19). However, because of conformational constraint caused by the pyrrolidine ring, proline can only offer restricted $\phi$ and $\psi$ angles (Tonelli, 1973). Therefore, compared to the other 19 natural amino acids which exist predominantly in the $trans$- configuration in polypeptides, proline exists in the $cis$- form in peptides and thus can disrupt the formation of normal $\alpha$-helix or $\beta$-sheet, which can eventually introduce a kink- or bulge-like region in these two conformations.
Moreover, in α-helical structure, proline works as a steric hindrance to prevent the normal formation of hydrogen bonds (MacArthur and Thornton, 1991). The α-nitrogen of proline in the amide bond is unable to form hydrogen bonding with the backbone carbonyl group of the amino acid four residues away, because of the lack of hydrogen...
donor on the α-nitrogen of proline. In addition, proline also causes the absence of hydrogen bonding between the carbonyl group of amino acid three residues away from the N-terminus of proline and the amide group of amino acid which is immediately adjacent to the C-terminus of proline (von Heijne, 1991). Therefore, the normal folding of helices is interrupted and a hinge of 20° or more is observed (Vanhoof et al., 1995). In β-sheet structure, proline can also significantly decrease the amount of inter-strand hydrogen bonds and thus destabilize the β-sheets (Blazyk et al., 2007b).

An impressive amount of effort has been dedicated to investigate the structural and functional influence of a proline residue incorporated in the α-helix-forming
antimicrobial peptides. Gaegurin is a 24-residue-long antimicrobial peptide that was initially isolated from a Korean frog *Rana rugosa* (Park et al., 1994). This peptide (P14) possesses a central proline residue in the sequence at position 14 and its single proline-to-alanine substituted analog (P14A) was designed and synthesized to study the effects of proline inclusion (Suh et al., 1999). Conformational and 3D structural studies using CD and NMR spectroscopic methods indicated that both P14 and P14A adopted α-helical structure, but P14 showed a pronounced kink in the helix near the central proline residue which differed significantly from the highly ordered helical structure of P14A. Thus P14 had a substantially lower helical character and stability compared to those of P14A. The P14 peptide displayed a wide range of antimicrobial activity against six different microorganisms including Gram-positive bacteria, Gram-negative bacteria, and fungi with MIC values ranging from 8 – 64 µg/ml (MIC, Minimum Inhibitory Concentration, which is defined as the lowest concentration of peptide that completely inhibits the growth of the microorganism) and was more effective against Gram-negative and Gram-positive bacteria than the P14A peptide, while they both showed low hemolytic activity against sheep red blood cells. Peptide-lipid interaction studies using fluorescence probing techniques revealed that P14 possessed a considerably lower affinity for neutral lipid PC although both peptides exhibited similar binding affinities for negatively charged lipid PG (Suh et al., 1999). These results along with the enhanced antimicrobial activity but similarly low hemolytic activity of P14 compared to P14A suggested that synthetic α-helical peptides with structural distortions induced by a single proline may provide insights into the design and identification of potent antimicrobial agents. In addition, this
hinge-like motif of P14 introduced by the proline residue was believed to play a critical role in the activation of the ion channel formation in planar lipid bilayers and thus might promote antimicrobial activity (Woolfson et al., 1991).

Given the advantages of single proline incorporation in α-helical peptides from the antimicrobial potency and selective toxicity standpoint, the introduction of proline residues into the synthetic simplified KL-containing peptides may lead to discovery of promising model peptides for further modifications. The effects of both L- and D-proline substitution on such KL-containing peptides were investigated with amphipathic α-helical derivatives on their structural properties and cellular selectivity (Song et al., 2004). A series of four analogs with 18 amino acid residues was designed and analyzed based on an amphipathic α-helical parent peptide (KLW) with non-cell selectivity, containing a sequence as KWKKLLKKLLKLLKKLLK-NH₂. An L- or D-proline substitution (KLW-L9P or KLW-L9p) of leucine at position 9 was incorporated within the hydrophobic helical face of KLW, and a L- or D-proline substitution (KLW-K11P or KLW-K11p) of lysine at position 11 was incorporated within the hydrophilic helical face. All these proline substitutions uniformly induced a kink region in the α-helices of peptides and reduced the level of helical content. The derivatives displayed improved antimicrobial and hemolytic activity compared to those of KLW, except for KLW-K11P. Generally the D-proline substituted peptides exhibited more substantial reductions in hemolytic activity and more significant disruption in α-helical structure than the L-proline containing analogs. In addition, the KLW-L9P/p peptides were more promising derivatives with better combinations of antimicrobial and hemolytic activity compared to the KLW-
K11P/p peptides (Song et al., 2004). These results indicated that the strategy employed with L- or D-proline replacements of nonpolar residue at the hydrophobic face of amphipathic peptides was useful for the design of novel antimicrobial agents. To be specific, this may be utilized to modify amphipathic β-sheet-forming peptides such as KL-repeat containing peptides that were intensively investigated in our laboratory.

Earlier work in our laboratory with proline incorporation in β-sheet-forming peptides started with a group of proline containing analogs of W2-KL-11. In an attempt to retain antimicrobial activity and optimize selectivity for bacterial cells of the parent peptide W2-KL-11, we applied a single leucine-to-proline substitution at strategic positions of W2-KL-11 (Blazyk et al., 2006). Profound differences were observed depending on the position of the substitution (Table 3), indicating that the proline incorporation can uniformly decrease the cytolytic activity toward human red blood cells (assessed by percentage of hemolysis of human red blood cells caused by W2,Pn-KL-11 peptides) while retaining reasonable antimicrobial activity vs. *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) when proline was placed at position 4 or 10 (antimicrobial activity was measured by the determination of the MIC values).
Table 3

MIC and Hemolysis Results of W2, Pn-KL-11 Family Peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>E. coli ATCC 25922</th>
<th>S. aureus ATCC 29213</th>
<th>P. aeruginosa ATCC 27853</th>
<th>% Hemolysis 100 µg/ml</th>
<th>% Hemolysis 500 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>W2-KL-11</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>79</td>
<td>100</td>
</tr>
<tr>
<td>W2,P4-KL-11</td>
<td>4</td>
<td>4</td>
<td>64</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>W2,P6-KL-11</td>
<td>32</td>
<td>32</td>
<td>&gt;64</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>W2,P8-KL-11</td>
<td>16</td>
<td>16</td>
<td>64</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>W2,P10-KL-11</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

Another attempt to investigate the effects of structural perturbations in our laboratory involved the replacement of L-leucine by D-leucine (L) (Blazyk et al., 2007a).

These analogs showed a dramatic decrease in their hemolytic activity, particularly when the D-leucine was placed at positions 6 or 8.

However, the effect of this modification on antimicrobial activity was highly position-dependent, with the best antimicrobial activity being observed for the L10 analog (Table 4). These results indicated that D-amino acids can induce structural perturbations in the highly ordered β-sheet of W2-KL-11 and dramatically lower hemolytic activity in analogs while retaining comparable antimicrobial activity.
Table 4

**MIC and Hemolysis Results of W2, Ln-KL-11 Family Peptides**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>MIC (µg/ml)</th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli ATCC 25922</td>
<td>S. aureus ATCC 29213</td>
</tr>
<tr>
<td>W2-KL-11</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>W2,L4-KL-11</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>W2,L6-KL-11</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>W2,L8-KL-11</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>W2,L10-KL-11</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

These results suggested that proline or D-leucine incorporation in β-sheet structure was potentially effective in suppressing hemolytic activity via the introduction of local distortions of the β-strands and, in the case of proline, a decrease in the amount of intermolecular hydrogen bonds. This peptide modifying principle was then applied to shorter KL-repeat containing peptides W4-KL-10 and W2-KL-9 in this project. The conformational constraint of proline was expected to alter the interactions between the W4-KL-10/W2-KL-9 analogs and cell membranes. Hopefully, this would result in decreased cytolytic activity toward mammalian cells, while not significantly lowering their antimicrobial potency.

In order to retain the potential electrostatic interactions between the peptide and the bacterial cell membrane, the positively charged lysine residues were not replaced in the peptide design. Inspired by the earlier work in our laboratory with nonpolar residue substitution (Jin et al., 2003; Jin and Blazyk, 2002; Blazyk et al., 2003; Blazyk et al.,
a single leucine-to-proline substitution was applied to the parent peptides W4-KL-10 and W2-KL-9 to generate two families of derivatives.

In addition, D-proline substitutions were also tested. Introduction of both L- and D-proline substitutions provides a rich set of analogs to study the functional significance and structural perturbations in the interaction between peptides and cell membranes.

H. Research Goals

In this project, two novel classes of peptide analogs (W4-KL-10 and W2-KL-9 families) were intensively studied. The specific aims for this project were to:

1) Determine the antimicrobial and hemolytic activity of the peptide variants containing strategic proline substitutions;

2) Measure the peptide-induced leakage in synthetic lipid bilayers and particular bacterial cell membranes;

3) Measure the ability of these peptides to bind to synthetic lipid bilayers of defined lipid composition;

4) Measure the percentage of killing against several bacterial strains by the potent analogs among these two peptide families;

5) Determine the ability of the potent analogs to kill the bacterial cells inside the established biofilm matrix structure from several bacterial strains.

For these specific aims, a series of experimental methods were employed to investigate these peptide analogs. At the beginning, the minimum inhibitory concentration (MIC) assay and hemolytic assay were applied to determine the antimicrobial and hemolytic activity respectively to achieve specific aim 1. Then calcein
leakage from large unilamellar vesicles (LUV) and o-nitrophenyl-β-D-galactopyranoside (ONPG) entry into *E.coli* ML-35 cells were measured to achieve specific aim 2. Peptide binding experiments measured by tryptophan fluorescence emission, acrylamide quenching, 10-doxylanodacane quenching and circular dichroism (CD) spectroscopy were performed to accomplish specific aim 3. LIVE/DEAD® BacLight bacterial viability assay by using nucleic acid stains was conducted to fulfill the purpose of specific aim 4. Colony counting and the absorbance of crystal violet staining assay based on polystyrene 96-well tissue culture plates were employed to complete specific aim 5.

This innovative project facilitates the identification and characterization of an array of small and potent antimicrobial peptides that are promising candidates for the future development of antimicrobial agents. The results from these experiments may provide insight into further understanding of peptide-membrane interactions.
CHAPTER 2: MATERIALS AND METHODS

A. Materials

All peptides were initially synthesized by Bio Synthesis Inc. (Lewisville, TX). Supplementary supplies of peptides were obtained from SynBioSci Co. (Livermore, CA).

1-palmitoyl-2-oleoyl-sn-glycero-phosphatidylglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-phosphatidylethanolamine (POPE), and 10-doxylnonadecane (10-DN) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Sephadex G-50, calcein, Triton X-100 and o-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from SIGMA-ALDRICH (St. Louis, MO). L-ascorbic acid was obtained from VWR International (West Chester, PA). Ammonium molybdate was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Ethylenediaminetetraacetic acid disodium salt (EDTA), sodium phosphate monobasic (NaH₂PO₄·H₂O), HEPES, 30% Hydrogen peroxide and crystal violet were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium azide was obtained from Eastman Organic Chemicals (Rochester, NY). BBL™ Mueller-Hinton broth and agar, and Trypticase soy broth and agar were purchased from Becton, Dickinson and Company (Sparks, MD). The LIVE/DEAD® BacLight™ bacterial viability kits were purchased from Molecular Probes, Inc. (Eugene, OR).

The bacterial strains Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Staphylococcus aureus (ATCC 29213), Staphylococcus hominis + (ATCC 25615), Staphylococcus hominis – (ATCC 27844) Staphylococcus warneri +
(ATCC 17917) and Staphylococcus warneri – (ATCC 49518) were obtained from American Type Culture Collection (ATCC, Manassas, VA). E. coli ML35, a genetically engineered strain, was a gift from Dr. Renato Gennaro from University of Trieste, Trieste, Italy.

B. Peptide Design

Two families of peptides were designed for this project, with different peptide lengths and positions of proline residues (Table 5). All of these analogs were derived from linear KL-repeat containing peptides (W4-KL-10 and W2-KL-9) and might possess a high potential to adopt β-sheet conformation upon binding to the lipid bilayers or the cell membrane.

W4, Pn-KL-10 family peptides uniformly contain a tryptophan residue at position 4 of their sequence. Proline residues were applied to each peptide to replace a leucine residue at position 2, 6, 8 and 10, respectively. The tryptophan residue is fixed at position 2 of the sequence of the W2, Pn-KL-9 family peptides. The leucine-to-proline substitution occurred at position 4, 6, and 8. Since W2-KL-8 is one leucine residue shorter than W4-KL-9, this family apparently lacks variants with a proline at position 10.

