Chapter 1

Antimicrobial Peptide
Chapter 1.1

Antimicrobial Peptide: Introduction
1.1.1 Antibiotic resistance and emergence of Antimicrobial peptides (AMPs):

The past five decades have witnessed the “antibiotic era” in which numerous natural, semisynthetic, and synthetic antibacterial drugs have been discovered with great achievement against life-threatening diseases.\[^1\] Penicillin was the first antibiotics and was discovered by Scottish scientist Alexander Fleming in 1928.\[^2\] However, the successful use of penicillin as a therapeutic agent was established only in 1940.\[^2\] But preceding its introduction, penicillinase synthesizing bacteria were reported in the 1940s.\[^2\] In the same manner, resistant strains of bacteria to sulfonamides and streptomycin were reported soon after its introduction as effective therapeutic agents.\[^3\] The number of multiple drugs resistant (MDR) bacteria was found to increase steadily with a decrease in the number of antibiotics being permitted by FDA (Figure 1).\[^4\]

![Figure 1. Accelerated increase in the existence of drug-resistant bacteria and creeping decrease in the approval of drugs by FDA (U.S.A). Taken from Ref\[^4\]\](image)

The growing emergence of these multi-drug resistant (MDR) bacterial strains has introduced the concept of membrane-perturbing cationic antimicrobial peptides (cAMP).\[^5\] The innovative discoveries of Melittin, Cecropins, Defensins, Magainins flourished as the new expectation of relief to the world.\[^6\]-\[^8\] These host defense antimicrobial peptides (AMPs) can be considered as a potential unique group of antibacterial agents because of their evolutionarily conserved role in innate immunity.\[^5, 9\] Since AMPs kill bacteria quickly by the physical disruption of cell membranes, they may not face the rapid emergence of resistance which traditional antibiotics face.\[^9\] They contain a cluster of positively charged amino acids (Lysine and Arginine) along with hydrophobic and aromatic residues to maintain amphipathicity. Hence at physiological pH, AMP imparts a net positive charge that helps the peptide to get attracted towards negatively charged bacterial membranes.

1.1.2 Key Characteristics of AMP that affect antimicrobial activity and specificity:

- Present in all forms of life (Plants, Insects, Animals)
- Short peptides (~10-40 amino acid long)
- Amphipathic in nature with a net positive charge
- A broad spectrum of activity against bacteria, fungi, parasites, Protozoa, cancer cells, etc.
Diverse mechanism of action
Neutralize endotoxins

1.1.3 Structural diversity of AMPs:

The AMPs are structurally divergent that they can be categorized based on the structures they adopt upon membrane interaction investigated by mostly solid and solution state NMR studies (Figure 2). The most prominent categories are described in Figure 2 and Table 1:

![Figure 2. An overview of the major structural classes of antimicrobial peptides (AMPs). (a) α-Helical Peptides, (b) β-sheet peptides and (c) extended peptides. Positively charged side chains are colored in blue, negatively charged side chains in red and remaining side chains in grey. PDB IDs: magainin 2, 2MAG; LL-37, 2K60; bovine lactoferricin, 1LFC; protegrin 1, 1PG1; human β-defensin-3, 1KJ5; tritrpticin, 1D6X; indolicidin, 1G89. Taken from Ref10]

<table>
<thead>
<tr>
<th>AMP(s)</th>
<th>Structure</th>
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<tr>
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<td>Linear cationic α-helical</td>
<td>Insects</td>
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<tr>
<td>HNP-1 (α-defensin), HBD-2 (β-defensin), Protegrin</td>
<td>β-sheet peptides</td>
<td>Human</td>
</tr>
<tr>
<td>Indolicidin, Tritrpticin</td>
<td>Extended Conformation</td>
<td>Cattle</td>
</tr>
</tbody>
</table>
1.1.4 Mode of action of AMPs in membrane:

Due to their amphipathic features, AMPs can interact and invade into cytosolic membranes of microorganisms by one or several mechanisms, e.g., barrel stave, toroidal pore, or carpeting which leads to cell lysis which is described below ((Figure 3).[^11][^12]

![Figure 3. Different modes of action of AMPs. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue. Taken from Ref [11, 12]](image)

**Carpet model:** In this model, the peptides disrupt the membrane by orienting parallel to the surface of the lipid bilayer and creating an extensive carpet-like layer. Examples: Dermaseptin, cecropin, melittin, etc.

**Toroidal Model:** In this model the attached peptides aggregate and induce the lipid monolayers to bend continuously through the pore so that both the inserted peptides and the lipid head groups line the water core. Examples: Magainin, MSI-78.

**Barrel-stave model:** In this model, the attached peptides aggregate and insert into the membrane bilayer so that the hydrophobic peptide regions align with the lipid core region and the hydrophilic peptide regions constitute the interior region of the pore. Examples: Alamethicin

1.1.5 Intracellular models of antimicrobial peptide killing and lysis

In early AMP studies, permeabilization of the bacterial cell membrane by AMP was thought as the primary mechanism of killing. So bacterial membrane was thought to be the primary target for AMPs. It was proposed that AMPs should be used at high concentrations so that they can destroy microorganisms by disturbing the membrane with sufficient channels and pores. However, extensive research shows that some AMPs were found to induce membrane permeabilization at concentrations lower than their MICs. Those AMPs can kill their target cells without causing membrane disruption indicates that those peptides have other intracellular targets such DNA, RNA, nuclear protein, etc. as shown below in Figure 4.
Figure 4. Mode of action for intracellular antimicrobial peptide activity. In this figure, *Escherichia coli* is shown as the target microorganism. Taken from Ref\cite{11}

Examples:
Mersacidin: Inhibit cell wall synthesis. Buforin II and tachyplesin: Binds nucleic acid
Pleurocidin, dermaseptin, PR-39, HNP-1, and indolicidin: Inhibit nucleic acid and protein synthesis
Seminalplasmin inhibits RNA polymerase and can stop RNA synthesis

1.1.6 Membrane composition:

Bacteria can be categorized as Gram-negative and Gram-positive bacteria depending on its cell wall membrane composition (Figure 5).

Figure 5: Outline of the Gram-positive (A) and Gram-negative (B) membrane cell wall. Taken from Ref\cite{13}
Gram-positive bacteria have one plasma membrane protected by a thick layer of cross-linked peptidoglycan which is penetrated by lipoteichoic acid and anchors the negatively charged teichoic acids which altogether contributes to the rigidity of the cell wall. In contrary, Gram-negative bacteria have both an outer and inner membrane divided by the periplasmic space where the thin peptidoglycan layer is present. Furthermore, the outer membrane holds porins and the negatively charged lipopolysaccharide (LPS) protruding from the membrane. The chemical structure of peptidoglycan is essentially the same for both gram-positive and gram-negative bacteria, but the layer is much thicker in Gram-positive bacteria where it can extend up to 80 nm in comparison to around 5 nm in most gram-negative bacteria.

1.1.7 Bacterial LPS: A potential target for AMP

Lipopolysaccharide (LPS) or endotoxin constitutes the outer leaflet of the outer membrane of the Gram-negative bacterial cell wall. LPS is composed of three distinct units: the hydrophobic conserved hexa-acylated lipid A moiety; the variable polysaccharide domain known as O-antigen and the core oligosaccharide section that covalently connects the two parts (Figure 6).[2] It has been shown that the negatively charged Lipopolysaccharide (LPS) coating of the outer membrane of Gram-negative bacteria prevents translocation of AMPs.[14] To achieve accessibility to the plasma membrane, AMPs should overcome the LPS permeability barrier first.

![Figure 6. A schematic view of LPS showing different domains (panel A) and a ball and stick representation of LPS molecule containing lipid A and inner core sugar (panel B). The acyl chains are shown as sticks whereas phosphate groups are depicted as a ball (panel B).](image)
Importantly, once released from dead bacteria or bacteria in the division, LPS forms micelles, which are the active form of the endotoxin. Initially, LPS is released into blood and binds to LPS binding protein (LBP). Next, it is carried to CD14 receptors present on the exterior of the immune cell membrane. CD14 takes LPS to myeloid differentiation protein-2 (MD2) that is associated with a toll-like receptor protein, TLR4. TLR4 upon binding to LPS activates the signaling cascade responsible for the cytokine burst which includes TNFα, IL-6, IL-8, and IL-1. High levels of TNF-α due to a prolonged stimulation of immune cells can cause the emergence of septic shock syndrome, in most serious cases (Figure 7). The six acyl chains and the phosphate head groups of the lipid A moiety of LPS are extremely crucial for inducing septic shock. A study conducted in the United States alone showed a steady increase in deaths associated with sepsis reaching a striking annual average mortality rate of 120 000. Thus, LPS establishes itself as a prime target for pharmacological intervention for the advancement of active antibacterial and antisepsis drugs. Thus, several AMPs having LPS binding and neutralizing capability would afford an elementary phase for the improvement of antibacterial drugs [17]. Therefore, high-resolution structural studies of AMPs in LPS in extremely significant to understand their activity and design next generation antibiotics and antisepsis candidates with reduced cytotoxicity and hemolytic property.

Figure 7. A schematic representation of LPS recognition by its receptor proteins LBP, CD14, MD2 and TLR4 leading to activation of the signaling cascade of macrophage cell. Taken from Ref[15]
1.1.8 Structural Elucidation of AMPs bound to LPS by trNOESY and STD NMR:

Transferred nuclear overhauser effect spectroscopy (trNOESY) is a robust technique to determine the three-dimensional structure of the ligands bound to the macromolecular receptor.\[16, 17\]

Let us consider a system where a ligand (L) binds to a receptor (P) to form a complex (PL).

\[ [P] + [L] \xrightarrow{K_{on}} [PL] \xleftarrow{K_{off}} \]

The equilibrium dissociation constant (K_D) and association constant (K_A) for this reaction is:

\[ K_D = \frac{K_{off}}{K_{on}} = \frac{[P] \times [L]}{[PL]} = \frac{1}{K_A} \]

where K_on and K_off are the rate constants of forward and backward process respectively.

In trNOESY experiment free and the bound form of ligand undergo chemical exchange, so that the bound ligand, when released from its binding site, retains its bound conformation for a particular period giving rise to intramolecular NOE constraints of the bound form (Figure 8).\[17\]

As a result, the measured transferred NOE (trNOE) can be used to determine the three-dimensional structure of the ligand in the bound state with a dissociation constant (K_D) in the range of micromolar to millimolar. The intramolecular trNOEs, which occurs at distances shorter than 5 Å, are key in defining the bound ligand conformation, whereas the intermolecular trNOE allows determining the orientation of bound ligand in the macromolecular binding site.