Both L-form and D-form proline substitutions were applied to the two parent peptides. The positioning pattern of the D-form proline was the same as that of the L-form. The D-form-proline containing variants were designed to study whether the stereochemical features of proline would affect the properties of peptides.
Table 5

*List of Designed W4, Pn-KL-10 and W2, Pn-KL-9 Peptides*

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MW</th>
<th>Length</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>W4-KL-10</td>
<td>KLKWKLKLKL-NH₂</td>
<td>1303</td>
<td>10</td>
<td>+6</td>
</tr>
<tr>
<td>P2, W4-KL-10</td>
<td>KPKWKLKLKL-NH₂</td>
<td>1282</td>
<td>10</td>
<td>+6</td>
</tr>
<tr>
<td>W4, P6-KL-10</td>
<td>KLKWKPKLKL-NH₂</td>
<td>1282</td>
<td>10</td>
<td>+6</td>
</tr>
<tr>
<td>W4, P8-KL-10</td>
<td>KLKWKLKPKL-NH₂</td>
<td>1282</td>
<td>10</td>
<td>+6</td>
</tr>
<tr>
<td>W4, P10-KL-10</td>
<td>KLKWKLKLKP-NH₂</td>
<td>1282</td>
<td>10</td>
<td>+6</td>
</tr>
<tr>
<td>D-P2, W4-KL-10</td>
<td>KPKWKLKLKL-NH₂</td>
<td>1282</td>
<td>10</td>
<td>+6</td>
</tr>
<tr>
<td>W4, D-P6-KL-10</td>
<td>KLKWKPKLKL-NH₂</td>
<td>1282</td>
<td>10</td>
<td>+6</td>
</tr>
<tr>
<td>W4, D-P8-KL-10</td>
<td>KLKWKLKPKL-NH₂</td>
<td>1282</td>
<td>10</td>
<td>+6</td>
</tr>
<tr>
<td>W4, D-P10-KL-10</td>
<td>KLKWKLKLKP-NH₂</td>
<td>1282</td>
<td>10</td>
<td>+6</td>
</tr>
<tr>
<td>W2-KL-9</td>
<td>KWKLKLKLK-NH₂</td>
<td>1190</td>
<td>9</td>
<td>+6</td>
</tr>
<tr>
<td>W2, P4-KL-9</td>
<td>KWKPKLKLK-NH₂</td>
<td>1169</td>
<td>9</td>
<td>+6</td>
</tr>
<tr>
<td>W2, P6-KL-9</td>
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<td>1169</td>
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</table>
C. Determination of Antimicrobial Activity

The antimicrobial efficacy of each peptide was measured by determination of the Minimum Inhibitory Concentration (MIC), which is defined as the lowest concentration of peptide that completely inhibits the growth of the microorganism. Eight bacterial strains, Gram-negative strains \textit{Escherichia coli} (ATCC 25922), \textit{Pseudomonas aeruginosa} (ATCC 27853), and Gram-positive strains \textit{Staphylococcus aureus} (ATCC 29213), \textit{Staphylococcus hominis} + (ATCC 25615), \textit{Staphylococcus hominis} – (ATCC 27844) \textit{Staphylococcus warneri} + (ATCC 17917) and \textit{Staphylococcus warneri} – (ATCC 49518) as well as the genetically engineered strain \textit{E. coli ML35}, were examined with peptides in this experiment.

The determination assay of MIC was performed utilizing sterile polystyrene 96-well tissue culture plates. The peptide stock solution was diluted with autoclaved water and prepared in serial two fold dilutions ranging from 1 to 128 µg/ml. Bacterial inoculums from overnight culture were grown in Mueller-Hinton broth (MHB) or Trypticase soy broth (TSB) to mid-logarithmic phase (measured by absorbance at 600 nm) and then diluted with broth to contain 1 x 10^6 colony-forming units (CFU)/ml. Aliquots of 100 µl each of the bacterial inoculum and peptide dilution were added to each well in microtiter plates. Therefore, the final concentration of bacteria was 5 x 10^5 CFU/ml in each well and the peptide concentration ranged from 0.5, 1, 2, 4, 8, 16, 32 to 64 µg/ml. The 96-well plates were incubated overnight and the MIC was determined by visual inspection of turbidity in each well.
D. Determination of Hemolytic Activity

As an index of cell lysis induced by antimicrobial peptides toward mammalian cells, hemolytic activity was used to assess the selectivity of peptides between bacterial vs. host cells. The hemolysis assay was conducted by measuring hemoglobin release from human red blood cells (hRBC) at an absorbance of 400nm using a Cary 100-Bio UV-Vis spectrophotometer (Varian Instruments, Walnut Creek, CA).

Approximately 4 ml of fresh blood was collected and suspended to 10 ml using phosphate-buffered saline (PBS, 35mM phosphate buffer with 150mM NaCl at pH 7.3-7.4). The adjusted blood suspension was centrifuged for 2-3 min at 2,000xg. The upper white blood cell and supernatant layers were removed, and then hRBC were resuspended in 10 ml PBS and centrifuged again for 2-3 min at 2,000xg. This washing process was repeated 5 times and the final hRBC concentration was adjusted to 10% v/v using PBS. Aliquots of peptide dilutions were mixed with aliquots of the hRBC so the hemolytic reaction occurred at a peptide concentration of 100, 250 or 500 \( \mu \)g/mL and a 5% suspension of hRBC.

The reaction mixtures were then incubated at 37°C for 30-60 min and centrifuged for 10 min at 10,000xg. Release of hemoglobin from hRBC was determined by monitoring the absorbance at 400nm. Complete hemolysis was determined by adding 0.2% Triton X-100 in place of the peptides, and 0% hemolysis was determined by adding PBS instead of peptides.
E. Determination of Phospholipid Concentration

In order to prepare artificial large unilamellar vesicles (LUV), phospholipid stock solutions need to be prepared beforehand with distilled chloroform. The concentration of phospholipid in the stock solution was determined by measuring the absorbance of a reaction product at 820 nm, which is formed between free phosphate and ammonium molybdate (VI) tetrahydrate.

The free phosphorus content of standard solution and stock lipid solution was released using an ashing procedure. To prepare the standard solutions, 0 µmoles (0µl) blank, 0.0325 µmoles (50 µl), 0.065 µmoles (100 µl), 0.114 µmoles (175 µl), 0.163 µmoles (250 µl), and 0.228 µmoles (350 µl) of a 0.65 mM KH2PO4 phosphorus standard solution were placed in 16 x 125 mm disposable glass test tubes in duplicates. To prepare sample tubes of phospholipid stock solution, 5 µl of stock solution were placed in glass culture tubes and the chloroform solvent was gently removed with N2. In order to break down the lipid samples to liberate inorganic phosphate, 0.45 ml of 8.9 N H2SO4 was added to each of the standard and sample tubes, and all tubes were heated at 200-215°C for 25 minutes.

The tubes were cooled and 150 µl of a 30% H2O2 solution was added to the bottom of each tube. They were returned to heat at 200-215°C for an additional 30 minutes and then cooled to room temperature.

3.9 ml of d H2O, 0.5 ml each of 2.5% w/v ammonium molybdate and 10% w/v ascorbic acid solution were added to each tube. Each tube was capped to prevent evaporation and heated at 100°C for 7 minutes. Using the 0 µmoles standard as the
reference, the absorbance of the reaction product was determined by measuring the five standards at 820 nm with an Ultrospec® 1000E spectrophotometer (Pharmacia Biotech, Piscataway, NJ). A standard curve was obtained by plotting the absorbance vs. concentration of the phosphate. Then the absorbance of the samples was measured at 820 nm and the phosphate concentrations of the samples were determined in mg/ml.

F. Preparation of Large Unilamellar Vesicles (LUV)

Large unilamellar vesicles (LUV) with defined composition, including POPG, POPC, varying ratios of POPE/POPG, and POPC/POPG, were prepared for peptide-induced leakage, peptide binding and peptide secondary structure experiments.

Aliquots of stock lipid solution were added to glass test tubes. The chloroform solvent was removed using N₂ and the sample was further vacuumed for 2-3 hours. Dried sample was suspended in appropriate buffer solutions corresponding to different experiments. The lipid dispersion was subjected to 3 repeated freeze/thaw cycles using liquid nitrogen, and then was extruded 10 times through a 0.05 µm or 0.1 µm pore polycarbonate membrane in an Avanti mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL). The lipid concentration of the LUV suspension was determined by the phosphorus assay as described above.

G. Measurement of Peptide-induced Calcein Leakage in LUV

The ability of the peptides to release a fluorescent dye, calcein, from LUVs of varying lipid compositions was compared. LUVs containing POPC (mimicking a mammalian membrane surface), POPG, and varying ratios of POPC/POPG, POPE/POPG (mimicking a bacterial membrane surface) were prepared as previously described. Dried
lipid was hydrated with 300 μl of 80 mM calcein, frozen and thawed 3 times in liquid nitrogen, and extruded. 200 μl of vesicles were loaded onto a 1x30cm Sephadex G-50 column. HEPES buffer (50 mM HEPES, 100 mM NaCl, 0.3 mM EDTA, pH 7.4) was used to facilitate the separation of calcein-bound vesicles from free calcein. The calcein-bound vesicles were collected in 4 ml and used for calcein leakage experiments.

The time-dependent increase in the fluorescence of calcein was measured using a Varian Cary Eclipse spectrofluorometer (Varian Instruments, Walnut Creek, CA) to determine calcein leakage induced by peptides. The excitation and emission wavelength was at 490 and 520 nm, respectively. This assay was performed using a 96-well plate with a white non-transparent base. An 180 μl aliquot of the LUV suspension and a 20 μl aliquot of peptide dilution were added to each well so the final concentration of lipid in the assay was fixed at 10 μM. Peptide stock solution was diluted to varying concentrations to achieve the desired lipid-to-peptide ratio of 2, 4, 8, 16, 32, 64, 128 and 256:1. The fluorescence of released calcein was monitored at 3 min following the addition of the peptide, representing at least 90% of the maximal induced leakage. The fluorescent intensities were converted to percentages according to negative and positive controls. 20 μl of water or 10% Triton X-100 were added in place of peptide to determine 0 % and 100 % leakage, respectively.

H. Determination of Peptide-induced Leakage in E. coli ML-35

This method is used to directly measure peptide-induced disruption of the bacterial cell membrane. E. coli ML-35 is a genetically engineered bacterial strain that possesses constitutive cytoplasmic β-galactosidase activity. The intact cell membrane of
*E. coli* ML-35 is able to prevent galactose-containing substrates from entering the bacteria. If the bacterial cell membrane is disrupted, the substrate would invade into the cytoplasm of bacteria and interact with the enzyme $\beta$-galactosidase to generate products with distinguish absorbance (Skerlavaj et al., 1990).

Assays were conducted using *o*-nitrophenyl-$\beta$-D-galactopyranoside (ONPG) buffer. When the bacterial cell membrane is perturbed by peptides, $\beta$-galactosidase inside *E. coli* ML-35 obtains access to substrate ONPG and together they produce a cleavage product, *o*-nitrophonl, which was detected at 405 nm and monitored at 37°C for 15 minutes. The ONPG buffer consists of 10 mM NaH$_2$PO$_4$.H$_2$O (monobasic sodium phosphate), 100 mM NaCl and 1.5 mM ONPG. A suspension of mid-logarithmic phase *E. coli* ML-35 at the concentration of $2 \times 10^{11}$ CFU/ml was mixed with 700 µl of ONPG buffer, followed by the addition of 200 µl of peptides with different concentrations varying from 4, 2, 1, 1/2, 1/4 to 1/8 x MIC value. The OD$_{405}$ of *o*-nitrophonl was recorded using a Cary 100-Bio UV-Vis spectrophotometer (Varian Instruments, Walnut Creek, CA) equipped with a temperature control module. The peptide was replaced with an equivalent volume of dH$_2$O for a negative control. The leakage value of a peptide (W2-KL-11) at its 4x MIC known to induce maximum leakage was used as the 100% control.

The ONPG cleavage rate was calculated from the slope of the increase of absorbance values at 405 nm over the time course. The maximal percent rate of cleavage induced by a particular peptide was calculated as 

\[
\frac{\text{Rate}_{\text{peptide}}}{\text{Rate}_{\text{100\% release control}}} \times 100\%.
\]
I. Peptide Binding with LUV Measured by Tryptophan Fluorescence Emission

Since all of these proline substitution peptides contain a single tryptophan residue, the interaction of these peptides with lipid bilayers was investigated by measuring the tryptophan emission spectrum as a function of lipid-to-peptide ratio and LUV composition.

In aqueous solution, the emission maximum of tryptophan is around 355 nm. If the indole side chain is protected from a polar environment, for instance, embedded within the lipid bilayers of LUV, the emission maximum can shift to lower wavelengths. This feature was utilized to investigate the extent of peptide binding to LUV of defined lipid composition.

This assay was performed using a Varian Cary Eclipse spectrofluorometer (Varian Instruments, Walnut Creek, CA) equipped with a manual polarizer accessory. At room temperature, fluorescence emission spectra of tryptophan were collected from 290 to 500 nm at 1 nm increments using an excitation wavelength of 280 nm at a signal-to-noise ratio of 500 and a 2 mm x 10 mm quartz cuvette. Excitation and emission slit widths were 10 nm and 5 nm, respectively (Ladokhin et al., 2000). LUVs were prepared as previously described using HEPES buffer. Peptide concentration was constant at 10 µM and lipid concentrations were varied to give desired lipid-to-peptide ratios of 1, 5, 10, 15, 20, 30 and 50:1. All spectra were collected using emission and excitation polarizers in vertical and horizontal orientations. As for final results, the tryptophan emission intensity at 330 nm was used to assess the extent of peptide-LUV interaction. The light scattering
effects of LUV were corrected from measured intensity values at each lipid concentration using 10 µM of tryptophan in the absence and presence of LUV.

J. Peptide Binding with LUV Measured by Acrylamide Quenching

In addition, acrylamide quenching experiments were performed to extend the understanding of the interaction between peptides and lipid vesicles. In aqueous solution, the fluorescence intensity of the tryptophan in the peptide sequence can be readily quenched by a neutral, water-soluble quencher, acrylamide. In the presence of LUV, the tryptophan residues can reside in the nonpolar interior of the lipid bilayers instead of the water environment, and lead to a significant decrease in the acrylamide quenching. Therefore, acrylamide quenching experiments were used to study the changes in accessibility of antimicrobial peptide to the aqueous phase in the absence and in the presence of vesicles with defined lipid compositions.

In the acrylamide quenching experiment, fluorescence intensity values of tryptophan were collected by a Varian Cary Eclipse spectrofluorometer (Varian Instruments, Walnut Creek, CA) from a wavelength range of 320 nm - 390 nm. LUVs were prepared as described above using HEPES buffer. The concentration of peptides was fixed at 10 µM and the lipid-to-peptide ratio was constant at 10:1. The acrylamide was varied from 0 mM – 200 mM. The quenching results in the absence of LUV were also obtained.

The extent of tryptophan-protection offered by LUV was assessed by determining the maximum fluorescence intensity of tryptophan from 310 nm to 390 nm, and calculating the ratio of this peak value in the absence of quencher (F₀) to that in the
presence of quencher (F). The accessibility of peptides to the aqueous phase in the presence of different lipid vesicles were further analyzed by comparing the slopes of the quenching curves plotted as function of $F_0/F$ vs. acrylamide concentration.

K. Peptide Binding with LUV Measured by 10-Doxylnonadecane Quenching

The 10-doxylnonadecane (10-DN) is a novel quencher of tryptophan residue, which is significantly different from acrylamide. This molecule is highly hydrophobic and can be incorporated into the interior of lipid vesicles. As a result, in contrast to the acrylamide quenching in aqueous phase, 10-DN can strongly quench the fluorescence of the tryptophan indole side chain if it is embedded close to the center of the lipid vesicle (Caputo and London, 2003). Therefore, in addition to the acrylamide quenching experiment, the 10-DN quenching assay was conducted to assess the depth of the tryptophan insertion into LUVs with varying lipid compositions.

Similar to the acrylamide quenching experiment, fluorescence intensity values of tryptophan in the 10-DN quenching assay were collected by a Varian Cary Eclipse spectrofluorometer (Varian Instruments, Walnut Creek, CA) from a wavelength range of 310 nm - 390 nm. To prepare 10-DN containing LUVs, aliquots of 10-DN solution in 100% ethanol were mixed with phospholipids to give quencher concentrations of 5 mol% (for POPC and POPG vesicles) or 10 mol% (for POPC/POPG and POPE/POPG vesicles). The concentrations of peptides and the lipids were constant and equivalent to those in the acrylamide quenching experiment. Since the 10-DN quencher had to be loaded with lipid vesicles, it is unnecessary and impossible to collect quenching data without LUV.
The interaction between peptides and lipid vesicles were analyzed by calculating
the ratio between the maximum fluorescence intensity in the presence of quencher (F)
and that in the absence of quencher (F₀). The F/F₀ values were then compared to the
results from the acrylamide quenching assay in order to further investigate the position of
W4, Pn-KL-10 and W2, Pn-KL-9 peptides in the lipid bilayers.

L. Determination of Peptide Secondary Structure by Circular Dichroism

Circular Dichroism (CD) spectroscopy was used to determine the peptide
conformation upon binding to lipid bilayers. CD measures the absorption difference of
the right and left circularly polarized light beams. The antimicrobial peptides designed in
this project are chiral molecules and thus can produce CD spectra.

CD spectra can be analyzed to identify different secondary structural types such
as α-helix, β-sheet, random coil and others (Figure 20). The α-helical structure normally
shows one positive peak at 190 nm and two negative bands at 208 nm and 222 nm. The
β-sheet is usually associated with a positive band at 195 nm and a negative band at 220
nm. Random coil presents a negative band near 200 nm.

CD spectra were measured using a Jasco J-715 spectropolarimeter (Jasco, Inc.,
Easton, MD). The spectra were collected from 250 nm to 190 nm at a sensitivity of 100
millidegrees, resolution of 0.5 nm, response of 8 s, bandwidth of 1.0 nm, a scan speed of
20 nm/min and averaged over 2 accumulations. Phosphate buffer was used in CD
spectroscopy, containing 5 mM potassium phosphate, 150 mM KCl, 1 mM EDTA at pH
7.0. The peptide concentration in buffer and trifluoroethanol (TFE)/buffer mixtures was
20 µM. The lipid-to-peptide ratio varied from 1, 5, 10 to 20:1. LUV were prepared from
aqueous dispersions of phospholipids as described above except phosphate buffer was used to disperse dried lipid sample and as a zero control in the phosphorus assay. The final CD results were expressed as molar ellipticity vs. wavelength.

Figure 20. CD spectra of typical peptide secondary structures (Adapted from Fasman, 1996).
M. Measurement of Percent Bacterial Killing by Peptide

The percentage of killing against various bacterial strains by the potent analogs among W4, Pn-KL-10 and W2, Pn-KL-9 family peptides using the LIVE/DEAD® BacLight™ bacterial viability assay were determined. In this assay, a two-color staining and fluorescence detecting procedure was performed to quantitatively distinguish live and dead bacteria.

The LIVE/DEAD® BacLight™ bacterial viability kits provide 2 fluorescent nucleic acid stains: the SYTO 9 (green-dye) and the propidium iodide (red-dye). Their spectral characteristics are significantly different: the excitation/emission maxima of SYTO 9 stain are 480/500 nm but those of the propidium iodide are 490/635 nm. Additionally, the abilities of these two stains to penetrate healthy bacterial cells differ profoundly. The SYTO 9 stain universally labels all bacterial cells with intact membranes and those with damaged membranes when individually used. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Therefore, with an appropriate mixture of the SYTO 9 and the propidium iodide stains, bacteria with intact/damaged cell membranes were stained as green/red, and then quantitatively analyzed by calculating the fluorescence intensity ratio between the colors.

The Gram-positive strain Staphylococcus aureus (ATCC 29213) and the Gram-negative strains Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853) were tested with peptides in this assay. The overnight culture of bacteria was grown to late log phase in MH broth and 25 ml of this bacterial culture was centrifuged at
10,000 xg for 15 minutes and the supernatant was removed. The pellets were resuspended in aliquots of sterile deionized water with the addition of 4x, 2x, 1x, 0.5x, 0.1x and 0.05x MIC value peptide dilutions. 20 ml of sterile deionized water (for live bacteria) or 20 ml of 70% isopropyl alcohol (for dead bacteria) were added in place of peptides, in order to be used as 100% and 0% controls, respectively. All the samples were incubated at room temperature for 1 hour and then were treated with 2 repeated cycles of concentration at 10,000 xg for 15 minutes and resuspension with sterile deionized water. The final pellets were adjusted to a total volume of 10 ml using sterile deionized water and 5 standard samples were prepared by mixing 5 different portions of 0% and 100% controls. A combined stain mixture with 1:1 v/v ratio between the SYTO 9 and propidium iodide was prepared. 96-well plates were used to accommodate stained bacteria and each well received aliquots of stain mixture and aliquots of standard sample/peptide treated bacteria. The plates were incubated at room temperature in the dark for 15 minutes.

The fluorescent intensities of stained live/dead bacteria were detected and monitored by a Varian Cary Eclipse spectrofluorometer (Varian Instruments, Walnut Creek, CA). A standard curve was obtained by plotting the ratio of 530nm (live)/630nm (dead) fluorescent peak values vs. the designated percentage of live bacteria. Finally the percent killing induced by peptides were determined based on the analysis of Live/Dead fluorescence ratios according to the standard curve.

O. Determination of Inhibitory Effects of Peptides on Bacterial Biofilm Formation

Besides killing or inhibiting the growth of planktonic bacterial cells, these peptide analogs may possess the ability to prevent bacterial biofilm formation in vitro, even at
sub-MICs. This potential inhibitory effect of peptides on bacterial biofilm formation was assessed by detecting and analyzing the absorbance of crystal violet stained bacterial biofilm, which was treated with peptides at varying concentrations.

*Pseudomonas aeruginosa* (ATCC 27853) was tested in this assay. The bacterial inoculum was prepared from overnight cultures at 37°C and diluted to 1:100 solutions. The peptide stock solutions were diluted to achieve 0.125x, 0.25x, 0.5x, 1x, 2x, 4x, 8x MICs. Sterile clear 96 well microtiter plates were used to establish the desired biofilm structure with each well receiving 100 μl aliquots of the above 1:100 inoculum solutions, and 100 μl aliquots of peptide dilutions.

After overnight incubation of the mixtures at 37°C, the free moving bacterial cells and the supernatant broth were removed, followed by washing with sterile PBS buffer. The bacterial biofilm was stained with 1% w/v crystal violet solution and incubated for 20 min (Overhage et al., 2008). After removing the free unbound dye and rinsing the stained biofilm with PBS buffer, crystal violet was extracted by adding 200 μl of 100% ethanol solution in each well of a 96-well plate and reacting for 20 min (Narisawa et al., 2005). The absorbance of crystal violet was measured using a BioTek EL308 plate reader (BioTek Instruments, Inc., Winooski, VT) at 630 nm. The 0% and 100% control were prepared by adding 200 μl of 100% ethanol and 200 μl of 1:100 diluted solution of bacterial inoculum without peptides.
P. Determination of Antimicrobial Efficacy of Peptides toward Bacterial Cells within Established Bacterial Biofilm

This assay is used to assess the efficacy of peptides to kill the bacterial cells inside an established biofilm matrix. Colony counting was performed as a measurement of this potential inhibitory effect.

The bacterial inoculum was prepared from overnight cultures at 37°C and diluted to 1:100 solutions. Sterile clear 96 well microtiter plates were used to establish the desired biofilm structure with each well receiving 200 μl aliquots of the above 1:100 inoculum solutions. After 2 hours or 24 hours of incubation of the inoculum at 37°C, the free moving bacterial cells and the supernatant broth were removed, followed by washing with sterile PBS buffer, and 100 μl aliquots each of the peptide dilution and fresh broth (MHB or TSB) were added into each well. The plates were returned to incubate for overnight at 37°C and the removal/washing processes was repeated. Each well received 50 μl aliquots of fresh broth (MHB or TSB) and the bacterial cells within the biofilm were scraped and harvested. 10 μl of the scraped solutions were diluted to 1:10⁴ solutions and streaked onto agar plates. The agar plates were incubated overnight at 37°C and bacterial growth was determined by the counting of colony numbers on each of the agar plates. The following types of bacteria were used in the assay: 1) *Staphylococcus hominis* +; strain ATCC 25615, 2) *Staphylococcus hominis* -; strain ATCC 27844, 3) *Staphylococcus warneri* +; ATCC 17917 and 4) *Staphylococcus warneri* -; strain ATCC 49518.
CHAPTER 3: RESULTS AND DISCUSSION

A. Peptide Design

Two families of peptides were designed for this project, which were derived from linear KL-repeat containing peptides (W4-KL-10 and W2-KL-9) with proline residues at different positions (Table 5, Chapter 2). All of these analogs were expected to adopt $\beta$-sheet conformation upon binding to the lipid bilayer or the target cell membrane but with a lower level of amphipathicity and a lower extent of $\beta$-sheet character compared to the parent peptides.

The membrane disruption of target cells induced by antimicrobial peptides is considered crucial to their antimicrobial potency and believed to be a result of peptide-membrane interactions that might include peptide self association via intermolecular hydrogen bonds.

The parent peptides in this project, W4-KL-10 and W2-KL-9, can form perfect amphipathic $\beta$-sheet structure with strong intermolecular interactions. With a single leucine-to-proline substitution, the total cationic charge of the analogs is not changed (none of the positively charged lysine residues is replaced) thus the electrostatic attraction between the peptides and the negatively charged surface of the bacterial cell membrane is not disrupted, but a distortion might be formed in the peptide monomers and structural perturbations can be incorporated into the strands of $\beta$-sheet. In addition, the $\alpha$-nitrogen of proline lacks a hydrogen donor and cannot form a normal hydrogen bond with adjacent peptide molecules. These structural perturbation effects can decrease the amphipathicity of the peptides (decrease the $\beta$-sheet content), reduce intermolecular interactions between
peptides and thus disrupt the peptide self association on the surface of cell membranes, which lowers the membrane disruption capacity of our peptides (particularly against membranes with neutral lipids as a major component) (Figure 21).

In addition, the hydrophobicity of leucine and proline are significantly different (Eisenberg, 1984). The hydrophobic values of leucine and proline are 1.1 and 0.12, respectively (Table 2, Chapter 1). Therefore, the replacement of leucine (highly nonpolar)
with proline (less nonpolar) can reduce the hydrophobic character of the nonpolar domain of peptides and thus lead to a decrease in the amphipathicity of peptides.

Since the other parent peptide, W2-KL-9, was one amino acid residue shorter than W4-KL-10, the possible leucine residues of W2-KL-9 for proline replacement are less and the number of derivatives in the W2, Pn-KL-9 family were less than that of the W4, Pn-KL-9 family. Similarly, in the W2, Pn-KL-9 family peptides, the L- or D-proline residue at the middle positions of the peptide sequence (position 4 or 6) were expected to introduce a greater decrease in the amphipathicity and the β-sheet content of the analogs compared to that caused by the leucine-to-proline substitution at position 8.

These analogs were expected to show different properties in their antimicrobial and hemolytic activity, the extent of secondary conformation and in peptide-lipid interactions. The comparison of these properties in each family might reveal the pattern of structural and functional impact caused by proline substitutions on KL-repeat β-sheet-forming peptides at different positions and provide guidance for further modification of antimicrobial peptides in the future.

B. Antimicrobial Activity

The antimicrobial activities of W4, Pn-KL-10 and W2, Pn-KL-9 peptides were measured by the determination of the Minimum Inhibitory Concentration (MIC), which is defined as the lowest concentration of peptide that completely inhibits the growth of the microorganism. Several bacterial strains from both the Gram-positive and Gram-negative bacteria classes were selected to be tested with our peptides. Generally, different strains
are associated with different diseases and possess different levels of resistance to one or multiple conventional antibiotics.

*Escherichia coli* (*E. coli*) is a group of Gram-negative, rod-shaped bacteria and most strains in this category are non-pathogenic. However, several virulent strains of *E. coli* are mainly responsible for a wide range of conditions such as intestinal diseases (gastroenteritis), diarrhea, and in severe cases, peritonitis, which can be life-threatening without proper treatment. Other extra-intestinal diseases caused by different *E. coli* strains include urinary tract infections (UTI), neonatal meningitis, hemolytic-uremic syndrome (HUS, kidney failure) and pneumonia (Salyers and Whitt, 2002). *E. coli* ML-35 (*ML-35*) is a genetically engineered bacterial strain that possesses constitutive cytoplasmic β-galactosidase activity. It was used in the experiment of peptide-induced o-nitrophenyl-β-D-galactopyranoside (ONPG) into living bacterial cells.