![Figure 8. Schematic illustration of a receptor-ligand complex [LP] in dynamic exchange with a large excess of the free ligand (L). Protons A and B exchange magnetization in the bound ligand but measurements are made in the free state with magnetization transfers achieved through combinations of chemical exchange and cross-relaxation mechanisms. The unbound protein molecules [P] are not shown here since the protein is saturated by a large excess of the ligand ([L]/[P] > 10-50) with a reasonable binding affinity (K_D = 10^{-3}-10^{-6} M) for the receptor-ligand complex. Taken from Ref\[18\]](image)

It is noteworthy to mention that, due to its high molecular weight of LPS micelles, transferred nuclear Overhauser effect spectroscopy (tr-NOESY) is an attractive strategy to determine 3D structures of AMPs bound to LPS.\[18-20\] However due to very low critical micelles
constant of LPS (CMC~1-2 µM) intermolecular NOE cross peaks can not be observed in the peptide-LPS complex.\cite{20}

On the other hand, Saturation Transfer Difference (STD) NMR, which also works almost on the same principle as tr-NOESY, is a widely used technique to identify the important groups or residues of a ligand molecule that are in close vicinity to the macromolecule.\cite{21, 22} In a typical STD NMR experiments, receptor: ligand molar ratio should be at least 1:100.\cite{23} At first, selective saturation of the receptor/protein ($t_{\text{sat}}$) is achieved at a frequency where no ligand signals are present which is either at the up-field methyl region ($\sim$ -1 ppm) or the down-field aromatic region ($\sim$7.0 ppm). Then with the help of spin diffusion, the saturation spreads in the whole receptor/protein (P*) magnetization. Next, the saturation also transfers to the binding ligands (L*) during the residence time ($t_{\text{res}}$) in the receptor-binding site. The acquired spectrum is known as the “on resonance” spectrum and the signal intensities are represented as $I_{\text{sat}}$. In next experiment, the irradiation frequency is set to a value where neither protein nor the ligand resonance signals is present, e.g., 40 ppm. The outcome is a normal NMR spectra of the ligand which is denoted as the “off resonance” spectrum with intensity $I_{\text{o}}$. Subtraction of these two spectra (on-resonance-off-resonance) by phase cycling leads to a difference spectra of signal intensities, ($I_{\text{sat}} – I_{\text{o}}$) called STD NMR spectrum (shown by gray background). In this spectra, the observed signals of the ligand proton are due to the transfer of saturation from receptor to ligand. It is also noteworthy to mention that based on the variance in the proximity of different atoms of ligand in receptor pocket group epitope mapping (GEM) can be performed from STD NMR data using differential intensity of ligand NMR signals.\cite{23}

Saturation Transfer Difference (STD) NMR method has been employed to identify key residues of AMPs that are in close vicinity in LPS micelles.\cite{21} This method utilizes group epitope mapping of several AMPs in the context of LPS to design optimized engineered AMPs. Therefore, the collective application of tr-NOESY and STD-NMR methods delivers a comprehensive representation of the atomic resolution structures and interacting residues of AMPs in the context of LPS micelles.\cite{14, 19}
1.1.9 References:

Chapter 1.2

Mechanistic Insights of Lipopolysaccharide Permeabilization and Antimicrobial Activity of Lactoferrampin (WR17)

This chapter was adapted from the following publication: A. Ghosh, A. Datta, J. Jana, R. K. Kar, C. Chatterjee, S. Chatterjee, A. Bhunia. (2014) Mol. BioSyst. 10, 1596-1612.
1.2.1 Introduction:

The World Health Organization (WHO) has recently raised serious concerns about the increasing worldwide spread of microbes that are resistant to the available anti-infective agents. However, despite that multi-drug resistant bacteria or superbugs have become a global life threat, the US Food and Drug Administration (FDA) has approved only three new antibiotics in the recent years. Many of the pathogenic Gram-negative bacterial strains like Pseudomonas aeruginosa, Klebsiella pneumoniae etc. have developed resistance to a variety of antibiotics rendering their bactericidal activity totally ineffective.[1, 2] Lipopolysaccharide (LPS), a glycolipid component present in the outer leaflet of the outer membrane of gram-negative bacteria plays an essential role in bacterial infections affecting human health.[3] Agents which can sequester and neutralize LPS would prove to be an invaluable therapeutic candidate.[4] LPS presents itself as an impermeable barrier in bacterial membrane, protecting them from a hoard of antibacterial agents and subsequently contribute to septicemia or endotoxic shock in human and animals.[5] Thus, LPS establishes itself as a prime target for pharmacological intervention towards the development of effective antibacterial and anti-sepsis drug.

In this context, cationic antimicrobial peptides (AMPs) which can lyse the bacterial membrane, are being extensively studied from the structure-function point of view, to gain insights into their mechanism of action. AMPs have been studied for a long time as a potential alternative to the conventional antibiotics due to their significant prevalence in nature and generalized mode of action, against micro-organisms.

In the present study, the interaction of bovine lactoferrampin (hereafter denoted as WR17) (Figure 1A) with LPS using various biophysical techniques like Circular Dichroism, fluorescence, isothermal titration calorimetry (ITC), dynamic light scattering and high-resolution NMR spectroscopy in conjunction with MD simulation was performed to provide structural insights into its mechanism of action. Further, investigation of the structure-activity relationships by designing shorter peptide analogs from WR17, based on its sequence (Figure 1B) to obtain residue-specific information was done. At the outset, the spectroscopic study yields not only the details of the binding phenomenon but also hints at the importance of the residues responsible for its noted activity.

1.2.2 Results and discussion:

1.2.2.1 Designing of peptides

The three-dimensional structure of the protein, lactoferrin, consists of several α-helices, β-sheets and β-turn or loop (Figure 1A).[6] The three-dimensional solution structure of lactoferrampin (WR17) (residues 268-284) (Figure 1B) or its analogues including longer version of lactoferrampin (residues 265-284) in the presence of perdeuterated lipid micelles such as zwitterionic DPC or negatively charged SDS clearly shows that the N-terminal part of WR17 (residues Trp1-Phe11) adopts a α-helical conformation while the final 6-residues, Gly12-Arg17, at the C-terminus region remains unstructured.[7] The flexible C-terminal end of WR17 is cationic in nature and is vital for antibacterial activity of the peptide. Structurally, the aromatic ring of Phe11 orients itself in the same plane as that of the indole ring of Trp1 which serves as an anchor for the insertion into lipid bilayer.[8] Solid-state NMR experiment in conjunction with MD simulation confirms the tilt angle of the N-terminal helix to be 45° with respect to the bilayer, which in turn facilitates the penetration
of the peptide into the lipid bilayer.\textsuperscript{[9]} Several other biophysical techniques such as fluorescence, differential scanning calorimetry (DSC) etc. were used to understand the mechanism of action of this peptide and its analogues in multilamellar vesicles. However, it is necessary to understand the high resolution structure of this peptide in the context of LPS because the AMPs interact with the outer membrane components first before gaining access to the inner membrane.\textsuperscript{[10]}

\textbf{1.2.2.2 Antimicrobial activity assay}

The antimicrobial activity of WR17 and its truncated analogues against Gram-negative \textit{P. aeruginosa ATCC 27853}, \textit{Xanthomonas campestris pv campestris} and Gram-positive \textit{Bacillus subtilis} were studied. WR17 inhibited \textit{P. aeruginosa}, \textit{X. campestris}, and \textit{B. subtilis} at 9.5, 10 and 20 µM, respectively. On the other hand, among the truncated analogues, only WG12 showed antimicrobial activity against \textit{X. campestris} at 75 µM. All the other analogues showed no activity against any of the pathogens tested.
1.2.2.3 Outer Membrane permeabilization assays through NPN dye uptake

Primarily, to understand whether WR17 or its analogues can permeabilize the *E.coli* cells, using 1-N-phenylnaphthylamine (NPN) dye uptake assay was performed.\(^{[11]}\) The fluorescence emission intensity of hydrophobic NPN molecules is quenched in the aqueous solution because NPN cannot enter into the cells as such. However, disruption of the outer membrane on treatment with AMPs allows the dye to enter into the hydrophobic environment of the cell membrane resulting in an enhanced emission of fluorescence intensity.

As shown in Figure 2A, WR17 showed a dramatic increase in the emission intensity of NPN by ~ 98 % at a concentration of 30 µM. Surprisingly, WG12, KG11, and KR12 could achieve only a maximum fluorescence of 67 %, 35 % and 28 %, respectively at the similar concentration of each peptide. WK10 and KK9 showed a negligible fluorescence intensity enhancement of only 15 % (Figure 2A). These results indicate that WR17 is capable of disrupting the intact outer membrane model efficiently. Nonetheless, its N- and C-terminal fragments alone are incapable of permeabilizing the cell membrane with only WG12 showing partial activity. Thus, it may be possible that Trp1 and Phe11 are crucial for the outer membrane permeabilizing activity of the intact peptide WR17.

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**Figure 2.** Permeabilization of outer- and inner membrane of the cell by WR17 and its fragments. (A) The plot shows the extent of permeabilisation of *E. coli* BL21 in the form of the percentage increase of fluorescence of NPN dye plotted against increasing concentrations of WR17 and its fragments WG12, KR12, KG11, WK10, and KK9. A significant reduction of permeabilization is observed in the case of fragments of WR17 when compared to itself showing that they have a reduced ability to induce outer membrane permeabilization. (B) The plot shows the efficiency of calcein dye leakage from small unilamellar vesicles (SUV) composed of 3:1 POPC: POPG lipids in the percentage of leakage as a function of the concentrations of peptides.

1.2.2.4 Calcein leakage assay to probe inner membrane permeabilization

In the next attempt, to determine the permeabilization of the inner membrane by native WR17 and its fragments WG12, KG11, WK10, KR12, KR8 and KK9 calcein dye leakage assay using bacterial inner membrane mimetic POPC: POPG (3:1 molar ratio) lipid vesicles was performed.\(^{[12]}\) The fluorescence intensity of calcein increases, due to the release of the dye from the lipid vesicle upon disruption caused by the addition of the peptide. Native
peptide WR17 showed a 36% increase in the fluorescence intensity as shown in Figure 2B. In comparison, the calcein leakage activity for WG12 was reduced to half of WR17. Peptide fragments WK10 and KG11 showed negligible activity, whereas KR12, KR8, and KK9 showed no dye leakage (Figure 2B).

1.2.2.5 Neutralization of endotoxin by LAL assay
Limonis amoebocyte lysate (LAL) assay is an extremely useful assay to identify the inhibition and neutralization activity of LPS with sensitivity as low as pico-molar. The experiment was carried out at three different LPS/endotoxin concentrations of 0.25, 0.5 and 1 EU/ml with six different peptide concentrations of 5, 10, 15, 25, 50 and 100 µM.

Table 1. LPS neutralization and depth of insertion by the designed peptides.

<table>
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<tr>
<th>Peptide name</th>
<th>Neutralization of LPS at 0.25 EU/ml</th>
<th>Neutralization of LPS at 0.5 EU/ml</th>
<th>Neutralization of LPS at 1 EU/ml</th>
<th>Distance of Trp from LPS head group (Å)</th>
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<td>10</td>
<td>15</td>
<td>7.4</td>
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<td>WG12</td>
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<td>50</td>
<td>50</td>
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</table>

WR17 was capable of neutralizing 0.25 EU/ml at a concentration of 5 µM and 1 EU/ml at a concentration of 15 µM (Table 1). On the other hand, similar endotoxin concentrations of 0.25 EU/ml and 1 EU/ml were neutralized by WG12 at 25 µM and 50 µM, respectively. All the other fragments were unable to neutralize the endotoxin.