*Pseudomonas aeruginosa* (*P. aeruginosa* or *P. aerug.*) is another family of Gram-negative, rod-shaped bacteria and has long been documented as an important pathogen in infectious diseases. *P. aeruginosa* is responsible for a broad spectrum of illnesses including dermatitis (skin infections caused by wounds), burn infections, ulcerative keratitis (eye infections in constant contact lens wearers), lung infections, and most significantly, the hospital-acquired infections such as urinary tract infections and septicemia. It is important to note that *P. aeruginosa* possesses the ability to form biofilms that can increase the bacterial resistance to antimicrobial compounds, and has been widely used as a model organism for biofilm formation, structure and prevention studies (Salyers and Whitt, 2002).
*Staphylococcus aureus* (*S. aureus*) is a class of spherical Gram-positive bacteria that is commonly found in an impressive variety of infectious diseases ranging from minor nasal and/or skin infections (Kluytmans et al., 1997) to osteomyelitis (infections of bone), endocarditis (infections of the heart), brain abscesses, pneumonia, septicemia and toxic shock syndrome that can be fatal if not treated promptly (Salyers and Whitt, 2002). *S. aureus* shows multi-resistance to antibiotics and contributes significantly to the mortality of hospitalized patients. Therefore, it has been recognized as one of the most common nosocomial infectious pathogens. Methicillin was once considered as a potent therapeutic agent against *S. aureus* related infections. However, in recent years, due to the increasing number of infections caused by methicillin-resistant *S. aureus* (MRSA) strains, the conventional clinical treatment has become problematic (the description “multiple resistant *S. aureus*” would be more precise as many *S. aureus* strains have developed resistance to the majority of antibiotics except vancomycin) (Salyers and Whitt, 2002; Tacconelli and Cataldo, 2007).

*Staphylococcus hominis* (*S. hominis* or *S.H.*) and *Staphylococcus warneri* (*S. warneri* or *S.W.*) are also members of the Gram positive, spherical bacterial genus *Staphylococcus* (Salyers and Whitt, 2002). These strains can be categorized into two classes based on their ability to produce an extracellular polysaccharide polymer - polyglutamic acid (PGA): the PGA-production-positive class *S. H.* + and *S. W.* + can generate this polymer whereas the negative class *S. H.* - and *S. W.* - lacks this property. Theoretically, PGA production can provide a protective effect and thus decrease the susceptibility of the bacterial strains to the antimicrobial agents (Otto, 2006). These
strains are common inhabitants of skin and were once thought to be harmless in most cases, but have proven to be capable of forming biofilms on the surface of plastic such as venous catheters and artificial heart valves (Vuong and Otto, 2002), which can significantly increase the virulence of the bacteria. Therefore, they have recently emerged as one of the leading causes of hospital-acquired infections as well as a broad collection of other illnesses such as endocarditis, urinary tract infections and sepsis (Salyers and Whitt, 2002).

The antimicrobial potency assessed by the MIC values of all the peptides including the parent peptides against the above bacterial strains are shown in Table 6 and 7. The results were selected from five repeated determinations.

For W4, Pn-KL-10 family peptides, the proline-substitution-containing analogs demonstrated significant differences in MIC values compared to the parent peptide, W4-KL-10. When L-proline was placed on position 6 or 8, the peptide derivatives W4, P6-KL-10 and W4, P8-KL-10 dramatically lost their antimicrobial potency, displaying MIC values 4-32 times higher than that of W4-KL-10 (Table 6). ATCC numbers of bacteria are shown in the row below the bacterial strains. The results are consensus values from five separate experiments.
In contrast, when L-proline was positioned close to either the amino or carboxyl terminus, the two derivatives P2, W4-KL-10 or W4, P10-KL-10 retained comparable antimicrobial activities. Although the difference of the MIC values between W4-KL-10 and P2, W4-KL-10 vs. Staphylococcus warneri –, W4-KL-10 and W4, P10-KL-10 vs. Pseudomonas aeruginosa and Staphylococcus warneri – were greater than a factor of 2, the two proline-containing variants showed substantial antimicrobial activity against all of the eight tested bacterial strains (Table 6). In general, there was no obvious preference to inhibit the growth of the Gram-negative or the Gram-positive bacteria. P2, W4-KL-10 was slightly more potent than W4, P10-KL-10 against most tested bacteria except Staphylococcus warneri +. A similar pattern of variation in MIC values was observed from the D-proline substituted W4-KL-10 analogs (Table 7). D-P2, W4-KL-10 and W4, D-P10-KL-10 demonstrated higher levels of antimicrobial activity against all eight

### Table 6

**MIC Values for W4, Pn-KL-10 Family Peptides (µg/ml)**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>P. aerug.</th>
<th>ML-35</th>
<th>S.H.+</th>
<th>S.H.-</th>
<th>S.W.+</th>
<th>S.W.-</th>
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<td>29213</td>
<td>27853</td>
<td>N.A.</td>
<td>25615</td>
<td>27844</td>
<td>17917</td>
<td>49518</td>
</tr>
<tr>
<td>W4-KL-10</td>
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<tr>
<td>W4, P8-KL-10</td>
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<tr>
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<tr>
<td>W4, D-P8-KL-10</td>
<td>32</td>
<td>32</td>
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<tr>
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<td>4</td>
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</tbody>
</table>
bacterial strains than W4, D-P6/D-P8-KL-10. Therefore, the analogs with D-/L-proline at position 2 or 10 were promising candidates for further investigations.

Since L- and D- proline substitutions in the W4, Pn-KL-10 family displayed an identical pattern of changes in the MIC values of the analogs, the L-proline substitutions in the W2, Pn-KL-9 family were expected to have impacts on the antimicrobial activity in a similar manner as the D-proline substitutions. For W2, Pn-KL-9 family peptides, the leucine-to-L-proline substitution dramatically increased the MIC values of the analogs and thus they uniformly lost their antimicrobial potency against any of the tested bacteria compared to the parent peptide W2-KL-9. The same result was observed for W2, D-P4-KL-9. Therefore, the possibility is extremely low for the other two D-proline substituted analogs in this family (W2, D-P6-KL-9 and W2, D-P8-KL-9) to retain the antimicrobial activity of the parent peptide and no further experiments were performed with these two.

Table 7

*MIC Values for W2, Pn-KL-9 Family Peptides (µg/ml)*

<table>
<thead>
<tr>
<th>Peptides</th>
<th><em>E. coli</em></th>
<th><em>S. aureus</em></th>
<th><em>P. aerug.</em></th>
<th><em>ML-35</em></th>
<th><em>S.H.</em>+</th>
<th><em>S.H.</em>-</th>
<th><em>S.W.</em>+</th>
<th><em>S.W.</em>-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25922</td>
<td>29213</td>
<td>27853</td>
<td>N.A.</td>
<td>25615</td>
<td>27844</td>
<td>17917</td>
<td>49518</td>
</tr>
<tr>
<td>W2-KL-9</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<td>W2,P8-KL-9</td>
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<td>&gt;32</td>
</tr>
<tr>
<td>W2,D-P4-KL-9</td>
<td>64</td>
<td>64</td>
<td>&gt;64</td>
<td>64</td>
<td>&gt;64</td>
<td>64</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>
C. Hemolytic Activity

The hemolytic activities of these peptides were determined and used to assess the cytolysis of peptides toward human red blood. The best results were selected from three repeated determinations of the hemolytic activities, and averages and standard deviations were calculated from the duplicated measurements of each peptide at three different peptide concentrations (Table 8).

For the W4, Pn-KL-10 peptides, the proline replacement uniformly decreased the hemolytic activities of the derivatives compared to their parent template W4-KL-10 (Table 8). Therefore, the four derivatives with either L- or D-proline substitutions at position 2 or 10 were good combinations of high antimicrobial activity and extremely low hemolytic activity.

By substituting a leucine of W2-KL-9 peptide at different positions for a proline, all the derivatives mildly decreased the hemolytic activity (Table 8). However, because of their lack of potent antimicrobial activity, the W2, Pn-KL-9 family peptides were not considered promising candidates for the development of antimicrobial agents.
Table 8

Hemolytic Activity of W2, Pn-KL-9 and W4, Pn-KL-10 Peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>% Hemolysis at peptide concentration of</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>500 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>W2-KL-9</td>
<td>15.5</td>
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<td>W2,P4-KL-9</td>
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<tr>
<td>W4-KL-10</td>
<td>22</td>
</tr>
<tr>
<td>P2,W4-KL-10</td>
<td>0</td>
</tr>
<tr>
<td>W4,P6-KL-10</td>
<td>0</td>
</tr>
<tr>
<td>W4,P8-KL-10</td>
<td>0</td>
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<td>W4,P10-KL-10</td>
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<tr>
<td>D-P2,W4-KL-10</td>
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<td>W4,D-P6-KL-10</td>
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<td>W4,D-P10-KL-10</td>
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D. Peptide-induced Calcein Leakage in LUV

Membrane perturbations are crucial to the activity of most antimicrobial peptides (Shai, 2002). Therefore, a series of lipid vesicles of defined lipid composition loaded with a fluorescent dye were treated with our peptides to investigate the potential membrane-lytic effects caused by these proline substituted peptides. The results of this kind of peptide-induced release of the fluorescent dye, calcein, from large unilamellar vesicles (LUV) of varying lipid compositions are shown in this section. LUVs are prepared from aqueous dispersions of the appropriate lipid systems, containing neutral POPC, or anionic POPG, or varying ratios of either POPC/POPG or POPE/POPG (mimicking the *E. coli* plasma membrane surface). For each type of vesicle, two sets of LUVs were prepared and treated with different peptides; each set was measured once with each peptide. The averages of the percent release of calcein induced by each peptide were calculated from the two measurements of these two sets of LUVs and the error bars were based on the standard deviations of these two measurements.

In order to better relate the leakage experimental results to the lipid composition of bacterial membranes, the level of leakage in lipid mixture LUV’s containing POPG as the anionic component and either POPC or POPE as the neutral component were tested. For instance, since the composition of *E. coli* membrane phospholipids is around 75% PE and 25% PG/DPG (Raetz, 1978), 2:1 POPE/POPG LUV is a reasonable mimic of the *E.coli* cell membrane phospholipid composition. LUVs composed of neutral (either POPE or POPC) and anionic (POPG) lipid mixtures with other neutral-to-anionic phospholipid ratios such as 1:1 and 4:1 were investigated, although these ratios were not
designed to necessarily represent the phospholipid composition of any particular bacterial strain.

For W2, Pn-KL-9 family peptides, only the parent peptide W2-KL-9 induced high rates of calcein release from the POPG LUV (Figure 22). All of the proline-containing derivatives induced less than 10% of calcein release even at the lowest lipid-to-peptide ratios (2:1).

Figure 22. Percent release of calcein from POPG LUV (W2, Pn-KL-9).
With POPC LUV, the level of calcein release induced by peptides was significantly lower than that in POPG LUV, even for the model peptide W2-KL-9. All of the peptides were unable to induce leakage (Figure 23). Since POPG or POPC LUVs were simplified models designed to mimic anionic vs. neutral cell membrane surfaces, and both the antimicrobial and hemolytic activity of the W2, Pn-KL-9 derivatives were relatively low, there was a good correlation between the antimicrobial and hemolytic activity and peptide-induced calcein leakage from POPG and POPC LUV.

Figure 23. Percent release of calcein from POPC LUV (W2, Pn-KL-9).
Generally, the trend of the results from mixtures of LUVs was a reduction in leakage induced by W2-KL-9 as the neutral-to-anionic lipid ratio increases, whereas extremely low leakage rates were induced by all the proline-containing derivatives in all six kinds of lipid mixtures (Figure 24-29).
Figure 24. Percent release of calcein from 1:1 POPC/POPG LUV (W2, Pn-KL-9).

Figure 25. Percent release of calcein from 2:1 POPC/POPG LUV (W2, Pn-KL-9).
Figure 26. Percent release of calcein from 4:1 POPC/POPG LUV (W2, Pn-KL-9).

Figure 27. Percent release of calcein from 1:1 POPE/POPG LUV (W2, Pn-KL-9).
Figure 28. Percent release of calcein from 2:1 POPE/POPG LUV (W2, Pn-KL-9).

Figure 29. Percent release of calcein from 4:1 POPE/POPG LUV (W2, Pn-KL-9).
These results indicated that the ability of the W2, Pn-KL-9 peptides to increase the permeability of LUVs were significantly weaker than that of the parent peptide W2-KL-9, which was also closely correlated to their low antimicrobial and hemolytic activities.

For W4, Pn-KL-10 family peptides, a similar experimental design was applied. However, these peptides showed strikingly different behaviors. With POPG LUVs (Figure 30), at a high lipid-to-peptide ratio such as 256:1, 128:1 and 64:1, none of the peptides were able to induce a substantial amount of calcein leakage. Among all the proline-containing derivatives, P2, W4-KL-10 and D-P2, W4-KL-10 demonstrated comparable antimicrobial potency compared to the parent peptide W4-KL-10, and they promoted a high percent to complete release of calcein at lipid-to-peptide ratios below 16:1. But their abilities to induce calcein leakage from LUVs faded away quickly at a higher lipid-to-peptide ratio (32:1) compared to W4-KL-10.

W4, P10-KL-10 caused considerable leakage at the lowest lipid-to-peptide ratio of 2:1, almost as half as W4-KL-10 induced, and then sharply lost its ability to induce leakage at ratios above 4:1. The only other peptide that demonstrated low MIC value, W4, D-P10-KL-10, surprisingly did not show a similar tendency as W4, P10-KL-10 to induce leakage even at the lowest lipid-to-peptide ratio. All of the other peptides with proline substitutions on position 6 or 8 were not able to induce more than 10% of calcein release at all lipid-to-peptide ratios.
The level of calcein release from POPC LUVs (Figure 31) induced by W4, Pn-KL-10 was uniformly much lower than that from POPG LUV. In fact, none of the peptides induced significant calcein leakage from POPC LUVs even at the lowest lipid-to-peptide ratio (2:1).