1.2.2.6 Interaction study using Fluorescence Spectroscopy and ITC
The presence of Trp residue in the peptides WR17, WG12, and WK10, was used to determine the binding affinities in LPS. The Trp residue of WR17, WG12, and WK10 in free solution showed an emission maximum at ~350 nm, which argues that the Trp residue is exposed to the aqueous environment (Figure 3A-C). However, successive addition of LPS into the peptide solution at an increasing molar ratio yielded a progressive blueshift of the emission maxima. The blue shift observed for the Trp residue of WR17/WG12/WK10 in the presence of LPS indicates the presence of Trp in the hydrophobic or non-polar environment (Figure 3A-C). The extent of the blue shift was greatest for WR17 with a shift of 16 nm, whereas its analogues, WG12, and WK10 recorded a blue shift of about 12 and 8.4 nm, respectively. The larger blue shift of the emission wavelength provides evidence for the deep insertion of the Trp residue of WR17 in the hydrophobic environment of LPS compared to that of the analogues WG12 and WK10.[13]

Also, the extent of the solvent exposure of Trp residues in WR17/WG12/WK10 was further investigated using static quenching with a neutral quencher acrylamide in free solution as well as in complex with LPS. All of the peptides exhibited much higher Stern-Volmer Quenching Constants ($K_{SV}$) in free-state compared to the LPS bound state indicating that the Trp residue is well embedded inside the LPS, which protects its accessibility to the quencher (Figure 3D). This quenching data is in good agreement with
the fact that the Trp has an indiscriminate preference for the interfacial region of the lipid bilayer.[14]

Figure 3. Different fluorescence experiments showing binding affinity and solvent accessibility of peptides towards LPS. (upper panel) Intrinsic tryptophan fluorescence emission spectra of (A) WR17, (B) WG12 and (C) WK10, respectively, in the presence of LPS at a molar ratio of 1 : 4. (D) Bar diagram showing the Stern–Volmer constant of the mentioned peptides in aqueous as well as in the presence of LPS. (E) Bar diagram demonstrating the equilibrium dissociation constant (K_D) of the peptides in LPS bound state derived using following changes in emission maxima with LPS concentrations. All fluorescence experiments were performed in 10mM sodium phosphate buffer (pH 6.0) at 298 K.

Additionally, the changes of fluorescence emission maxima of Trp in WR17/WG12/WK10 in the presence of LPS yielded an equilibrium dissociation constant (K_D) of 2.4 ± 0.6, 11.8 ± 0.3 and 8.6 ± 0.6 µM, respectively (Figure 3E). Altogether, the larger blue shift and lower K_D values observed in the case of WR17 is attributed to the strong electrostatic interaction between the positively charged residues, Lys, and Arg at the C-terminal region of WR17 and the negatively charged phosphate head group of LPS. Due to the lack of Trp residues in the analogues KG11, KK9, KR12 and KR8, the fluorescence-based assays in the presence of LPS could not be carried out. Further, fluorescence quenching studies were carried out using two spin-labeled lipids, 5-DSA, and 16-DSA. The position of Trp residues from the center of the LPS player for WR17 and WG12 was found to be around 7.4 and 7.1 Å, respectively (Table 1). This distance information indicates that the Trp residue of both the peptides is well buried in the LPS bilayer and forms strong van-der-Waals interaction with the acyl chains of LPS.
Next, isothermal titration calorimetry (ITC) experiments were performed to obtain the equilibrium dissociation constant ($K_D$) and the binding energy of the active peptides WR17, WG12, and KR8 with LPS (Figure 4).

![Figure 4. Isothermal titration calorimetric (ITC) profiles of WR17, WG12 and KR8. The upper panel shows the endothermic heat of reaction vs. time (minute) upon interaction with LPS for peptides (A) WR17, (B) WG12 and (C) KR8, respectively. The lower panels of the figures A, B and C show enthalpy change per mole of peptide injection vs. molar ratio (peptide: LPS) for peptides WR17, WG12, and KR8, respectively, upon interaction with LPS. 10 mM of LPS were titrated against 250 mM of peptides. All peptides and LPS were dissolved in 10 mM phosphate buffer at pH 6.0.](image)

As a control experiment, the binding interaction of the C-terminal KR8 (peptide without Trp1 and Phe11) with LPS was also performed. It is noteworthy to mention that, KR8 did not show any binding to the LPS micelle (Figure 4C) which shows KR8 can neither penetrate the outer membrane of the cell nor bacterial inner membrane. The binding of WR17-LPS or WG12-LPS is a spontaneous, entropy driven process ($\Delta G = -8.7/8.6$ kcal mol$^{-1}$) with a dissociation constant ($K_D$) of about 0.4 and 0.5 µM, respectively (Table 2). This data corroborates well with other AMP-LPS based studies performed by ITC, where it was reported that the interaction is strongly entropy driven and has an upward ITC profile.$^{[12]}$

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>WG12</th>
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<tr>
<td>$\Delta H$ (kcal.mol$^{-1}$)</td>
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<td>$6.3 \pm 0.7$</td>
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<td>14.9</td>
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<tr>
<td>$\Delta G$ (kcal.mol$^{-1}$)</td>
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<td>-8.6</td>
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<tr>
<td>$K_D$ (µM)</td>
<td>0.4</td>
<td>0.5</td>
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1.2.2.7 Secondary structure of peptides in LPS shown by CD spectroscopy

Figure 5 shows the far-UV CD spectra of the peptides WR17, WG12, WK10, KG11, KK9 and KR12/KR8 in the absence and presence of LPS, respectively. In the aqueous solution, native WR17 as well as its C- and N-terminal truncated analogues showed a strong negative band at 200 nm, indicating that the free peptides adopt a disordered or random-coil conformation (Figure 5). LPS induces a drastic change in the CD spectra of WR17, WG12, and WK10. The negative CD ellipticity at ~200 nm observed for the free peptides disappeared upon addition of LPS, with a concomitant increase in the intensity of a positive peak at ~195 nm and two negative peaks with maxima at ~208 nm and ~222 nm. This CD pattern demonstrates the formation of the alpha-helical conformation of these peptides in LPS micelle (Figure 5A-C). Interestingly, the minima observed at ~222 nm for WR17 in the presence of LPS is broadened, signifies the greater extent of the dynamics taking place in some part of the alpha helical region. On the contrary, the WG12 peptide in LPS micelle showed two minima at ~208 and ~222 nm with equivalent intensities.

![Figure 5. Secondary structures of peptides in free and LPS bound forms by circular dichroism. Far-UV CD spectra of native WR17 (A), WG12 (B), and WK10 (C) (upper panel), KG11 (D), KK9 (E), KR8 (F) (lower panel) in the absence (solid line) and the presence (dashed line) of E. coli 0111:B4 LPS. All CD spectra were performed in 10 mM sodium phosphate buffer (pH 6.0) at 298 K.](image)

WK10 showed a partial loss of the helical structure, in the presence of LPS micelle. Other analogues, KG11, KK9 and KR8 in the presence of LPS did not show any significant changes in the secondary structure (Figure 5D-F). Moreover, the slight changes in the intensity near the negative maxima around ~200 nm for KK9 and KR8 can be attributed to the electrostatic interaction between the positively charged side chains of the terminal amino acids and negatively charged phosphate groups of the LPS moiety. Overall the CD data identifies the structural changes taking place in the parent and the truncated analogues of WR17 upon binding to LPS.

1.2.2.8 NMR Studies of Peptides in free and LPS Micelle

One-dimensional $^1$H NMR spectra of WR17 and its N-terminal analogues, WG12 and WK10 as well as the C-terminal analogues, KR12 or KR8 show a large dispersion for the amide proton resonances (7.7 – 8.6 ppm) (Figure 6). In contrast, the control peptides, KG11 or KK9 showed a
severe signal overlap in the one-dimensional $^1$H NMR spectra, suggesting a random coil conformation of the peptide.

![Figure 6. Interaction of peptides with LPS using NMR.](image)

Interestingly, the addition of LPS even at a low concentration of $\sim 5\text{-}20 \mu M$ to the sample containing the peptides ($\sim 1 \text{ mM}$) showed extensive line-shape broadening in the proton dimension for WR17, WG12, WK10 and KR12 without causing any chemical shift perturbation (Figure 6).
This result is a clear evidence of the peptide undergoing conformational exchange between the free and the LPS bound form, in the fast to the intermediate time scale.\textsuperscript{[15]} In addition to the line broadening effect seen for the amide protons of WR17/WG12/WK10, the most downfield resonances of the indole ring protons (N$^\varepsilon$H) of Trp also showed a substantial line broadening effect (data not shown). Further, three-dimensional structures of all the peptides in the presence of LPS was carried out using \textit{Transferred} Nuclear Overhauser effect spectroscopy (\textit{trNOESY}) experiments.\textsuperscript{[11]} The complete sequence-specific proton resonance assignments for all the free peptides in solution were determined with the help of both two-dimensional total correlation spectroscopy (TOCSY) and NOESY.\textsuperscript{[16]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Analyses of \textit{trNOESY} spectra of peptides in the LPS micelle. Selected aromatic region of two-dimensional $^1$H--$^1$H \textit{trNOESY} spectra of WR17 (A), WG12 (B) and WK10 (C) showing aromatic ring proton connectivities of Trp1 and Phe11 with aliphatic side chains of Leu3/Leu4 and Ala7 depicting proximity between those residues in the presence of LPS. Fingerprint region of two-dimensional $^1$H--$^1$H \textit{trNOESY} spectra of KR12 (D) and KR8 (E) in the presence of LPS. \textit{trNOESY} experiments were carried out at 500 MHz and 298 K, with a mixing time of 150 ms.}
\end{figure}
The NOESY spectra for all the peptides showed only a weak intro- and sequential NOE between the backbone and side chain proton resonances. Also, the aromatic amino acid residues such as Trp1 and Phe11 did not show any notable NOE between the side chain of the hydrophobic amino acids and their aromatic ring protons (data not shown). The lack of NOEs indicates that the peptides are highly flexible in solution and do not adopt any folded conformation. This result is in good agreement with the CD spectra, where it was demonstrated that all the peptides considered here adopt random coil or unstructured conformation.

On the other hand, the addition of LPS even in small quantity to WR17, WG12 and WK10 showed a marked increase in the number of NOEs, because peptide adopts a folded conformation in the presence of LPS. As LPS forms a high molecular weight micelle even at a very low concentration (<1 µM), the peptide that interacts with this large LPS micelle also adopts a conformation that is different from that of the free peptide, giving rise to unique intermolecular NOE patterns. In general, intermolecular trNOE between the ligand and the LPS could not be detected due to the significantly lower concentration of LPS being used for the experiment.\(^{(17)}\)

In addition to the sequential \(\alpha(N)(i, i+1)\) trNOEs obtained for the peptides, we could also see a large number of medium range trNOEs of the type \(\alpha(N)(i, i+3/i+4)\) in the presence of LPS. The aromatic ring protons of Trp1 showed an ample number of trNOEs with the side chains of neighboring aliphatic amino acids such as Leu3 and Leu4 (Figure 7). The side chain of Ala7 also showed trNOEs with the aromatic ring protons of Phe11 (Figure 7). Furthermore, the indole ring protons (N\(\varepsilon\)H) of Trp1 are found to make additional contacts with the adjacent residues of WR17 (Figure 7). The medium range trNOEs \(\alpha(N)(i, i+3/i+4)\) for WR17 were unambiguously identified between the residues from Trp1 to Phe11, suggesting that the N-terminal part of the peptide adopts a helical conformation in the presence of LPS (Figure 8A and 8D). Apart from this, several short range trNOEs \(\alpha(N)(i, i+2)\) were also observed for WR17 (Figure 8A and D). The presence of Gly at the 12\(^{th}\) position breaks the helix due to which the C-terminal region remains unstructured. WG12 showed almost similar trNOEs to that of WR17 (Fig. 6).

All the amino acid residues of WG12 showed a short range \(\alpha(N)(i, i+2)\) as well as medium range trNOEs \(\alpha(N)(i, i+3/i+4)\) bound to LPS. Also, the aromatic ring protons of Phe11 showed trNOEs with the side chain of Ala7 in the case of WR17 and WG12 with LPS (Figure 7A and 7B). In contrast, the trNOE of the aromatic ring proton of WK10 to the neighboring side chain protons was much less in comparison to either WR17 or WG12 (Figure 7C). Only one \(\alpha(N)(i, i+4)\) and five \(\alpha(N)(i, i+3)\) trNOEs were observed for WK10 in LPS (Figure 7C and F). A close inspection of the NOE distribution per residue indicated that the Trp1-Phe11 of WR17 was well characterized by a large number of trNOE contacts, whereas the C-terminal region (G12-R17) of WR17 showed only sequential NOEs (Figure 8A and D). The pattern of NOE distribution per residue for WK12 was almost similar to that of the N-terminal region of WR17 (Figure 8). Overall the NOE distribution per residue for WK10 was much less in comparison to either WR17 or WG12. Especially, the medium range NOEs at the C-terminal region of WK10 was negligible in the presence of LPS, indicating the C-terminal region of WK10 may not be structurally defined. It is noteworthy to mention that neither KR12 nor KR8 in the presence of LPS exhibited any detectable medium range trNOEs \(\alpha(N)(i, i+3/i+4)\) except for a few short range trNOE \(\alpha(N)(i, i+2)\) between Gly12-Asn14 (Figure 7D-E). The spectrum analysis of the peptides KG11 or KK9 in LPS micelle could not be determined due to severe signal overlap (data not shown).
Figure 8. A summary of NOESY contacts of peptides in the LPS micelle. (Left panel) Bar diagram summarizing type (sequential, medium-range, and long-range) and a number of NOE contacts in the trNOESY spectra of WR17 (A), WG12 (B) and WK10 (C) in the presence of LPS among backbone–backbone and backbone–side chain resonances. The thickness of the bars indicates the intensity of the NOESY peaks which are assigned as strong, medium, and weak. The primary amino acid sequences of each peptide are shown at the top. (Right panel) Histogram showing the number and type (intra, sequential, and medium) of trNOEs of WR17 (D), WG12 (E), and WK10 (F) as a function of the residue number in complex with the LPS micelle.
1.2.2.9 Three-dimensional structure of Peptides bound to LPS Micelle

The three-dimensional structure of the peptides, WR17, WG12, WK10 and KR8 in LPS micelle were determined solely based on the distance constraints obtained from the tRNOESY experiments. The superposition of the backbone atoms (N, Cα and C’) for an ensemble of 20 lowest energy structures of WR17, WG12, WK10, and KR8 were shown in Figure 9 (upper panel). The average backbone RMSD values of WR17, WG12, WK10 and KR8 peptides were 1.43 ± 0.53, 0.1 ± 0.05, 0.34 ± 0.13 and 1.06 ± 0.25 Å, respectively (Table 3).

Table 3. A Summary of Structural Statistics for the 20 Final NMR Structures of peptides in LPS micelle.

<table>
<thead>
<tr>
<th>Distance restraints</th>
<th>WR17</th>
<th>WG12</th>
<th>WK10</th>
<th>KR8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-residue (i–j = 0)</td>
<td>36</td>
<td>20</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td>Sequential ([i–j] = 1)</td>
<td>52</td>
<td>41</td>
<td>32</td>
<td>17</td>
</tr>
<tr>
<td>Medium-range (2≤</td>
<td>i–j</td>
<td>≤4)</td>
<td>57</td>
<td>53</td>
</tr>
<tr>
<td>Long-range (</td>
<td>i–j</td>
<td>≥5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>145</td>
<td>114</td>
<td>101</td>
<td>33</td>
</tr>
</tbody>
</table>

Angular restraints

| Φ                           | 16     | 11     | 9      | 7     |
|                             | 16     | 11     | 9      | 7     |
| Ψ                           | 16     | 11     | 9      | 7     |

Deviation from mean structure (Å)

<table>
<thead>
<tr>
<th>Average backbone to mean structure</th>
<th>WR17</th>
<th>WG12</th>
<th>WK10</th>
<th>KR8</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal region “Trp1-Gly12”</td>
<td>1.43±0.53</td>
<td>0.10±0.05</td>
<td>0.34±0.13</td>
<td>1.06±0.25</td>
</tr>
<tr>
<td></td>
<td>0.05±0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average heavy atom to mean structure</td>
<td>2.23±0.65</td>
<td>0.55±0.08</td>
<td>1.21±0.29</td>
<td>2.13±0.42</td>
</tr>
</tbody>
</table>

Ramachandran plots for mean structure

| % Residues in the most favourable and additionally allowed regions | 100 | 100 | 100 | 100 |
| % Residues in the generously allowed Region | 0 | 0 | 0 | 0 |
| % Residues in the disallowed region | 0 | 0 | 0 | 0 |

The helical conformation of WR17 was found to be disrupted at the residues Phe11-Gly12 due to the non-helical backbone dihedral angle of Gly (Figure 9). The role of Gly as a helix breaker has been known for a long time. We could not detect any long range tRNOEs between the N-terminal helical segment of WR17 and its unstructured C-terminal region, suggesting a high flexibility at C-terminus (Figure 9). The average backbone RMSD for the residues Trp1-Gly12 is 0.05 ± 0.01 Å for WR17 in the presence of LPS (Table 3). The C-terminal region of the peptide is flexible and controlled by the Trp1 residue situated at the beginning of N-terminus. Solution Dynamics of WG12 was found to be identical to WR17. Whereas, in WK10, the absence of Phe11 perturbed the helicity partially, indicating the important role of this residue in maintaining the structural dynamics from randomness to ordered helical conformation. The same observation has been noted for KR8 where Trp1 and Phe11 were removed from the sequence (Figure 9). The crowding of the positively charged residues like Lys and Arg over the entire structure enabled the peptide to interact strongly with the negatively charged phosphate group of LPS. On the other hand, the hydrophobic residues such as Trp1, Leu2, Leu3, Ala7, and Phe11 form a rigid architecture that facilitates the penetration of the LPS membrane (Figure 9).

The structure of WR17, WG12, and WK10 in the presence of LPS is found to be stabilized by the hydrophobic packing between aromatic side chains and the hydrophobic residues of Trp1,
Leu3, Leu4, Ala7, and Phe11. The opposite face of the helix is characterized by the charged and the polar residues such as Lys2, Lys6, Glu9, and Lys10.

Figure 9. NMR-derived three-dimensional structures of WR17, WG12, WK10 and KR8 in the LPS micelle. (Upper panel) superposition of backbone atoms (N, C\(^\alpha\), and \(\tilde{\mathcal{C}}\)) of the twenty lowest energy structures of WR17 (A), WG12 (B), WK10 (C), and KR8 (D) bound to the LPS micelle, obtained from CYANA 2.1. (Middle panel) Ribbon representative average conformations of WR17 (A), WG12 (B), WK10 (C), and KR8 (D). (Lower panel) Cartoon representative conformations of LPS-bound WR17 (A), WG12 (B), WK10 (C), and KR8 (D) showing side-chain positioning and backbone topology. These figures were produced using PyMOL and Chimera.

It is to be noted that the Lys6 and Glu9 of the peptides, WR17, WG12, and WK10 form potential salt bridge/hydrogen bond in LPS micelle. However, the unique feature of the structure of WR17 and its N-terminal analogues lies within their K\(^2\)xxxK\(^6\)xxxK\(^{10}\) motifs, which tunes the peptide structure in such a way that the Lys residues can interact with the negatively charged phosphate groups of LPS. Side by side, the interaction between the highly hydrophobic “xxx” tripeptide sequences and the LPS also takes place resulting in the complete burial of the peptide inside the LPS groove.

1.2.2.10 Dynamics of Peptides bound to LPS Micelle

Red Edge Excitation Shift (REES) was employed to investigate the dynamics of the environment surrounding the tryptophan residue of WR17 and analogues, in the presence of LPS micelle.\(^{[13]}\) REES is well defined by a shift of the emission maximum of fluorophore towards the longer wavelength caused by a shift in the excitation.\(^{[18]}\) A stepwise increment in excitation wavelengths ranging from 280 to 310 nm, does not show any substantial change in the fluorescence emission maxima for WR17, WG12, WK10 in their free states, suggesting a mobile aqueous environment surrounding the Trp residue (Figure 10A-C). In contrast, when bound to LPS, a significant REES of 22, 23 and 15 nm in emission maxima of WR17, WG12, and WK10, respectively was observed (Figure 10A-C). This shows that in the peptide-LPS complex, Trp residues are experiencing an
environment where the dynamics of the water molecules have been restricted. This study monitored the presence of the Trp residue at the interfacial region of the LPS micelle, which is characterized by the bulk aqueous phase outside and the hydrophobic membrane environment inside.

Figure 10. Probing restricted dynamics of peptides (WR17, WG12, and WK10) in the LPS micelle using REES and fluorescence anisotropy experiments. The effect of a change in excitation wavelengths on the emission maxima of WR17 (A), WG12 (B), and WK10 (C) in their free (blue circles) and LPS bound forms (red squares) (Upper panel). A plot showing the change in anisotropy values of WR17 (D), WG12 (E), and WK10 (F) as a function of the concentration of LPS (lower panel).

Fluorescence anisotropy elucidates the global rotational freedom and the local dynamics surrounding the Trp residues in protein. The effect of the macromolecular size of LPS on a peptide can be well correlated by measuring their anisotropy values. As the free peptides in the solution rotate and tumble rapidly, the rotational diffusion rate will be faster compared to the emission rate of the excited state. Hence, the emitted light will be depolarized to the maximum, and the anisotropy will average to zero. Whereas, in LPS, the movement of the peptide will be restricted due to the formation of large molecular weight assembly, which increases the rotational correlation time. This, consequently polarize the emitted light to the extent the anisotropy is present (Figure 10D-F). Fluorescence anisotropy technique exploits this degree of polarization of the emission, caused by the increase in correlation time of the complex. In our case, the degree of anisotropy measured, demonstrates the formation of peptide-LPS with longer correlation time depicting the restricted motion of the peptide.