*Figure 30. Percent release of calcein from POPG LUV (W4, Pn-KL-10).*
The ability of W4, Pn-KL-10 peptides to increase the permeability of LUVs with mixed lipid compositions was also tested. In POPC/POPG LUVs (Figure 32-34), W4-KL-10 was able to induce a considerable amount of calcein leakage (around 20%) at the lowest lipid-to-peptide ratio of 2:1 and the percent leakage decreased gradually until it was almost undetectable at the highest lipid-to-peptide ratio of 256:1. It is intriguing to note that this ability was almost the same for 1:1 and 2:1 POPC/POPG LUVs but reduced dramatically from 4:1 POPC/POPG LUVs.

Figure 31. Percent release of calcein from POPC LUV (W4, Pn-KL-10).
Among the proline-containing analogs, only P2, W4-KL-10 and D-P2, W4-KL-10 showed comparable potency to induce calcein leakage, similar to W4-KL-10. The pattern of variations of their ability between 1:1, 2:1 and 4:1 POPC/POPG LUVs was the same as their parent peptide. The remaining proline-containing derivatives including W4, P10/D-P10-KL-10, were much less active than W4-KL-10 in POPC/POPG vesicles (Figure 34).

The experimental data slightly changed when POPC was replaced by POPE (Figure 35-37), although they are both neutral phospholipids. The parent peptide W4-KL-10 demonstrated a higher potency to induce calcein release (around 30%) from 1:1 POPE/POPG than it did from 1:1 POPC/POPG at the lowest lipid-to-peptide ratio of 2:1, and P2, W4-KL-10 and D-P2, W4-KL-10 were less active than W4-KL-10 (Figure 35).

However, in 2:1 POPE/POPG these three displayed almost the same potency, but it was obvious that the calcein leakage induced by these peptides were lower than those from 1:1 POPE/POPG. The other derivatives, including P10 and D-P10, were inactive in both 1:1 and 2:1 POPE/POPG at all lipid-to-peptide ratios (Figure 35 and 36). When the ratio of neutral component PE was further increased (4:1 POPE/POPG LUVs), all the peptides were unable to induce calcein leakage (Figure 37).
Figure 32. Percent release of calcein from 1:1 POPC/POPG LUV (W4, Pn-KL-10).

Figure 33. Percent release of calcein from 2:1 POPC/POPG LUV (W4, Pn-KL-10).
Figure 34. Percent release of calcein from 4:1 POPC/POPG LUV (W4, Pn-KL-10).

Figure 35. Percent release of calcein from 1:1 POPE/POPG LUV (W4, Pn-KL-10).
Figure 36. Percent release of calcein from 2:1 POPE/POPG LUV (W4, Pn-KL-10).

Figure 37. Percent release of calcein from 4:1 POPE/POPG LUV (W4, Pn-KL-10).
In general, the potency of W4, Pn-KL-10 peptides to induce calcein leakage from LUVs, was gradually reduced as the neutral lipid component POPE or POPC increased. PG is an anionic phospholipid while PE and PC are neutral phospholipids. Therefore, the electrostatic interaction between the cationic peptides and the PG lipids may lead to an increased amount of peptide binding on the surface of the POPG LUVs and result in greater calcein leakage from POPG vesicles.

Among the proline-containing derivatives, only P2 and D-P2 peptides exhibited a similar leakage-inducing activity pattern compared to that of W4-KL-10. P10 and D-P10 peptides possess comparable antimicrobial activities as W4-KL-10, which are similar to P2/D-P2 peptides as described in the previous two sections of antimicrobial and hemolytic activity. However, P10 and D-P10 analogs did not induce as much calcein leakage as the P2 and D-P2 analogs. These results suggest that although peptide-induced membrane leakage may play a role in the process of membrane disruption, massive membrane leakage or pore-formation may not be a prerequisite for the antimicrobial activity. It has been proposed that the perturbation of the bacterial cell membrane protein complexes by means such as electron transport channels, or cell wall and lipid components may lead to bacterial cell death in the absence of permeability changes (Pag et al., 2008).

It is notable that the LUVs that were used in our experiments are only models and do not really mimic bacterial membranes. Given the diversity of the lipid and protein compositions of the cell membranes of different bacterial strains, it is unreasonable to expect that this group of simplified lipid systems would fulfill the task of precisely and
accurately mimicking peptide-induced membrane leakage in living bacterial cells. Nevertheless, this experiment exhibited significant differences in the behavior of the W4, Pn-KL-10 peptides and provided insight into the further investigation of peptide-membrane interactions.

In addition, it is also important to address how comparable are the above three assays in terms of lipid-to-peptide ratio. For example, in the assay for antimicrobial activity, we used *Escherichia coli* inoculums with a concentration of 5 x 10⁵ cells/ml. There are ~5 x 10⁶ lipids on the exterior of the *E. coli* plasma membrane. If the MIC value of an antimicrobial peptide against *E. coli* is 2 μg/ml, the lipid-to-peptide ratio is about 1:400 (Blazyk, 2007a). In the hemolytic assay, we used a human red blood cell (hRBC) suspension with a concentration of 5 x 10⁸ cells/ml. There are ~2 x 10⁸ lipids on a single hRBC cell surface. At a peptide concentration of 500 μg/ml, the lipid-to-peptide ratio in this assay is around 1:2 (Blazyk, 2007a). Therefore, the lipid-to-peptide ratios in the MIC, hemolytic and calcein leakage experiments are significantly different and cannot be directly correlated to compare the peptide-membrane interactions without taking this difference into consideration.

E. Peptide-induced Leakage in the Plasma Membrane of *E. coli* ML-35

In addition to the ability of W4, Pn-KL-10 and W2, Pn-KL-9 peptides to induce leakage from artificial lipid systems, peptide-induced disruption of the living bacterial cell membranes can be directly monitored. The genetically engineered bacterial strain *E. coli* ML-35 was selected for testing because of its constitutive cytoplasmic β-galactosidase activity and lactose permease deficiency. Normally, the substrate *o-*
nitrophenyl-β-D-galactopyranoside (ONPG) is impermeable to the plasma membrane. If the membrane is disrupted by peptides, ONPG can enter the cytoplasm and undergo cleavage by cytosolic β-galactosidase to generate o-nitrophenol, which absorbs at 405 nm. This provides a direct measurement of the ability of our peptides to induce leakage in the plasma membrane of a living bacterial cell.

The parent peptide W4-KL-10 and the P2/P10, D-P2/D-P10 analogs can inhibit the growth of *E. coli* ML-35 at a concentration of 2 - 4 µg/ml whereas the MIC values against *E. coli* ML-35 of other derivatives are all at 16 µg/ml.

Each column in Figure 38 represents the average value of duplicate measurements of ONPG cleavage and the error bar is calculated based on the standard deviation of these two measurements. The leakage value of the reference peptide, W2-KL-11, at its 4x MIC, where it is known to induce maximum leakage, was used as the 100% control. The peptide was replaced with an equivalent volume of dH2O for the negative control.
Figure 38. Leakage of extracellular ONPG into the cytoplasm of *E. coli* ML-35 cells following the addition of W4, Pn-KL-10 family peptides.
As shown in Figure 38, the parent peptide W4-KL-10 can induce a high rate of leakage, above 80% at its 4x MIC value (8 µg/ml). W4-KL-10 was slightly less effective at its 2x and 1x MIC values (4 and 2 µg/ml), but the leakage rate is still around 70%. When the peptide concentration reached values lower than its MIC, the leakage-inducing ability of W4-KL-10 decreased dramatically, showing a leakage rate around 10% ~ 15% (the first three bars from left to right in the results of W4-KL-10 in Figure 38).

Compared to W4-KL-10, the analogs with proline at position 2 or 10 showed potent activity in inducing leakage of *E. coli* ML-35 cells at their 4x MIC values (leakage rate around 60% ~ 70%), only slightly lower than that of W4-KL-10. However, at the peptide concentration of their 2x MIC values, they were significantly less effective than W4-KL-10. P2, W4-KL10 and D-P2, W4-KL-10 were able to induce a leakage rate near 50% at 4 µg/ml while W4, P10-KL-10 and W4, D-P10-KL-10 were less active at 8 µg/ml, inducing a leakage rate of 20% ~ 25%. At peptide concentrations ≤ their MIC values, no obvious differences were observed between these peptide pairs and they were not able to induce a significant cytoplasmic membrane disruption of *E. coli* ML-35 cells, showing leakage rates all lower than 20% (Figure 38).

For the derivatives with proline residues at position 6 or 8, the leakage of extracellular ONPG into *E. coli* ML-35 cells was uniformly lower than 20%. The differences of leakage percentage depending on peptide concentration were not observed among these peptides as they were unable to induce significant leakage even at 4x MIC values (Figure 38).
It is surprising to observe that at their MIC values, the P2/D-P2 and P10/D-P10 analogs demonstrated decreased activity compared to that of their parent peptide W4-KL-10. Although they all share similar MIC values against *E. coli* ML-35 (2 - 4 µg/ml), apparently they possess a significantly lower ability to induce leakage of living *E. coli* ML-35 cell membranes at least to ONPG molecules. It is important to note that the size of the ONPG molecule (MW = 301.249) is larger than some small molecules or ions that are essential for cell metabolism, such as Na\(^+\), K\(^+\), etc. Therefore, it is possible that the peptides that are inactive in the ONPG assay may cause cell death by inducing membrane leakage and loss of ion gradients.

These results also suggest that membrane disrupting abilities of these proline-containing peptides were concentration dependent but not stereochemically specific, based on the same pattern of the leakage-inducing results of P2, W4-KL-10 and D-P2, W4-KL-10. Similar observations between the pair of P10 and D-P10 analogs were also observed in this assay.

It is also notable that the other proline-containing peptides in the W4, Pn-KL-10 family were relatively poor at inducing leakage in the *E. coli* ML-35 cytoplasmic membranes even at 4x MIC. Despite the fact that their MIC values were much higher than that of W4-KL-10, their failure to induce a significant leakage at 4x MIC suggests that their antimicrobial activity may involve a different mechanism than simply membrane permeabilization.
F. Peptide Binding with LUV Measured by Tryptophan Fluorescence Emission

The tryptophan residue, which can serve as a fluorescent probe, was uniformly incorporated in the W4, Pn-KL-10 and W2, Pn-KL-9 peptides. The influence of peptide-lipid association on the tryptophan fluorescence spectra includes a blue shift of the emission maxima from 355 nm to 330 nm and an increase in the intensity of the emission spectrum. If the imidazole ring is transferred from an aqueous to a hydrophobic environment, the tryptophan fluorescence will increase in intensity and shift to a higher frequency. In this section, a qualitative assessment of the degree of peptide-lipid interactions was obtained by comparing the intensity of emission at 330 nm in the absence and presence of lipid vesicles with defined lipid composition. LUVs with varying lipid compositions (POPG, POPC, POPE/POPG and POPC/POPG) similar to those in calcein leakage experiments were used. The change in tryptophan fluorescence emission intensity induced by each peptide was monitored as a function of the lipid-to-peptide (L/P) ratio and the maximal interaction was observed at 50:1 L/P ratio. The average and standard deviation of duplicated results from two sets of lipid vesicles are shown in Figures 39 – 41.

In the presence of LUV containing only neutral POPC, the fluorescence enhancement was relatively small for all the proline containing analogs, including the parent peptides W2-KL-9 and W4-KL-10 (Figures 39 – 41, the last group of columns on the right side of each figure).
For W2, Pn-KL-9 peptides, in the presence of LUV containing only anionic POPG, the parent peptide W2-KL-9 demonstrated a large increase in tryptophan fluorescence emission intensity with nearly a 3-fold enhancement. POPG LUVs also induced an increase in tryptophan intensity with all the proline-containing analogs but the enhancements were smaller compared to that of W2-KL-9. In the presence of LUVs with mixtures of 1:1 POPE/POPG or POPC/POPG, the increase in fluorescence intensity of W2-KL-9 was significantly decreased compared to that in the presence of POPG LUVs, with about a 2-fold enhancement. The other proline-containing peptides showed intensity enhancements ranging from 1.3 ~ 1.7-fold, which were smaller than that of W2-KL-9.
with 1:1 PEPG or 1:1 PCPG. When the ratio of the neutral phospholipid PE or PC to the negatively charged phospholipids PG increased to 2:1 in the LUVs with lipid-mixture, smaller enhancements in intensity were observed, especially for the parent peptide W2-KL-9 (Figure 39).

These results indicated that the position of the tryptophan residue of W2, Pn-KL-9 peptides was in a less polar environment in the presence of POPG LUVs, implying that these peptides can bind and insert tryptophan residue into the lipid bilayers of POPG LUV. Apparently, W2-KL-9 associated more strongly with POPG LUV than the proline-containing analogs. The extent of binding between the LUVs and the W2, Pn-KL-9 family peptides were closely associated with the percentage of negatively charged phospholipid PG in the lipid system. Generally when there was more PG, the binding was stronger. Proline substitutions uniformly weaken the binding of the analogs to the LUVs.

For W4, Pn-KL-10 peptides, in the presence of LUVs containing only POPG and at the maximal L/P ratio 50:1, the result of each peptide differed significantly (Figure 40). The parent peptide W4-KL-10 demonstrated a 4-fold enhancement in intensity. P2, W4-KL-10 and W4, P10-KL-10 peptides displayed slightly lower increases compared to that of W4-KL-10, exhibiting enhancements around 3.6- and 3-fold. W4, P6/P8-KL-10 peptides showed the lower increase compared to the other three peptides, which were near 2.3-fold. These results suggested that the extent of binding of peptides to POPG LUVs was in this order: W4-KL-10>W4, P2/P10-KL-10>W4, P6/P8-KL-10.
In the presence of 1:1 POPE/POPG and POPC/POPG LUVs, the increase in intensity was uniformly smaller compared to that of POPG LUVs but showed a similar order: W4-KL-10 ≥ W4, P2/P10-KL-10 > W4, P6/P8-KL-10 (Figure 40). When the POPE-to-POPG or the POPC-to-POPG ratio increased to 2:1, the fluorescence enhancements were below 2-fold for all W4, Pn-KL-10 family peptides, indicating that the binding of peptides to LUVs was weaker than that in 1:1 POPE or 1:1 PCPG (Figure 40). A similar pattern of change was observed among the increase of fluorescence intensity of D-proline-containing analogs with these lipid systems (Figure 41).
In general, the fluorescence increase of the W4, Pn-KL-10 analogs in the presence of lipid vesicles, was gradually reduced as the neutral lipid component POPE or POPC increased, if we consider POPG LUVs as 0% POPE or POPC LUVs. The analogs with potent antimicrobial activity in the W4, Pn-KL-10 family demonstrated better binding capabilities compared to the less active proline-containing analogs, while the proline substitutions uniformly decrease the binding of the analogs to the LUVs in the W2, Pn-KL-9 family.