1.2.2.11 Disaggregation and fragmentation of LPS by the peptides monitored by DLS and $^{31}$P NMR
Structural perturbation of LPS micelle by WR17 and its truncated peptides is examined using Dynamic light scattering (DLS) and $^{31}$P NMR techniques as described below. Here, we measured
the size distribution of the particle size of free LPS micelles, and peptide bound LPS using DLS. It was observed that in the absence of AMPs, LPS micelles had a broad distribution of particle sizes with a hydrodynamic diameter of about \( \sim 534 \) nm (Figure 11A). The high polydispersity of \( \sim 72\% \) suggests that the LPS in free form gets aggregated (Figure 11A). However, in the presence of WR17 at a molar ratio of 1:1, there is a drastic change in the aggregation pattern of LPS with a reduced hydrodynamic diameter of about \( \sim 175 \) nm (Figure 11B). Also, the polydispersity also decreased to around \( \sim 40\% \). Such observations demonstrate that addition of WR17 causes an extensive disaggregation of large LPS micelles into smaller fragments.

![Bar diagrams showing hydrodynamic diameter (nm) versus intensity (AU) of scattered light for LPS in the absence of peptides (A) and in the presence of peptides, WR17 (B), WG12 (C), WK10 (D), KK9 (E), and KR12 (F) at a 1 : 1 molar ratio in 10 mM sodium phosphate buffer (pH 6.0) at 298 K. DLS measurements showed disaggregation of the LPS micelle in the presence of WR17, WG12, and WK10 from the particle size distribution pattern.](image)

Figure 11. Structural deformation of the LPS micelle by peptides revealed by DLS and \(^{31}\text{P}-\text{NMR}.\) (left panel) Bar diagrams showing hydrodynamic diameter (nm) versus intensity (AU) of scattered light for LPS in the absence of peptides (A) and in the presence of peptides, WR17 (B), WG12 (C), WK10 (D), KK9 (E), and KR12 (F) at a 1 : 1 molar ratio in 10 mM sodium phosphate buffer (pH 6.0) at 298 K. DLS measurements showed disaggregation of the LPS micelle in the presence of WR17, WG12, and WK10 from the particle size distribution pattern. (right panel) Stacked plot of the one-dimensional \(^{31}\text{P}-\text{NMR} \) spectrum of 0.5 mM \( E. \text{coli} \) LPS (0111: B4) without the peptide and in the presence of different concentrations of WR17 (G), WG12 (H) and KR12 (I) ranging from 0.5 to 1.5 mM. The changes in chemical shift (marked by arrows) as well as the line broadening of \(^{31}\text{P} \) resonances of LPS portraying significant structural perturbation of the LPS head group in the presence of WR17 and WG12 in contrast to KR12.
Similarly, LPS micelle also undergoes structural perturbation in the presence of both WG12 and WK10, with an apparent shift of hydrodynamic diameter to ~200 nm and 230 nm, respectively (Figure 11C-D). This is also evident from the fact that the polydispersity values was reduced to 67 % and 55 % for WG12 and WK10, respectively, suggesting a modest disaggregation of the LPS micelle. In the case of KG11, KK9, and KR12, we found a minimal disruption of aggregated state in LPS with negligible change in the polydispersity (Figure 11E-F). Therefore, these DLS results undoubtedly point towards the fact that disruption of LPS occurs as a result of interaction between native WR17 and its shorter active forms WG12 and WK10 with LPS.

Further, $^{31}$P NMR experiment was performed to investigate the plausible interaction between the phosphate groups of LPS with the positive charge amino acid of WR17 or/and its analogues (Figure 11G-I). As evident, the addition of WR17 to LPS showed a chemical shift perturbation as well as line broadening of the phosphate groups of LPS. Similar structural perturbation was also observed for the naturally occurring AMPs such as Paradaxin and melittin derived AMPs.[21, 22] However, similar titration of WG12, with LPS showed negligible chemical shift perturbation for phosphate groups of LPS. The C-terminal fragment, KR12 was unable to change neither the chemical shift of the phosphate head groups of LPS nor any line broadening was seen. Thus, the breakdown of large LPS micelle into smaller fragments as found in the case of WR17, WG12 and WK10 can be considered to be responsible for the plausible conformational exchange taking place between these two species of LPS at an intermediate time scale (millisecond to the microsecond). We can safely exclude the alternate way of interpreting the line-broadening effect, as a consequence of the LPS aggregation because results from DLS experiments inferred a decrease in the hydrodynamic radius for LPS on the addition of WR17. It is noteworthy to mention that the capability of WR17 and its truncated peptides to perturb the structure of LPS are well correlated with the antimicrobial and antiendotoxin properties of AMPs.

1.2.2.12 Docking-based interaction analysis of peptides with LPS

Molecular docking was carried out to understand the structure-function relationship by analyzing the arrangement of the key amino acids of the peptide fragments (WR17, WG12, WK10, and KR8) over LPS moiety. Figure 12 shows the docked conformation of all peptides (WR17, WG12, WK10, and KR8) over LPS moiety. Figure 12 shows the docked conformation of all peptides (WR17, WG12, WK10, and KR8) over LPS moiety. WR17, which is found to be most active peptide from experimental results, showed a unique orientation over LPS with a curvature of 45° in its structural architecture (Figure 12A).

![Figure 12. Docked pose of peptides with LPS.](image-url)
N-terminal of WR17 is aligned diagonally on the long axis of LPS, whereas, the unstructured C-terminal end was oriented over Glucosamine I and Glucosamine II of lipid A. Residues such as Lys10, Asn14, and Arg17 are involved in strong hydrogen bonding with the phosphate groups of LPS (data not shown). Such interaction is believed to be crucial for the peptide fragment to get initially attached to the bacterial outer membrane via “K13NKSR17,” the molecular anchor which, subsequently enables the N-terminal part of the peptide to penetrate through the outer membrane architecture of Gram-negative bacteria. Crucial hydrophobic/van-der-Waal interactions were also found between Trp1, Leu3, Leu4, and Ala7 and acyl chain of LPS, which in turn is responsible for the activity and stabilization of peptide structure in LPS (Figure 12A).

WG12 was also stabilized by hydrogen bonds and salt bridge interaction with Lipid A part of LPS, in addition to the hydrophobic interactions existing between Trp1, Leu3, Leu 4 and the acyl chain of LPS. Interestingly, the lack of “K13NKSR17” region in WG12 assisted salt bridge/hydrogen bond formation between Lys2, Lys6 and Lys10 and the phosphate group of Lipid A (Figure 12B).

On the other hand, WK10 orients preferentially in a diagonal manner over the long axis of LPS (Figure 12C). Two hydrogen bonds were found between the residues Gln8 and Glu9 with LPS, where the former acts as H-bond donor and latter acts as H-bond acceptor with specific functional groups of LPS. Similar to WR17, pronounced hydrophobic interactions were also seen in the case of WK10, with the acyl chain of LPS. The shortest fragment KR8 showed strong polar contacts between the phosphate groups of LPS and Lys13, Asn14, Lys15 and Arg17 (Figure 12D). It is noteworthy that we did not find any crucial hydrophobic interaction between this fragment and LPS. Overall, the docking study agrees well with our experimental results that suggest that the hydrophobic interaction with LPS is crucial for peptide activity.

1.2.2.13 The role of ‘KNKSR’ fragment as LPS motif anchoring

The structural information as obtained from NMR-derived structure calculation and other biophysical experiments suggest that the bioactive fragment WR17, associated with the LPS micelle, by getting anchored through KNKSR portion. In contrast, the absence of such residues in WG12, WK10, and KR8, has rendered peptides relatively inactive. The docked pose for WR17 with LPS revealed many key polar contacts, in which, the unstructured C-terminal is found to interact only with the GlcN I and GlcN II of lipid A. Residues which are interacting with the negatively charged phosphates such as Lys10 and Arg17 have been shown to maintain the hydrogen bonding throughout the course of the simulation. The terminal residue, Arg17 showed a consistent hydrogen bonding within the range of 2.5-3.1 Å, which highlights the strong nature of this polar interaction (data not shown). Based upon the initial anchoring provided by the C-terminal part, structured N-terminal part is found to establish hydrophobic interaction with the acyl chains of LPS. This interaction is believed to help in subsequent penetration and disintegration of the micelle. Glycine residue is known to be a helix breaker, which resides at the center of the WR17 sequence divides the fragment into a structured region responsible for the hydrophobic interactions and an unstructured region responsible for the polar interactions. Comparison of the individual RMSD for each of the atoms suggests minimal deviation for Gly12 residue, which preserves a 45° degree tilt architecture. Similarly, Lys10 also showed a lesser RMSD deviation by forming a hydrogen bond with the phosphate group that ranges in between 1.9-3.0 Å, which brought the fragment near the corresponding atoms of LPS (data not shown).
1.2.3 Conclusions:

The molecular mechanism by which WR17 permeabilizes the membrane and exert potent antimicrobial activity has been addressed in this work in an ad-hoc manner using various biophysical and spectroscopic techniques. The use of fluorescence-based biophysical techniques such as calcein leakage, NPN dye uptake, and endotoxin neutralization assay could establish that the N-terminal region of WR17 is responsible for the membrane permeabilization. With the N-terminal and C-terminal truncated analogues of WR17 it is further confirmed above hypothesis and found Trp1 and Phe11 to be the essential residues for stabilization of the N-terminal helical structure and activity of WR17. Also with the high-resolution NMR structures of WR17 and its analogues, two unique motif’s namely, “K2xxxK6xxxK10” and “A1xxH3xxH6H7xxxA11” were identified which are involved in the polar and hydrophobic interactions with the phosphate group and acyl chain of LPS, respectively. Also, computational studies suggest that the motif “K13NKSR17,” present at the C-terminal part act as a “structured molecular anchor” at the initial stages of the interaction. This anchoring step is necessary for the N-terminal helical region to interact subsequently with LPS and permeabilize the outer membrane, which in turn is responsible for its antibacterial and endotoxin activity (Figure 13). This novel anchoring “K13NKSR17” motif is currently being utilized in ongoing research to design novel anti-endotoxic molecules.