Although our LUVs with varying lipid compositions are only crude models to represent bacterial and mammalian cell membranes, it is reasonable to conclude that the
association between the W4, P2/P10-KL-10 (or W4, D-P2/D-P10-KL-10) and the lipid bilayers of the cell membranes is quite similar to that of the parent peptide W4-KL-10, whereas the binding of the other non-active analogs to the lipid bilayers of the cell membranes was clearly much weaker. These distinguishing properties may be representatives of the mechanism for these four proline-containing analogs (P2, P10, D-P2 and D-P10) to achieve the potent antimicrobial activities compared to their parent peptide W4-KL-10 while the other derivatives from the W2, Pn-KL-10 and W4, Pn-KL-10 families lose their antimicrobial efficacy, if peptide-membrane interactions are essential for antimicrobial activity of peptides.

However, it is difficult to directly correlate the uniformly decreased hemolytic activities of these proline-containing analogs to their binding capabilities with our LUVs, since none of the peptides showed especially strong binding to POPC, which is a major lipid component on the outer surface of human red blood cells.

G. Peptide Binding with LUV Measured by Acrylamide and 10-DN Quenching of Tryptophan Fluorescence

Although the tryptophan fluorescence enhancement experiment provides some insight into the interaction between peptides and the lipid bilayers of cell membranes, it is possible to gain further knowledge of the location of tryptophan-containing peptides using aqueous and membrane-bound quenchers of tryptophan fluorescence. This section describes acrylamide and 10-Doxylnonadecane (10-DN) quenching of tryptophan fluorescence.
Water-soluble acrylamide can quench the tryptophan fluorescence of peptides in solution, while 10-DN is intercalated into the hydrophobic core of lipid bilayers and thus can quench tryptophan fluorescence of peptides if the tryptophan residue in the peptide resides in proximity to the hydrophobic interior of lipid bilayers (Caputo and London, 2003). In other words, the relative steady-state distribution of peptides embedded in membranes vs. free in the aqueous environment can be measured by acrylamide quenching of tryptophan emission intensity, and the degree of penetration of the peptides into the lipid bilayers can be estimated by 10-DN quenching of tryptophan fluorescence. Therefore, an analysis of the two quenchers can provide an estimate of the relative binding and location of the peptide in the membrane (Figure 42).

*Figure 42. Schematic diagram of tryptophan fluorescence quenched by acrylamide and 10-DN (Blazyk et al., 2009).*
1. Acrylamide Quenching of Tryptophan Emission Intensity

In aqueous solution (no lipid), the fluorescence intensity of tryptophan in the peptide can be readily quenched by acrylamide. In the presence of LUV, if the tryptophan residues insert into the nonpolar interior of the lipid, the peptide may be protected from acrylamide quenching effects. Therefore, acrylamide quenching experiments were used to study the changes in accessibility of the peptides to the aqueous phase in the absence and in the presence of vesicles with defined lipid compositions.

The acrylamide quenching of tryptophan fluorescence occurs by a collisional mechanism. Upon contact, this collisional quenching deactivates the excited state and returns the fluorophore to the ground state without allowing a photochemical reaction (fluorescence emission). Acrylamide quenching of tryptophan fluorescence is described by the Stern-Volmer equation (Lakowicz, 2006):

\[ \frac{F_0}{F} = 1 + K_{sv} [Q] \]

\( F_0 \): the fluorescence intensity in the absence of quencher;
\( F \): the fluorescence intensity in the presence of quencher;
\( [Q] \): the quencher concentration;
\( K_{sv} \): the Stern-Volmer constant (the slope of plot of \( \frac{F_0}{F} \) vs. \([Q]\), unit: \(1/\text{mM}\)).

In the acrylamide quenching experiment, the concentration of peptides and lipid were fixed at 10 µM and 100 µM, respectively (i.e., a 10:1 lipid-to-peptide ratio). The acrylamide concentration was varied from 0 mM – 200 mM. The extent of protection from quenching provided by LUVs was assessed by determining the maximum fluorescence intensity of tryptophan between 310 nm and 390 nm in the absence and
presence of LUVs. The Stern-Volmer plots for the W2, Pn-KL-9 and W4, Pn-KL-10 peptides are shown in Figure 44 – 48 and 51 – 59. Representative results were selected from two measurements of two different sets of vesicles of each kind of lipid composition.

Theoretically, the slope ($K_{sv}$) of the plot of $F_0/F$ vs. acrylamide concentration is proportional to the accessibility of the peptide to the aqueous phase; that is to say, the lower the degree of the protection afforded by peptides inserting into the lipid vesicles, the closer the slope will be to the control without lipid present. In contrast, if the lipid vesicles offer complete protection for the tryptophan residue, the slope of the line will be zero. The $K_{sv}$ values of each peptide with different lipid vesicles that were calculated based on the results in Figures 44 – 48 and 51 – 59 are shown in Tables 9-11 and the relative percentages of protection from acrylamide quenching were converted according to the following equation:

$$\%\text{protection} = \left(1 - \frac{K_{sv \text{ with lipid}}}{K_{sv \text{ no lipid}}}\right) \times 100\%$$

For W2-KL-9, nearly complete protection from quenching is afforded by lipid vesicles containing only anionic POPG. In contrast, very little protection from quenching was observed in the presence of POPC, 2:1 PEPG and 2:1 PCPG vesicles. When the ratio of the neutral phospholipid PE or PC to the negatively charged phospholipid PG was decreased to 1:1, however, the degree of the protection was comparable to that observed for pure POPG vesicles (Figures 43 – 44, Table 9).
Table 9

*K*<sub>sv</sub> Values and Relative Percentage of Protection from Acrylamide Quenching Effects of W2, Pn-KL-9 Family Peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>W2</th>
<th>W2P4</th>
<th>W2P6</th>
<th>W2P8</th>
<th>W2DP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>K&lt;sub&gt;sv&lt;/sub&gt;</td>
<td>0.0122</td>
<td>0.0147</td>
<td>0.0125</td>
<td>0.0152</td>
</tr>
<tr>
<td>Lipid</td>
<td>%Protection</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>POPG</td>
<td>K&lt;sub&gt;sv&lt;/sub&gt;</td>
<td>0.0016</td>
<td>0.0068</td>
<td>0.0067</td>
<td>0.0065</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>87</td>
<td>54</td>
<td>46</td>
<td>57</td>
</tr>
<tr>
<td>1:1</td>
<td>K&lt;sub&gt;sv&lt;/sub&gt;</td>
<td>0.0034</td>
<td>0.0085</td>
<td>0.0102</td>
<td>0.0098</td>
</tr>
<tr>
<td>PEPG</td>
<td>%Protection</td>
<td>72</td>
<td>42</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>1:1</td>
<td>K&lt;sub&gt;sv&lt;/sub&gt;</td>
<td>0.0029</td>
<td>0.0089</td>
<td>0.0102</td>
<td>0.0088</td>
</tr>
<tr>
<td>PCPG</td>
<td>%Protection</td>
<td>76</td>
<td>39</td>
<td>18</td>
<td>42</td>
</tr>
<tr>
<td>2:1</td>
<td>K&lt;sub&gt;sv&lt;/sub&gt;</td>
<td>0.0116</td>
<td>0.0120</td>
<td>0.0116</td>
<td>0.0150</td>
</tr>
<tr>
<td>PEPG</td>
<td>%Protection</td>
<td>5</td>
<td>18</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>2:1</td>
<td>K&lt;sub&gt;sv&lt;/sub&gt;</td>
<td>0.0110</td>
<td>0.0129</td>
<td>0.0115</td>
<td>0.0150</td>
</tr>
<tr>
<td>PCPG</td>
<td>%Protection</td>
<td>10</td>
<td>12</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>POPC</td>
<td>K&lt;sub&gt;sv&lt;/sub&gt;</td>
<td>0.0108</td>
<td>0.0135</td>
<td>0.0128</td>
<td>0.0154</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>11</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 43. Percentage of protection from acrylamide quenching effect of W2, Pn-KL-9 in the absence and presence of LUVs with defined lipid compositions.
Figure 44. Tryptophan fluorescence of W2-KL-9 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
For the L-proline-substituted analogs in the W2, Pn-KL-9 family, in the presence of POPC vesicles they showed similar results compared to that of their parent peptide W2-KL-9. However, the slope of $F_0/F$ vs. acrylamide concentration in the presence of POPG LUVs was significantly higher than that of W2-KL-9, indicating less binding of proline-containing peptides to POPG vesicles in comparison to W2-KL-9 (Table 9). With the 1:1 PEPG and 1:1 PCPG LUVs, the acrylamide quenching results of these analogs showed greater $K_{sv}$ values and lower degree of protection from acrylamide quenching compared to that in the presence of POPG LUV. When the ratio of the neutral lipid PE or PC to the negatively charged lipid PG was increased to 2:1, no significant changes in accessibility of peptide to the aqueous phase was observed compared to that in the no lipid environment. (Figure 43, Figure 45 – 47, Table 9).

A similar pattern of changes in the degree of protection from acrylamide quenching offered by lipid vesicles was observed for W2, D-P4-KL-9 in the presence of LUVs with varying lipid compositions (Figure 43, Figure 48 and Table 9), although with 2:1 mixtures W2, D-P4-KL-9 showed slightly higher percentages of protection than other proline-containing analogs. In general, the change in protection from acrylamide quenching of each W2, Pn-KL-9 peptide followed this order: neutral POPC < 2:1 mixtures < 1:1 mixture < anionic POPG. These results showed a relatively lower degree of protection for the analogs with each lipid vesicle compared to W2-KL-9, indicating that proline substitution in this family uniformly weakened the peptide-lipid binding and decreased the amount of peptides penetrating into the lipid bilayers.
Figure 45. Tryptophan fluorescence of W2, P4-KL-9 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 46. Tryptophan fluorescence of W2, P6-KL-9 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 47. Tryptophan fluorescence of W2, P8-KL-9 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 48. Tryptophan fluorescence of W2, D-P4-KL-9 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
For W4, Pn-KL-10 family peptides, the parent peptide W4-KL-10 showed almost identical results compared to the other parent peptide W2-KL-9 (Figure 43 – 44, Figure 49 – 51, Table 9 – 11); but among the W4, L-Pn-KL-10 analogs, strikingly different behaviors were observed. None of these analogs showed notable protection from acrylamide quenching in the presence of POPC, 2:1 PEPG or 2:1 PCPG LUVs, which were similar to their parent peptide, W4-KL-10. With 1:1 PEPG or 1:1 PCPG LUVs, the analogs with L-proline at position 2 or 10 afforded substantial protection from the quenching of acrylamide (although the degrees of protection were lower than those of W4-KL-10), whereas W4, P6-KL-10 and W4, P8-KL-10 were readily quenched. In the presence of lipid vesicles containing only POPG, all the analogs except W4, P8-KL-10 demonstrated similar degrees of protection (Figure 49, Figure 52 – 55, Table 10).

A similar pattern of changes in the degree of protection of peptides from acrylamide quenching was observed for the D-proline-substituted analogs in this family in the presence of LUVs with varying lipid compositions (Figure 50, Figure 56 – 59, Table 11). However, with anionic POPG vesicles, W4, D-P8-KL-10 showed a significantly lower degree of protection compared to W4-KL-10 and other analogs.

Generally, in the W4, Pn-KL-10 family, the analogs with proline at position 2 or 10 in the presence of 1:1 PEPG or 1:1 PCPG vesicles showed substantially less binding than the parent peptide W4-KL-10 but considerably more than the less antimicrobially active analogs with proline at position 6 or 8.
Table 10

$K_{sv}$ Values and Relative Percentage of Protection from Acrylamide Quenching Effects of W4, Pn-KL-10 Family Peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>W4</th>
<th>P2W4</th>
<th>W4P6</th>
<th>W4P8</th>
<th>W4P10</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>0.0126</td>
<td>0.0113</td>
<td>0.0124</td>
<td>0.0171</td>
<td>0.0144</td>
</tr>
<tr>
<td>Lipid</td>
<td>%Protection</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>POPG</td>
<td>$K_{sv}$</td>
<td>0.0021</td>
<td>0.0024</td>
<td>0.0030</td>
<td>0.0058</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>83</td>
<td>79</td>
<td>76</td>
<td>66</td>
</tr>
<tr>
<td>1:1</td>
<td>$K_{sv}$</td>
<td>0.0033</td>
<td>0.0062</td>
<td>0.0101</td>
<td>0.0156</td>
</tr>
<tr>
<td>PEPG</td>
<td>%Protection</td>
<td>74</td>
<td>45</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>1:1</td>
<td>$K_{sv}$</td>
<td>0.0027</td>
<td>0.0063</td>
<td>0.0101</td>
<td>0.0156</td>
</tr>
<tr>
<td>PCPG</td>
<td>%Protection</td>
<td>79</td>
<td>44</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>2:1</td>
<td>$K_{sv}$</td>
<td>0.0103</td>
<td>0.0108</td>
<td>0.0103</td>
<td>0.0161</td>
</tr>
<tr>
<td>PEPG</td>
<td>%Protection</td>
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<td>4</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>2:1</td>
<td>$K_{sv}$</td>
<td>0.0112</td>
<td>0.0100</td>
<td>0.0118</td>
<td>0.0167</td>
</tr>
<tr>
<td>PCPG</td>
<td>%Protection</td>
<td>11</td>
<td>12</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>POPC</td>
<td>$K_{sv}$</td>
<td>0.0122</td>
<td>0.0119</td>
<td>0.0117</td>
<td>0.0143</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>3</td>
<td>0</td>
<td>6</td>
<td>16</td>
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</table>
Figure 49. Percentage of protection from acrylamide quenching effect of W4, L-Pn-KL-10 in the absence and presence of LUVs with defined lipid compositions.
Table 11