Figure 13: A schematic representation of mechanism of LPS permeabilization of WR17.
1.2.4 References:

Chapter 1.3

NMR structure and binding of esculentin-1a (1–21)NH2 and its diastereomer to lipopolysaccharide: Correlation with biological functions

This chapter was adapted from the following publication: A. Ghosh, S. Bera, Y. Shai, M. L. Mangoni, A. Bhunia. (2016) BBA 1858 800–812.
1.3.1 Introduction:

Esculentin-1 peptides (46 amino acids long) were first isolated from the skin secretion of the European frog *Rana esculenta* (currently known as *Pelophylax lessonae/ridibundus*).\(^{[1]}\) Lately, it was demonstrated that in contrast to the majority of mammalian AMPs, a derivative of the frog skin AMP esculentin-1a, named esculention-1a(1-21)NH\(_2\) [Esc(1-21), GIFFSKLAGKKIKNLLISGLKG-NH\(_2\)] and corresponding to the first 20 amino acids of esculentin-1a plus a glycineamide at its C-terminus (Scheme 1) \(^{[2,3]}\) has a rapid bactericidal activity at high ionic strength, against both free-living and biofilm forms of the gram-negative bacterium *Pseudomonas aeruginosa*, with a concomitant membrane-perturbation process.\(^{[4-8]}\)

Previous studies performed with the N-terminal 1-18 fragment of esculentin-1b, which differs from Esc(1-21) by a single amino acid at position 11 and lacks the C-terminal tail Leu-Lys-Gly, indicated that in the presence of zwitterionic micelles, which mimic the electrically-neutral membrane of mammalian cells, only the C-terminal fragment of esculentin-1b(1-18) was expected to fold in a helical conformation, this structure is known to be important for peptide’s cytotoxicity.\(^{[9-11]}\) Since the carboxyl tail of Esc(1-21) contains two achiral glycine residues (Gly\(_{18}\)-Leu-Lys-Gly\(_{21}\)) it is unlikely that this portion of the peptide folds into a stable alpha-helical structure. Therefore, replacement of two L-amino acids with the corresponding D-enantiomers in positions 14 and 17 of Esc(1-21) is expected to be sufficient to break a turn of the expected helix at the C-terminal half of the peptide. This, in turn, should reduce its cytotoxicity as confirmed by in vitro assays in different types of mammalian cells.\(^{[7]}\) The designed diastereomer of Esc(1-21), named Esculentin-1a(1-21)-1cNH\(_2\) [Esc(1-21)-1c], (Scheme 1), was found to exhibit potent anti-Pseudomonal activity by reducing ≥ 3 log bacterial cells within 30 min at 4 μM.\(^{[7]}\) However this concentration was 4-fold higher than that needed by the wild-type peptide Esc(1-21).\(^{[7]}\) Furthermore, both peptides disaggregated and detoxified *P. aeruginosa* LPS (Scheme 1), although to a less extent for the D-amino acid containing isomer.

Herein, to understand the molecular mechanism underlying the interaction of the two peptides with LPS leading to their anti-endotoxin activity, we investigated the three-dimensional structure of Esc(1-21) and its diastereomer Esc(1-21)-1c in *P. aeruginosa* LPS micelles by NMR spectroscopy. Also, diffusion ordered spectroscopy (DOSY), saturation transfer difference (STD) NMR, \(^{31}\)P NMR and ITC experiments in conjunction with docking analysis provided the mode of binding of the peptides to LPS at an atomic resolution. The structural insights should assist the search and design of new potent and selective AMPs with anti-sepsis properties.
1.3.2 Results and discussion:

1.3.2.1 Interaction of LPS with Esc(1-21) and Esc(1-21)-lc by ITC

Isothermal titration calorimetry (ITC) is a versatile tool to elucidate different thermodynamic information such as enthalpy change (ΔH), entropy change (ΔS) and Gibbs free energy change (ΔG) along with the binding affinity (Kd) for a ligand-receptor binding phenomenon. The interaction between LPS and Esc(1-21) peptide was characterized by an exothermic heat released, as indicated by downward trends of the ITC thermograms and consequent negative ΔH (Figure 1).
For both peptides, the processes were enthalpy driven ($\Delta H < 0$), and equilibrium dissociation constants ($K_D$) were in the micromolar range (Table 1).

**Table 1: Different thermodynamic parameters from ITC.** Thermodynamic parameters ($\Delta H$, $\Delta S$, $\Delta G$ and $K_D$) derived from the ITC experiment for the interaction of Esc(1–21) (Left panel) and Esc(1–21)-lc (Right panel) peptides with LPS.

<table>
<thead>
<tr>
<th>Thermodynamic Parameters</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_A$ ($M^{-1}$)</td>
<td>Esc(1-21)</td>
</tr>
<tr>
<td>$\Delta H$ (cal mol$^{-1}$)</td>
<td>$(2.48 \pm 0.91) \times 10^5$</td>
</tr>
<tr>
<td>$\Delta S$ (cal mol$^{-1}$ deg$^{-1}$)</td>
<td>-2976 $\pm$ 189.6</td>
</tr>
<tr>
<td>$T\Delta S$ (kcal mol$^{-1}$)</td>
<td>-64.6</td>
</tr>
<tr>
<td>$\Delta G$ (kcal mol$^{-1}$)</td>
<td>-7.36</td>
</tr>
<tr>
<td>$K_D$ ($\mu M$)</td>
<td>4.03</td>
</tr>
</tbody>
</table>

![Figure 1. ITC experiments showing the interaction between AMPs and LPS. Isothermal titration calorimetric (ITC) profiles of Esc(1-21) (left panel A) and Esc(1-21)-lc (Right panel B) to show the thermodynamics of binding with LPS. The upper panel shows plot of heat of reaction ($\mu$cal.sec$^{-1}$) vs. time (minute) upon interaction with LPS micelle for Esc(1-21) (A) and Esc(1-21)-lc (B) peptides, respectively. The lower panels show corresponding enthalpy change per mole of peptide injection vs. molar ratio (peptide: LPS) for peptides Esc(1-21) and Esc(1-21)-lc, respectively. All experiments were performed in 10 mM phosphate buffer at (pH 7.2) at 298 K using VP-ITC (iTC200) MicroCalorimeter (GE Healthcare).](image-url)
The relative binding affinity of Esc(1-21) to LPS was quite stronger than Esc(1-21)-lc binding to LPS. The $K_D$ was ~8 times higher in magnitude ($K_D \approx 31 \mu M$) for Esc(1-21)-lc binding to LPS (Table 1). However, the sign of $\Delta H$ and $\Delta S$ reflected the dominance of electrostatic/ionic interactions between the negatively charged phosphate head groups of LPS and the positively charged Lys side chains of both peptides.\textsuperscript{[15]} Overall, these findings indicate that the two peptides have an appreciable binding affinity to \textit{P. aeruginosa} LPS, in the micromolar range, although this is weaker for the diastereomer Esc(1-21)-1c.

\textbf{1.3.2.2 NMR Studies of Peptides in free form and LPS Micelle}

The solution conformation of Esc(1-21) and Esc(1-21)-1c peptides was elucidated using two-dimensional $^1$H–$^1$H nuclear Overhauser effect spectroscopy (NOESY) and total correlation spectroscopy (TOCSY). The NOESY spectra of both peptide isomers predominantly consisted of intra-residue (CαH$_i$/NH$_i$) and sequential (CαH$_i$/NH$_{i+1}$) NOEs involving amide proton and CαH resonances (Figure 2). Apart from these NOEs, there was no medium and long range ($i \text{ to } i+2/i+3/i+4$) NOE cross peaks, suggesting that the peptides have a highly dynamic structure in aqueous solution and hence do not adopt any folded conformation. These results are in good agreement with previous circular dichroism (CD) spectra showing a random coil conformation of the peptides in aqueous solution.\textsuperscript{[7]}

![Figure 2](image.png)

**Figure 2:** Selected amide region of two-dimensional $^1$H–$^1$H NOESY spectrum of Esc (1-21) (A) and Esc(1-21)-lc (C) in a free state (upper panel) and presence of LPS (lower panel B and D). The lack of medium and long range NOEs precludes formation of any folded conformation, rather adopting a flexible conformation in a free state. In the presence of LPS, there are several NOE peaks demonstrating folded conformation in the presence of LPS. NOESY experiments were performed on Bruker AVANCE III 500 MHz at 288 K, with a mixing time of 150 ms.
We then studied the three-dimensional structure of the peptides in complex with *P. aeruginosa* LPS, by transferred nuclear Overhauser effect spectroscopy (tr-NOESY) \[^{[16]}\]. Tr-NOESY is a potent technique to elucidate conformations of AMPs in LPS micelles where binding affinity lies in the micromolar range \[^{[14, 17-19]}\]. It is noteworthy to mention that the critical micelles concentration of LPS is very low (CMC~14 µg/mL or 1-1.6 µM)\[^{[18]}\]. This allows the formation of large molecular weight micellar assemblies of LPS in solution \[^{[20, 21]}\]. However, intermolecular trNOE peaks between LPS micelle and the peptide could not be observed due to the considerably lower concentration of LPS used for the NMR experiment.

---

**Figure 3. Interaction of peptides with LPS using \(^1\)H NMR.** LPS-induced line broadening of Esc(1-21) (upper panel A) and Esc(1-21)-lc (lower panel B), for the amide proton resonances. In both cases, 1 mM peptide was used whereas LPS concentration was varied between 0 to 10 µM. The NMR experiments were carried out in aqueous solutions (pH 4.5) on a Bruker Avance III 500 MHz spectrometer at 288 K.
Figure 3 shows the amide proton region of the one-dimensional spectrum of Esc(1-21) and Esc(1-21)-1c peptides in absence or presence of different concentrations of LPS. The addition of a small amount of LPS to Esc(1-21) and Esc(1-21)-1c (~1 mM) showed a concentration dependent line-shape broadening of amide proton resonances without causing any chemical shift perturbation. The extent of broadening was estimated to be within 6–8 Hz. The broadening phenomenon can be explained regarding fast to intermediate conformational exchanges between the free and the LPS bound state of the peptides within NMR time frame.\textsuperscript{[22]}

Figure 4. The fingerprint region of 2D trNOESY spectra of Esc(1-21) and Esc(1-21)-1c in LPS. Fingerprint region of two-dimensional $^{1}$H-$^{1}$H tr-NOESY spectra of Esc(1-21) (upper panel A) and Esc(1-21)-1c (lower panel B) showing NOE cross-peaks between CαH/HN resonances. For the sake of clarity, only medium range NOE cross peaks (i to i+2/i+3/i+4) are indicated by green color. The trnoesy experiment was performed in aqueous solution (pH 4.5) containing LPS at a mixing time of 150 ms and at 288 K. Bruker Avance III 500 MHz spectrometer was used for data acquisition.
Interestingly, the trNOESY spectrum of Esc(1-21) in the presence of LPS was characterized by the presence of a sufficient number of medium range NOE cross-peaks meaning an LPS-induced conformational transition, from a random coil state to a well-ordered conformation (Figure 2). Figure 4 shows some medium range (i to i+2/i+3/i+4) NOE cross peaks for Esc(1-21) as well as Esc(1-21)-1c peptides in the context of LPS. Also, few medium-range side chain/side chain NOEs as well as diagnostic backbone NH/NH (i to i+2) NOEs were also detected. Unfortunately, aromatic ring protons of Phe3 did not show any NOEs with the neighboring side chains of amino acid residues, attributing a flexible side chain orientation of Phe3 residue in the context of LPS micelle.