<table>
<thead>
<tr>
<th>Peptides</th>
<th>$K_{sv}$</th>
<th>W4</th>
<th>DP2W4</th>
<th>W4DP6</th>
<th>W4DP8</th>
<th>W4DP10</th>
</tr>
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<tbody>
<tr>
<td>No</td>
<td></td>
<td>0.0126</td>
<td>0.0138</td>
<td>0.0128</td>
<td>0.0130</td>
<td>0.0142</td>
</tr>
<tr>
<td>Lipid</td>
<td>%Protection</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>POPG</td>
<td>$K_{sv}$</td>
<td>0.0021</td>
<td>0.0023</td>
<td>0.0024</td>
<td>0.0078</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>83</td>
<td>83</td>
<td>82</td>
<td>40</td>
<td>90</td>
</tr>
<tr>
<td>1:1 PEPG</td>
<td>$K_{sv}$</td>
<td>0.0033</td>
<td>0.0080</td>
<td>0.0098</td>
<td>0.0131</td>
<td>0.0096</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>74</td>
<td>42</td>
<td>23</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>1:1 PCPG</td>
<td>$K_{sv}$</td>
<td>0.0027</td>
<td>0.0082</td>
<td>0.0106</td>
<td>0.0125</td>
<td>0.0092</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>79</td>
<td>41</td>
<td>17</td>
<td>4</td>
<td>35</td>
</tr>
<tr>
<td>2:1 PEPG</td>
<td>$K_{sv}$</td>
<td>0.0103</td>
<td>0.0146</td>
<td>0.0120</td>
<td>0.0137</td>
<td>0.0146</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>18</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2:1 PCPG</td>
<td>$K_{sv}$</td>
<td>0.0112</td>
<td>0.0131</td>
<td>0.0113</td>
<td>0.0141</td>
<td>0.0137</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>11</td>
<td>5</td>
<td>12</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>POPC</td>
<td>$K_{sv}$</td>
<td>0.0122</td>
<td>0.0119</td>
<td>0.0107</td>
<td>0.0148</td>
<td>0.0166</td>
</tr>
<tr>
<td></td>
<td>%Protection</td>
<td>3</td>
<td>14</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 50. Percentage of protection from acrylamide quenching effect of W4, D-Pn-KL-10 in the absence and presence of LUVs with defined lipid compositions.
Figure 51. Tryptophan fluorescence of W4-KL-10 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 52. Tryptophan fluorescence of P2, W4-KL-10 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 53. Tryptophan fluorescence of W4, P6-KL-10 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 54. Tryptophan fluorescence of W4, P8-KL-10 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 55. Tryptophan fluorescence of W4, P10-KL-10 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 56. Tryptophan fluorescence of D-P2, W4-KL-10 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 57. Tryptophan fluorescence of W4, D-P6-KL-10 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 58. Tryptophan fluorescence of W4, D-P8-KL-10 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
Figure 59. Tryptophan fluorescence of W4, D-P10-KL-10 quenched by acrylamide in the absence and presence of LUVs with defined lipid compositions.
2. 10-DN Quenching of Tryptophan Emission Intensity

Since 10-DN partitions into the hydrophobic core of lipid bilayers, the penetration of the peptides into the lipid bilayers can be estimated by 10-DN quenching of tryptophan fluorescence (Caputo and London, 2003). Since 10-DN is localized within lipid vesicles, no 100% quenching control is possible as in the acrylamide quenching assay. The concentration of peptide and lipid were fixed at 10 µM and 100 µM, respectively, identical to the 10:1 lipid-to-peptide ratio that was used in the acrylamide quenching experiments. The 10-DN concentration was 5 mol% (for POPC and POPG vesicles) or 10 mol% (for POPC/POPG and POPE/POPG vesicles). The degree of tryptophan quenching by 10-DN was assessed by comparing the maximal fluorescence intensity value in the presence of quencher (F) to that in the absence of quencher (F₀) (Figure 60 – 62). Each column represents the average value of two measurements and the error bars are based on the standard deviations of these two measurements.

Theoretically, if the peptide penetrates deep into the hydrophobic region of the lipid bilayer, the tryptophan residue may be close enough to undergo collisional quenching with 10-DN, resulting in a reduction of fluorescence intensity. As the distance between tryptophan and 10-DN decreases (tryptophan resides closer to the center of lipid bilayers), more quenching should be observed.

In the W2, Pn-KL-9 family, none of the peptides were appreciably quenched by 10-DN with any of the LUVs except POPG. Interestingly four proline-containing analogs were quenched by 10-DN in the presence of POPG LUV compared to their parent peptide W2-KL-9 (Figure 60).
In the W4, Pn-KL-10 family, no changes in the F/F₀ value of the peptides were observed with any 10-DN-containing LUVs except POPG. All W4, Pn-KL-10 peptides were substantially quenched by 10-DN in the presence of POPG LUV including the parent peptide W4-KL-10 (Figure 61). It is surprising to note that the two analogs with high MIC values (W4, P6-KL-10 and W4, P8-KL-10) demonstrated greater quenching by 10-DN compared to the other three peptides. Similar results were observed with D-proline-substituted W4, Pn-KL-10 peptides in the presence of POPG LUVs (Figure 62).

Figure 60. Tryptophan fluorescence of W2, Pn-KL-9 quenched by 10-DN in the presence of LUVs with defined lipid compositions.
The results from the fluorescence experiments (tryptophan fluorescence intensity enhancement, acrylamide and 10-DN quenching) revealed differences in the peptide-lipid interactions among the proline-containing peptides. For W2, Pn-KL-9 peptides, the proline substitutions uniformly reduced the binding capabilities of the analogs but once effective binding occurred, the presence of proline appeared to promote the entry of the peptides into the hydrophobic core of the lipid bilayers in comparison to the parent peptide W2-KL-9.

More striking differences were observed for the W4, Pn-KL-10 peptides. Although the antimicrobial activity and binding capabilities of the analogs with the

Figure 61. Tryptophan fluorescence of W4, L-Pn-KL-10 quenched by 10-DN in the presence of LUVs with defined lipid compositions.
proline residue at position 6 or 8 were significantly compromised compared to their parent peptide W4-KL-10, these peptides are more susceptible to quenching by 10-DN in anionic POPG LUVs compared to W4-KL-10 and the P2/P10 or D-P2/D-P10 derivatives with potent antimicrobial activity. It is apparent that the W4, Pn-KL-10 peptides show a difference in both initial binding, and once bound, in location within the bilayer. These results are supported by earlier observations indicating the analogs with proline residue at position 6 or 8 were unable to induce leakage in both calcein-loaded LUVs and E. coli ML-35 membranes.

*Figure 62.* Tryptophan fluorescence of W4, D-Pn-KL-10 quenched by 10-DN in the presence of LUVs with defined lipid compositions.
H. Peptide Secondary Structure Determined by Circular Dichroism

Circular dichroism (CD) spectroscopy was used to determine the peptide conformation upon binding to lipid bilayers. Peptide concentration was fixed at 20 μM in all cases and the highest lipid concentration was 400 μM. CD spectra of W4, Pn-KL-10 and W2, Pn-KL-9 peptides in the absence and presence of LUVs with varying lipid compositions at the lipid-to-peptide ratio of 20:1 are shown in Figures 63 - 66.

Characteristic CD spectra of peptide secondary structure were described before (Chapter 2, Part L, and Figure 20). With buffer containing no lipid, the CD results showed random coil structure with a negative band below 200 nm for all the peptides including W2-KL-9 and W4-KL-10 (Figure 63). This is consistent with the design of our peptides: they were expected to adopt this random structure in aqueous solution.

![CD spectra of W2, Pn-KL-9 and W4, Pn-KL-10 in the absence of lipid.](image)

*Figure 63. CD spectra of W2, Pn-KL-9 and W4, Pn-KL-10 in the absence of lipid.*
For W2, Pn-KL-9 family peptides (Figure 64), with neutral POPC LUVs, the CD spectra also showed no discernible structure for any of these peptides. With negatively charged POPG LUVs, only the parent peptide W2-KL-9 demonstrated a β-sheet structure with a peak near 200 nm and a single minimum around 220 nm. The proline-containing analogs all exhibited random coiled structure that was not significantly different from the spectra of this group of peptides in buffer or with POPC LUV.

Most bacterial cell membranes contain a considerable amount of anionic phospholipids and thus POPG LUV crudely mimics the outer surface of bacterial cell membranes. In other words, POPG LUV provides a model that exhibits the maximal extent of secondary structure upon binding to the membrane surface. No significant β-sheet conformation with POPG LUV was observed for all four proline-containing analogs. Therefore, only W2-KL-9 and one analog, W2, P4-KL-9 were tested with lipid mixture vesicles.

With the lipid mixtures 1:1 PEPG and 1:1 PCPG, W2-KL-9 showed less β-sheet structure compared to POPG LUVs, whereas W2, P4-KL-9 exhibited no discernible structure (Figure 64).

In the presence of 2:1 PEPG and 2:1 PCPG, neither of these two peptides demonstrated any defined structure (Figure 64).
Figure 64. CD spectra of W2, Pn-KL-9 in the presence of LUVs with defined lipid compositions.
For W4, Pn-KL-10 peptides containing L-proline substitutions, with neutral POPC LUV, the CD spectra showed random coil structure for all of the peptides. With anionic POPG LUV, the parent peptide W4-KL-10 and one analog, P2, W4-KL-10 exhibited \(\beta\)-sheet structure, although the magnitude of the latter one was less than that of W4-KL-10. W4, P10-KL-10 showed some structure (non-random coil), but it cannot be defined as \(\beta\)-sheet conformation. The other two L-proline-containing analogs all exhibited random coil structure with POPG. In the presence of 1:1 PEPG and 1:1 PCPG LUV, W4-KL-10 showed a large maximum near 200 nm, although the signal was slightly weaker than that with POPG LUV. P2, W4-KL-10 adopted \(\beta\)-sheet structure but the character of \(\beta\)-sheet was substantially reduced compared to that with POPG LUV. In contrast, none of the other three derivatives showed any discernible structure and no clear differences were observed among these peptides (Figure 65).

With 2:1 PEPG and 2:1 PCPG LUVs, only W4-KL-10 and P2, W4-KL-10 were tested since the other analogs already showed no apparent structure in the 1:1 mixtures. The spectra of W4-KL-10 and P2, W4-KL-10 showed one single minimum below 200nm, indicating that they both were unstructured in the presence of these LUVs (Figure 65).

Similarly, the secondary structure of the D-proline-containing peptides was assessed using CD spectroscopy (Figure 66). An identical trend in the change of \(\beta\)-sheet signal with different lipid vesicles was observed for W4-KL-10 and W4, D-P2/D-P10-KL-10 as compared to the L-proline-containing analogs. The other two derivatives showed random coil structure with all four LUVs.
Figure 65. CD spectra of W4, L-Pn-KL-10 in the presence of LUVs with defined lipid compositions.
Figure 66. CD spectra of W4, D-Pn-KL-10 in the presence of LUVs with defined lipid compositions.
The proline-containing analogs possess a significantly different capability to form β-sheet structure compared to their parent peptides.

In the W2, Pn-KL-9 family, the proline substitution uniformly caused the analogs to lose β-sheet-forming potential with any lipid vesicles. These analogs showed much higher MIC values compared to their parent peptide, W2-KL-9, in the antimicrobial potency assay.

In the W4, Pn-KL-10 family, the analog with a proline residue at position 2 showed a reasonable ability to form β-sheet structure with POPG and 2:1 lipid mixtures in comparison to W4-KL-10 while the analog with a proline residue at position 10 also exhibited much less structure. These analogs actually showed reasonable MIC values compared to W4-KL-10 in the antimicrobial potency assay. The analogs with a proline at position 6 or 8 lost the ability to form β-sheet structure and failed to demonstrate antimicrobial potency compared to P2/D-P2 or P10/D-P10 analogs.

These results indicated that the β-sheet structure appeared to be linked to antimicrobial activity for these KL-repeat-containing peptides and the location of proline in the peptide primary sequence has a significant influence on the β-sheet-forming ability of 10-mers. When proline is close to the N-terminus of 10-mer, the β-sheet structure was only moderately perturbed. When proline is close to the C-terminus of 10-mer, the β-sheet structure was significantly perturbed. When proline is placed in the middle of the sequence of 10-mers, the β-sheet structure was completely disrupted. However, this proline-positional dependence of β-sheet-forming ability was not observed in the analogs of the 9-mers.
I. Measurement of Percent Bacterial Killing by Peptide using The Live vs. Dead Bacterial Viability Assay

In the assessment of antimicrobial activity of our proline-containing peptides, the minimum peptide concentration to completely inhibit the growth of bacteria was determined. However, the peptides may inhibit growth without actually killing bacterial cells. Therefore, in this section, bacterial cell killing by the antimicrobially active analogs among W4, Pn-KL-10 family peptides was measured using the LIVE/DEAD® BacLight™ bacterial viability assay (see method described previously in Chapter 2, Part M). The average values and standard deviations for duplicate measurements are shown in Figures 67 – 69. The third column from the left in each data set corresponds to the MIC value for each peptide. The Gram-positive bacteria strain *Staphylococcus aureus* (*S. aureus*, ATCC 29213) and the Gram-negative strains *Escherichia coli* (*E. coli*, ATCC 25922) and *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853) were tested in this assay.