Altogether, the NOE pattern is diagnostic for helical conformation, as also reinforced by CaH chemical shift deviation value (Figure 5A). The deviation of CaH chemical shifts from standard random coil chemical shift value is a valuable indication of secondary structures of proteins and peptides [23]. In helical structures, at least four consecutive amino acid residues show upfield deviation for the CaH chemical shifts. Conversely, downfield deviation trend from standard random coil chemical shift values is observed for beta sheet conformation. The ΔCaH resonances of residues 1-21 of Esc(1-21) showed a marked negative divergence from random coil chemical shifts except for Phe3 and Leu15 (Figure 5A and C). Collectively, the NOESY spectra revealed that Esc(1-21) peptide mainly adopts alpha-helical conformations in P. aeruginosa LPS bound state with some extended portion in the N and C-terminal ends. To further understand whether there was any structural difference between P. aeruginosa and E. coli LPS, we also performed trNOESY experiments with Esc(1-21) in the presence of E. coli LPS. The fingerprint region of Esc(1-21) in the presence of both types of LPS micelles showed almost identical NOE cross-peaks (data not shown), suggesting an identical structure of the peptide in both types of LPS. However, the aromatic region of the NOESY spectra of Esc(1-21) bound to E. coli LPS was enriched with several medium-range NOE connectivities involving aromatic ring protons of Phe3 with aliphatic side chains of Lys5 (i+2), Leu6 (i+3) and Ala7 (i+4) (data not shown). The additional NOE connectivities could be rationalized regarding pronounced hydrophobic packing interaction among Phe3, Lys5, Leu6 and Ala7 of Esc(1-21) in E. coli LPS.

In contrast, Esc(1-21)-1c peptide lacked similar kind of NOE patterns as compared to Esc(1-21), and it had a relatively lesser number of medium range NOE cross peaks (Figure 5B) and irregular deviation of CaH chemical shift (Figure 5B). The N-terminal peptide fragment Gly1-Lys12 contained some medium range NOEs (i to i+2/i+3/i+4), indicating a helical conformation in this region (Figure 5B and D). However, the C-terminal region (Asn13-Gly21) lacked any diagnostic medium as well as long range NOE connectivity suggesting an extended conformation in solution. It has to be noted that the Lys residues of either Esc(1-21) or Esc(1-21)-1c at the N-terminal represented as K^xK^9K^10xK^12 (where x is any on Lys amino acid residue), i.e., i, i+3/i+4 fashion, could be helpful to interact with the negatively charged phosphate groups of LPS. Collectively, both Esc(1-21) peptides interact with the phosphate groups as well as with the acyl chains of LPS moiety via hydrophobic and hydrophilic interactions, respectively. This in turn, facilitates their closer association with LPS micelle.
Figure 5. Summary of NMR structural parameters of Esc(1-21) and Esc(1-21)-lc peptides in the context of LPS micelles. (Left panel) Bar diagram summarizing type (sequential, medium range, and long range) NOE contacts and deviation in chemical shifts of each residues from the random coil values for the CαH resonances, in the trNOESY spectra of Esc(1-21) (A) and Esc(1-21)-lc (B) in the presence of LPS. The thickness of the bars indicates the intensity of the NOESY peaks, which are assigned as strong, medium, and weak. (Right panel) A histogram showing the number and type (intro, sequential, medium) of trNOEs of Esc(1-21) (C) and Esc(1-21)-lc (D) as a function of residue number in complex with LPS micelle.

1.3.2.3 Structure determination of peptides in LPS micelles

The three-dimensional structures of the LPS bound Esc(1-21) and Esc(1-21)-lc peptides were calculated by the distance constraints resulting from trNOESY experiments. For Esc(1-21) peptide, 38 intra-residue, 84 sequential and 59 medium range NOEs were used for structure calculation. Whereas 37 intra-residue, 74 sequential and 30 medium range NOEs were used for Esc(1-21)-lc (Table 2). Figure 6 and 7 show a superposition of an ensemble of twenty lowest energy structures of Esc(1-21) and Esc(1-21)-lc in LPS micelle.
Figure 6. Three-dimensional solution structures of Esc(1-21) peptide in LPS micelle. (A) Superposition of backbone atoms (N, Cα, C') of the 20 lowest energy structures of Esc(1-21), bound to LPS. (B) Cartoon representation of side chain orientation of a representative NMR structure of Esc(1-21) bound to LPS showing different residues. (C) Electrostatic potential surface of Esc(1-21) showing the distribution of polar and non-polar residues. The positively charged, neutral and negatively charged amino acid residues are indicated by blue, white and red, respectively. These images were produced using the PyMOL software.

The three-dimensional structure of Esc(1-21) peptide was well defined with average backbone atoms (N, Cα, and C') and the heavy atoms root mean square deviation (RMSD) of 0.28 ± 0.10 and 0.81 ± 0.09Å, respectively (Table 2). In contrast, the structure of Esc(1-21)-1c was not well defined and denoted by 1.45 ± 0.42 and 1.98 ± 0.36 Å RMSD values for backbone and heavy atoms, respectively (Table 2).

The confirmation of Esc(1-21) in LPS micelles was found to be mainly alpha helical except for the terminal ends (Figure 6). However, the helix tilts at the central region around Asn13-Gly18 residues. Previously another shorter fragment of this peptide Esc(1−18) was shown to adopt a similar helical conformation in the N-terminal part of membrane-mimicking TFE/water mixture as well as zwitterionic DPC or DPC:SDS (3:1) micelles, which correlates nicely with our structural features in LPS micelles.[10, 24] Interestingly, the first four residues of Esc(1-21) namely Gly1, Ile2, Phe3, and Ser4 are in very proximity facilitating hydrophobic interaction between the phenyl ring of Phe3 and the neighboring aliphatic side chains. Such a combination appears to support a well-organized extended hydrophobic surface at the N-terminal part of the peptide. Moreover, nonpolar van der Waals packing interaction could be maintained among residues Phe3, Leu6, Ala7, Ile11, Leu14, Leu15, Leu19, forming a close mutual hydrophobic cluster. On the other hand, the central segment of the Esc(1-21) peptide is enriched with polar residues i.e. Lys5, Lys9, Lys10, Lys12, Asn13, and Lys20 all forming a hydrophilic architecture. Collectively, the amphipathic surface of Esc(1-21) is tuned in such a way that the hydrophobic and hydrophilic interactions govern the peptide stabilization in LPS micelle. Indeed, the orientation of the five positively charged Lys residues in the structural framework of the peptide would create a positively charged microenvironment with a distance of ~12-14Å among Lys5, Lys9, Lys12, and Lys20 (Figure 6). This distance well-matched with the inter-phosphate distance of lipid A moiety of LPS.[14] Importantly, the electrostatic potential surface shows the distribution of polar and hydrophobic residues on opposite sides of the helix domain (Figure 6). This segregation would facilitate the electrostatic interaction between the hydrophilic amino acids and the phosphate head groups of lipid A moiety while the hydrophobic hub of Esc(1-21) would be responsible for the van der Waals interactions.
interaction with the acyl chains of LPS.

In contrast, Esc(1-21)-lc adopts an alpha-helical conformation only at the N-terminal region (Ser4-Lys12) with an average backbone RMSD of 0.06 ± 0.01 Å in that region (Figure 7A, B, and C). However, the helix breaks in the central region around Asn13-Gly18 residues, likely due to the presence of D-amino acids in a stretch of Asn13-DLeu14-Leu15-Ile16-DSer17. It is worthy to mention that D-amino acids showed a lower propensity for alpha-helical conformation [25], which is well supported by the absence of medium range NOE connectivities. Surprisingly, this peptide also maintains an amphipathic character in LPS micelle by segregating polar and non-polar residues on opposite sides of its structure. Notably, Lys5, Lys9, and Lys12 form a hydrophilic face, while Ile2, Phe3, Ala7, Ile11 and Leu15 constitute an hydrophobic segment. In summary, Esc(1-21)-lc adopts similar helical conformation (Ser4-Lys12) as Esc(1-21) at the N-terminal region while the C-terminal region is mostly flexible in nature (Figure 7D). Interestingly, the amphipathicity is maintained in both the structures of Esc(1-21) and Esc(1-21)-lc to interact with the LPS micelle.

**Figure 7. Three-dimensional solution structures of Esc(1-21)-lc peptide in LPS micelle.** (A) Superposition of backbone atoms (N, Ca, C') of the 20 lowest energy structures of Esc(1-21)-lc bound to LPS. (B) Cartoon representation of the average conformations of Esc(1-21)-lc, showing helical segment at the N-terminal region Ser4-Lys12. (C) Cartoon representation of side chain orientation of a representative NMR structure of Esc(1-21)-lc bound to LPS. (D) Overlaid structures of Esc(1-21) (red) and Esc(1-21)-lc (blue). The N-terminal helical segment (Ser4-Lys12) is superimposed nicely with a backbone RMSD of 0.06.

**Table 2. Summary of Structural Statistics for the 20 Final NMR Structures of Esc(1-21) and Esc(1-21)-lc in LPS micelle.**

<table>
<thead>
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<th>Distance restraints</th>
<th>Esc(1-21)</th>
<th>Esc(1-21)-lc</th>
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</thead>
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<tr>
<td>Intra-residue (i–j = 0)</td>
<td>38</td>
<td>37</td>
</tr>
<tr>
<td>Sequential (</td>
<td>i–j</td>
<td>= 1)</td>
</tr>
<tr>
<td>Medium-range (2≤</td>
<td>i–j</td>
<td>≤4)</td>
</tr>
<tr>
<td>Long-range (</td>
<td>i–j</td>
<td>≥5)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>181</td>
<td>141</td>
</tr>
<tr>
<td>Angular restraints</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Φ</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Ψ</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Distance restraints from violations (≥ 0.3Å)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Deviation from mean structure (Å)

<table>
<thead>
<tr>
<th></th>
<th>Average backbone to mean structure</th>
<th>Average heavy atom to mean structure</th>
<th>Ramachandran plots for mean structure</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.28 ± 0.10</td>
<td>0.81 ± 0.09</td>
<td>% Residues in the most favorable and additionally allowed regions 100 (89.7+10.3) 99.7 (65.9+33.8)</td>
</tr>
<tr>
<td></td>
<td>1.45 ± 0.42</td>
<td>1.98 ± 0.36</td>
<td>% Residues in the generously allowed Region 0 0</td>
</tr>
<tr>
<td></td>
<td>0.06 ± 0.01</td>
<td></td>
<td>% Residues in the disallowed Region 0.0 0.3</td>
</tr>
</tbody>
</table>

Further, pulsed-field gradient stimulated echo (PFG-STE) NMR experiments were performed to correlate the three-dimensional structure of the peptides in LPS with and their binding to LPS micelles. The calculated diffusion coefficients for Esc(1-21) and Esc(1-21)-lc peptides in free and LPS bound state are shown in Table 3. Interestingly, both peptides showed almost similar diffusion coefficients in aqueous solution whereas in the presence of LPS their diffusion coefficient decreased. This can be rationalized regarding slow diffusion of the peptides in the bulk core of LPS compared to their free state. It has to be noted that Esc(1-21) has a lower diffusion coefficient compared to Esc(1-21)-lc; this signifies that the motion of Esc(1-21) peptide is more rigid than its diastereomer, due to a stronger binding interaction with LPS micelle by its compact helical conformation. On the other hand, due to the presence of a flexible C-terminal region, Esc(1-21)-lc, might encounter some flexibility in LPS micelles providing the peptide a relatively higher diffusion. From ITC experiments, we also observed that Esc(1-21) has a stronger binding affinity to LPS in comparison to Esc(1-21)-lc.