For the Gram-negative strain *E. coli*, it is clear that the proline-containing analogs demonstrated different killing behaviors compared to their parent peptide W4-KL-10 although they all shared similar MIC values (Figure 67). At 4x MIC, these peptides all showed a high percentage of killing, ranging from 70% - 85% of the entire *E. coli* population. As the peptide concentration decreased to 2x MIC, the killing percentages of the four proline-analogs were significantly reduced to below 60% while W4-KL-10 was able to maintain a relatively high percentage of killing around 75%. At MIC, W4-KL-10 caused nearly 70% killing whereas the percentage of killing of the analogs was further
decreased. It is intriguing to note that W4-KL-10 and P2/D-P2 analogs shared identical MIC values (2 µg/ml), but their killing behaviors at this concentration were significantly different. As the peptide concentration further decreased, obviously the efficacy of peptides to kill bacteria faded away including the parent peptide W4-KL-10, especially at low peptide concentrations such as 0.1x and 0.05x MIC. However, at 0.5x MIC, W4-KL-10 still showed a substantial killing percentage around 60%.

Figure 67. Percentage of killing of *E. coli* by active W4, Pn-KL-10 analogs using live vs. dead bacterial viability assay.
For the other Gram-negative strain *P. aeruginosa*, that was tested, the proline-containing analogs showed a similar percentage of killing at both 4x and 2x MIC, whereas W4-KL-10 showed a significantly lower percentage of killing at 2x MIC compared to that at 4x MIC (~30% lower). At MIC, the analogs exhibited a considerably higher percentage of killing compared to that of W4-KL-10, which was opposite to the observation for *E. coli*. As the peptide concentration decreased, the killing potency of the analogs faded away quickly, except for W4, P10-KL-10 at 0.5x MIC (near 50% of killing) (Figure 68).

*Figure 68. Percentage of killing of *P. aeruginosa* by active W4, Pn-KL-10 analogs using live vs. dead bacterial viability assay.*
In the case of the Gram-positive strain *S. aureus*, at 4x MIC the two analogs with proline at position 10 demonstrated lower percentages of killing compared to those of W4-KL-10 and P2/D-P2 analogs (Figure 69). These results were unusual compared to the observations from the above two Gram-negative strains, i.e., the latter showed comparable percentages for all peptides at the highest 4x MIC. The killing potency of the analogs dramatically faded away as the peptide concentration decreased from 4x MIC with the magnitude of decrease between peptide concentrations substantially greater than that in the case of *P. aeruginosa*. However, in the comparison of *S. aureus* and *P. aeruginosa*, W4-KL-10 appeared to act in a similar way as the peptide concentration changes.

![Figure 69: Percentage of killing of *S. aureus* by active W4, Pn-KL-10 analogs using live vs. dead bacterial viability assay.](image)
These results provided insight into the actual bacterial killing by peptides at different concentrations. At the minimum inhibitory concentration, our peptides appeared to kill a considerable proportion of bacterial cells, but the percentage of killing is not necessarily close to 100%. In other words, it is clear that at the MIC, the peptides are not actually killing the entire bacterial population.

The percentage of bacterial killing by our proline-containing analogs was not necessarily consistent with that of the parent peptide W4-KL-10, even though they shared similar MIC values. Given the diversity of the composition and structure of bacterial cell membranes, it is difficult to compare and determine whether the parent peptide W4-KL-10 or our analogs are more potent in general to kill bacterial cells. However, we are able to conclude that the antimicrobially active proline-containing peptides can retain reasonable killing rates at their MICs compared to W4-KL-10.

J. Determination of the Inhibitory Effects of Peptides on Bacterial Biofilm Formation

As described in the introduction (Chapter 1, Part F), bacterial biofilms can be produced by almost all the bacterial strains and can greatly increase the resistance of bacteria toward antimicrobial agents. Therefore, in addition to the ability to kill or inhibit the growth of planktonic microorganisms, the potential inhibitory effects of the proline-containing analogs on the formation of bacterial biofilms were assessed.

This assay was performed according to the previous description (Chapter 2, Part O) and *Pseudomonas aeruginosa* (ATCC 27853) was tested with the four antimicrobially active analogs in the W4, Pn-KL-10 family. The results are presented as a function of absorbance of crystal violet stain on the biofilm vs. the peptide concentration. The
average values and standard deviations for duplicate absorbance measurements are shown in Figure 70. The first column on the left represents the absorbance of untreated biofilm and the fourth column from the left in each data set corresponds to the peptide concentration at its MIC. Higher absorbance values indicate a greater biofilm density. Lower absorbance values indicate a greater inhibitory effect on biofilm formation.

*Figure 70.* Absorbance of crystal violet stained *P. aeruginosa* biofilm treated by active W4, Pn-KL-10 family peptides.

At sub-MICs, the parent peptide, W4-KL-10, demonstrated some inhibitory effect on biofilm formation. At higher concentrations, biofilm density was reduced by about
70%. In contrast, none of the proline-containing analogs showed significant ability to inhibit biofilm formation at concentrations below the MIC. At MIC, where W4-KL-10 reduced biofilm by about 50%, biofilms treated by the proline-containing analogs were unaffected. Substantial reduction was only observed at concentrations much greater than the MIC values of the peptides.

The results indicate that the proline-containing analogs were less able to inhibit bacterial biofilm formation compared to W4-KL-10. As the peptide concentration increased, however, the inhibitory effect on biofilm formation of the proline-containing analogs was evident. Compared to the killing rates in the previous section, it appears that biofilm formation inhibits the action of the proline-containing peptides.

K. Determination of Antimicrobial Efficacy of Peptides toward Bacterial Cells within Established Bacterial Biofilm

Once the biofilm structure was established by bacteria, the effectiveness of the peptides to kill or inhibit the growth of the bacterial cells inside the biofilm may decrease compared to their antimicrobial activity that was determined against planktonic states of bacteria. Therefore, the antimicrobial efficacy of the active proline-containing peptides in the W4, Pn-KL-10 family was tested against the bacterial cells inside established bacterial biofilms. Bacterial biofilms of *Staphylococcus hominis* + (*S. hominis* +, ATCC 25615), *Staphylococcus hominis* – (*S. hominis* –, ATCC 27844), *Staphylococcus warneri* + (*S. warneri* +, ATCC 17917) and *Staphylococcus warneri* - (*S. warneri* -, ATCC 49518) were formed and tested with our peptides. These strains are closely associated with biofilm formation in hospital-acquired infections (Chapter 2, Part B).
The results were evaluated as a function of the number of bacterial colonies counted after peptide treatment vs. the peptide concentration. Average values and standard deviations for duplicates are shown in Figures 71 - 78. The single column on the left represents the bacterial colony number inside normally grown biofilm without peptide. The larger the value, the more bacterial cells have survived the peptide treatment, which indicates the lower extent of antimicrobial effectiveness of peptides toward bacterial cells inside biofilms. Incubation times of 2 hours and 24 hours for biofilm establishment were used in this assay.

For biofilms with 2 hours of incubation, the bacterial colony number of all four strains began to moderately decrease at sub-MICs of W4-KL-10 and all antimicrobially active analogs (Figure 71 - 74). At high peptide concentrations of 16, 32 and 64 µg/ml, colony numbers close to zero were observed.

For biofilms with 24 hours of incubation, however, the total colony number of the 100% control greatly increased except for *S. warneri* -, and the concentration for peptides necessary to cause notable decreases in bacterial colony numbers were over their MICs for all four strains (Figures 75 - 78).
**Figure 71.** Number of *S. hominis* + colonies counted within 2-hour-biofilm after W4, Pn-KL-10 peptide treatment.

**Figure 72.** Number of *S. hominis* - colonies counted within 2-hour-biofilm after W4, Pn-KL-10 peptide treatment.
Figure 73. Number of *S. warneri* + colonies counted within 2-hour-biofilm after W4, Pn-KL-10 peptide treatment.

Figure 74. Number of *S. warneri* - colonies counted within 2-hour-biofilm after W4, Pn-KL-10 peptide treatment.
Figure 75. Number of *S. hominis* + colonies counted within 24-hour-biofilm after W4, Pn-KL-10 peptide treatment.

Figure 76. Number of *S. hominis* - colonies counted within 24-hour-biofilm after W4, Pn-KL-10 peptide treatment.
Figure 77. Number of *S. warneri* + colonies counted within 24-hour-biofilm after W4, Pn-KL-10 peptide treatment.

Figure 78. Number of *S. warneri* - colonies counted within 24-hour-biofilm after W4, Pn-KL-10 peptide treatment.
In order to evaluate the antimicrobial efficacy of these analogs against the bacterial cells inside biofilms in a more comparative way, the results for each peptide at their MIC were converted to decrease % of colony number based on the 100% control in each strain, and the percentages are shown in Figure 79 - 80. Generally, for all tested strains, the percentage of decrease with the 24-hour-biofilms was universally lower than that in the 2-hour-biofilms, which indicated that 24-hour-biofilms provided more protection to bacteria from antimicrobial peptides. The proline-containing analogs with reasonable antimicrobial activity also showed a comparable potency to decrease the colony number at their MICs in comparison to W4-KL-10, in most cases. Although some variations were observed, we can conclude that the analogs with proline at position 2 or 10 were able to retain the antimicrobial efficacy of W4-KL-10 toward bacterial cells within established bacterial biofilms.

The bacterial strains *S. hominis* + and *S. warneri* + can generate polyglutamic acids (PGA) and, theoretically, they may have lower susceptibility to antimicrobial agents, whereas the other two strains *S. hominis* - and *S. warneri* - lack this property (Otto, 2006). However, the observations from 24-hour-biofilms between *S. hominis* + and *S. hominis* – were not consistent with this hypothesis.
Figure 79. Percent decrease of colony numbers within 2-hour-biofilm treated by W4, Pn-KL-10 peptides at MIC.

Figure 80. Percent decrease of colony numbers within 24-hour-biofilm treated by W4, Pn-KL-10 peptides at MIC.
CHAPTER 4: CONCLUSIONS

In this project, two groups of peptides, W4, Pn-KL-10 and W2, Pn-KL-9 were designed to investigate the structural and functional effects of single proline substitution on the antimicrobial activity and selectivity between bacterial and mammalian cells of small linear cationic antimicrobial peptides with amphipathic character as β-sheets.

Proline substitution was confirmed in this project to be a promising modification of antimicrobial peptides to decrease the cytolytic activity toward mammalian cells. In both W4, Pn-KL-10 and W2, Pn-KL-9 families, proline replacement uniformly reduced the hemolytic activity of the analogs toward human red blood cells to a negligible level, which was significantly lower than that of the parent peptides, W4-KL-10 and W2-KL-9.

In the W4, Pn-KL-10 family, the single leucine-to-proline substitution at position 2 or 10 retained reasonable antimicrobial activity in comparison to W4-KL-10. As a result, four antimicrobially active analogs with low hemolytic activity were identified:

- P2, W4-KL-10 and D-P2, W4-KL-10 (P2 and D-P2)
- W4, P10-KL-10 and W4, D-P10-KL-10 (P10 and D-P10)

These proline-containing analogs (especially P2 and D-P2) showed MIC values near 1 µg/ml against various tested bacterial strains, which was close to our target of MIC values close to 1 µg/ml with no hemolytic activity at 100x MIC. In addition, these analogs demonstrated substantial inhibitory effects on bacterial biofilm formation and in attacking established biofilms. Therefore, these analogs are promising candidates for further modifications as therapeutic antimicrobial agents.
In the W4, Pn-KL-10 family, the analogs with L- or D-proline residue at position 6 or 8 showed low antimicrobial and lytic activity, and an inability to form β-sheet structure. As described earlier (Chapter 2, Part A), the structure of β-sheet-forming peptides is dependent upon intermolecular bonding between peptide monomers and other peptide-peptide or peptide-membrane interactions. Therefore, these findings suggest that proline substitutions at different positions in the sequence of the peptide can generate a variety of structural perturbations that could influence peptide function at different levels. Apparently for KL-repeat containing peptides with 10 amino acids, proline substitutions in the middle of the primary sequence perturb the amphipathic β-sheet structure significantly (W4, P6/D-P6-KL-10 and W4, P8/D-P8-KL-10 actually lost the ability to form β-sheet structure even with purely anionic POPG vesicles), while proline substitutions close to the N- or C-terminus induce more moderate alterations in β-sheet formation, which allows for reasonable antimicrobial activity comparable to the parent peptide, W4-KL-10. These results also suggest that the activity of these proline-containing peptides were position-dependent but not stereochemically specific, which was supported by similar observations in peptide activity changes of D-proline substituted analogs.

An increasing amount of evidence suggests that small cationic peptides may achieve their antimicrobial activity through mechanisms other than simply membrane permeabilization. In this project, the four active analogs demonstrated substantially different properties compared to the parent peptide, W4-KL-10, in leakage-inducing experiments, tryptophan fluorescence enhancement and quenching experiments, and CD
spectroscopy, while showing only minor differences in antimicrobial potency. These results suggested that it is likely that these peptides may kill or inhibit the growth of microorganisms via multiple mechanisms of action.

In summary, the results in this project reveal the structural and functional impact caused by proline substitutions on linear cationic amphipathic β-sheet-forming peptides and provide guidance for further modification of this class of antimicrobial peptides.
CHAPTER 5: FUTURE WORK

Although our proline-containing peptides were screened for antimicrobial and hemolytic activity, their potential ability in modulating the innate immune response has not been assessed. Further experiments can be performed with various types of cells from the human immune system to test the effects of the peptides in real biological environments.

It is widely accepted that proline incorporation can introduce a kink region in the helical structure of α-helix-forming peptides. In contrast, only limited experimental data about the local structure has been collected for proline-containing, β-sheet-forming peptides. To improve our understanding of the exact nature of local distortions caused by proline substitution in the β-sheet backbone, more structural studies could be pursued in the future. Answers to the above questions may also reveal the orientation and position of peptides in lipid bilayers upon binding to lipid vesicles or cell membranes, and provide insights into the mechanisms of action other than membrane permeabilization.

The antimicrobial and the biofilm-formation inhibitory activity of peptides are not necessarily overlapped; that is to say, some peptides with weak antimicrobial potency may act efficiently against bacterial biofilm formation. In addition to the antimicrobially active analogs that were tested in this project, other proline-containing derivatives might be tested against bacterial biofilms to assess their potential anti-biofilm ability.
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