Table 3. Diffusion coefficients obtained for Esc(1-21) and Esc(1-21)-lc in their free state as well as LPS bound form.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Diffusion coefficient (D) (m².s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D_free (×10⁻¹⁰)</td>
</tr>
<tr>
<td>Esc(1-21)</td>
<td>4.08 ± 0.12</td>
</tr>
<tr>
<td>Esc(1-21)-lc</td>
<td>4.60 ± 0.24</td>
</tr>
</tbody>
</table>

1.3.2.4 Localization of peptides in LPS by STD NMR:
Saturation transfer difference (STD) NMR experiments have been used to reveal the proximity of AMPs with LPS micelles. It is noteworthy to mention that intrinsic fluorescence of Trp residues is often used to explore membrane or micelle localizations of AMPs. Since both peptides lack Trp residues, this rules out the possibility of performing fluorescence experiments. We, therefore, used one-dimensional STD NMR experiments to probe the localization of peptides in LPS micelles as well as residue specific binding epitope. The interaction between Esc(1-21) and Esc(1-21)-lc peptides with LPS satisfied the conditions for STD NMR experiments, which are proved by the micromolar K_D value as obtained from ITC experiments. Figure 8 depicts the one-dimensional reference NMR spectra of both peptides in the presence of LPS (Figure 8, upper panel) and the corresponding STD NMR spectrum (Figure 8, lower panel). In the STD spectrum of Esc(1-
almost all the proton resonances were observed with different intensities similar to that of reference $^1$H NMR spectrum meaning a closer association of the entire peptide molecule with LPS micelle.[28] However, the strongest STD effects were achieved for the side chain methyl resonances of Leu and Ile residues of the peptides proving their closest proximity to the LPS. Also, all the non-labile methylene protons (CβHs and CγHs) of Lys residues along with alkyl protons of Leu and Ile (CβHs and CγHs) showed moderately strong STD effects implying their proximity to the acyl chains of LPS micelle. The CβHs of Ser (Ser4, Ser17) residues and CαH protons of Gly (Gly1, Gly8, Gly18, and Gly21) residues showed almost similar STD effects. The group epitope mapping of the peptides in the context of LPS at an atomic resolution was not possible due to severe signal overlap in 1D STD as well as 2D STD TOCSY spectra (data not shown).[31, 32]

![Figure 8](image)

Figure 8. STD NMR studies of the interaction between Esc(1-21) and Esc(1-21)-lc with LPS. The reference $^1$H NMR spectra for Esc(1-21) and Esc(1-21)-lc peptide are shown in panel (A) and (B) (Top). The STD NMR spectra for Esc(1-21) and Esc(1-21)-lc are shown in the bottom (A) and (B), respectively. STD experiments were performed at a peptide concentration of 1.0 mM in presence of 5 µM LPS in D$_2$O, pH 4.5 at 288 K showing non-exchangeable proton resonances.

Interestingly, aromatic amino acid residue Phe3 showed almost similar STD effect for both Esc(1-21) and Esc(1-21)-lc in LPS micelle. In the contrary, the CβHs of Asn13, which were unambiguously assigned, showed different STD effects for two peptides Esc(1-21) and Esc(1-21)-lc in the presence of LPS. These data portray the difference between structural arrangements of the peptides in LPS micelles. This result is well supported by the trNOESY derived structural distinction between the two peptides. Although both peptides possess similar helical conformation in the N-terminal region, the helicity is disturbed in C-terminal part for Esc(1-21)-lc as described in the previous section. Notably, the STD control spectra of peptides in the absence of LPS did not show any observable STD signals. This establishes the selective saturation of high molecular weight receptor signal which proliferates to the bound ligand via spin diffusion.[32] Taken together,
STD NMR results propose an intimate association of many residues including aromatic, hydrophobic and polar amino acids between Esc(1-21) derived peptides and LPS micelle.

1.3.2.5 Docking-based interaction analysis of peptides with LPS
To achieve a structural model of LPS and peptide interactions, the NMR-derived structure of the peptides was docked into LPS (Figure 9A and 9C). Both the LPS/Esc(1-21) and LPS/Esc(1-21)-lc complexes are stabilized by polar interactions among the anionic phosphate groups of the lipid A moiety and the positively charged side chains of Lys residues.

![Figure 9. Proposed docked pose of Esc(1-21) and Esc(1-21)-lc in complex with LPS.](image)

(A) And (C) Docked pose showing predominant electrostatics interactions, created by Lys residues with the two phosphate groups of LPS (represented by CPK model blue color) for Esc(1-21) and Esc(1-21)-lc respectively. Cartoon representation depicts the peptide backbone. (B) And (D) Hydrophobic and hydrophilic packing constituted by the respective residues in Esc(1-21) and Esc(1-21)-lc. Also, the positively charged residues constitute a cluster of inter-residue distance, similar to the distance between two phosphate head groups of LPS (~12 Å). These figures were created using PyMOL.
For instance, the ammonium side chains of Lys5 and Lys9 are near the diphosphate groups at the O1 position of Glucosamine I (GlcN I). On the contrary, the ammonium side-chain of Lys20 is in close contact with the O4 monophosphate group of GlcN II. Also, Asn13 and Ser17 side chains are near the O4 monophosphate group of GlcN II thereby facilitating hydrogen-bonding contacts for Esc(1-21). Analysis of inter-residue distance revealed that the four residues Lys5, Lys9, Lys12 and Lys20 of Esc(1-21) constitute a hydrophilic network with an inter-residue spacing of 12-15 Å which is geometrically compatible with the inter-phosphate distance of the lipid A moiety (Figure 9B). In the case of Esc(1-21)-1c peptide, Lys5, Lys9 and Lys10 form a hydrophilic triad with 6-12 Å inter-residue spacing (Figure 9D). In particular, Lys5 and Lys10 may interact with the diphosphate and monophosphate groups of LPS, respectively, having an inter residues distance of ~ 13 Å. The other Lys residues i.e. Lys12 and Lys20 are in the flexible C-terminal region (Figure 9D). Apart from that, several hydrophobic/van-der-Waal contacts were observed between the side chain methylene groups of Lys5, Leu6, Leu14 and Ile16 of Esc(1-21) and Ile2, Phe3, Lys5 and Leu6 of Esc(1-21)-1c with the acyl chain of LPS moiety. These mutual non-covalent interactions may potentially stabilize the LPS-peptide complex triggering a disruption of LPS micelles and a perturbation of the LPS layer at the bacterial outer membrane. Taken together, our docking analysis is well correlated with the STD NMR studies, which ultimately pointed out that few hydrophobic, as well as hydrophilic interactions, are necessary for the stabilization of the Esc(1-21) and Esc(1-21)-1c peptides in LPS micelles.

1.3.2.6 LPS micelle disaggregation by $^{31}$P NMR studies:
$^{31}$P NMR is a versatile technique to elucidate alteration of LPS micelles organization upon AMP interaction $^{[29,30,33]}$. The possible mechanism of action by which AMPs neutralize LPS is breaking its large micelles into smaller ones, as it has been proposed by several researchers $^{[34]}$. Therefore, $^{31}$P NMR experiments were performed to investigate the mechanistic details of LPS micelle disaggregation by Esc(1-21) or Esc(1-21)-1c peptides. $^{31}$P NMR of LPS consists of two well resolved $^{31}$P resonances at -0.85 and -0.3 ppm (Figure 10) due to the presence of diphosphate and monophosphate groups in lipid A moiety, respectively $^{[35]}$.

There was a drastic change of $^{31}$P resonances of LPS upon addition of Esc(1-21) peptide in a dose-dependent manner. The chemical shift perturbation and line width broadening were observed for the phosphate groups even at 1:1 molar ratio which was subsequently more prominent with higher molar ratios of the peptide. On the other hand, a different effect at the $^{31}$P resonances of LPS was observed in the presence of the diastereomer, up to a 1:2 (LPS: peptide) molar ratios. However, at 1:4 (LPS: Peptide) molar ratio, there was some amount of broadening effect with a minor change in chemical shift perturbation. These data are also in agreement with previous DLS experiments showing a stronger efficacy in disrupting LPS micelle by the wild type peptide compared to Esc(1-21)-1c $^{[7]}$. This finding is also by the higher ordered alpha-helical conformation of Esc(1-21) peptide and higher LPS detoxification activity compared to the diastereomer $^{[36]}$.

Some other naturally occurring or synthetic AMPs such as paradaxin, temporins, bovine lactoferrampin, MSI 594, Indolicidin, etc. also showed similar structural perturbation of LPS micelle $^{[14, 21, 29, 30, 33, 37]}$. It can be noted that two $^{31}$P resonances are perturbed to a different extent upon binding with the peptide which precludes the possibility of line broadening induced by LPS aggregation.
Figure 10: Structural perturbation of LPS micelle by peptides revealed by $^{31}$P-NMR. One-dimensional $^{31}$P NMR spectra of 0.5 mM LPS in the presence of different concentrations of both peptides up to 1:4 molar ratio (LPS: Peptide). Left and Right panels are showing spectra after additions of different concentrations of peptides, Esc(1-21) (left panel A) and Esc(1-21)-lc (right panel B), demonstrating the changes in chemical shifts and line widths of $^{31}$P resonances of LPS. The monophosphate and diphosphate groups are represented by red and blue arrows. The NMR experiments were carried out in aqueous solutions of pH 4.5, on a Bruker Avance III 500 MHz spectrometer at 298 K.
1.3.3 Conclusions:

The three-dimensional structure of the peptides determined in the presence of LPS micelles has clearly provided an explanation for their different anti-endotoxin activity. Esc(1-21) completely folds into a helical conformation which extends from its N- to C-terminal end. In contrast, Esc(1-21)-1c adopts an alpha-helical conformation only at its Ser4-Lys12 N-terminal fragment, whereas the C-terminal portion is completely flexible. Presumably, a distance of 12-14 Å between positively-charged amino acids in the peptide moiety, which is geometrically comparable to the distance between the negatively charged phosphate groups of lipid A, plays a crucial role for (i) the peptide's interaction with LPS, and for (ii) the peptide's efficacy in disrupting the structure of LPS; two essential requirements to prevent LPS/LBP complex formation and therefore the activation of immune cells for the production of pro-inflammatory cytokines. The presence of multiple "optimal" distances between cationic residues located at both the N-terminal and C-terminal helical portions of Esc(1-21) should make it easier for this peptide to establish complementary interactions with the phosphate groups of LPS molecules compared to Esc(1-21)-1c, where the “flexible C-terminal arm” hampers a 15 Å distance between K20 and K9 or K12. This would account for the lower binding affinity of the diastereomer to LPS compared to the wild type peptide, as well as for the weaker ability to disaggregate the LPS structure and therefore to neutralize its toxic effect. This is well supported by DLS and $^{31}$P NMR studies.[7]

Isothermal titration calorimetry (ITC) revealed that the interaction of Esc(1–21) with LPS is an exothermic process associated with a dissociation constant of ~4 μM. In contrast, Esc(1–21)-1c had almost 8 times weaker binding affinity to LPS micelles. The weaker binding affinity of Esc(1-21)-1c to LPS as well as the minor perturbation of LPS structure compared to Esc(1-21) may also account for the lower antibacterial efficacy of the diastereomer on the parental peptide. In bacteria, the LPS-outer membrane forms a permeability barrier to both hydrophilic and hydrophobic molecules preventing them from diffusing through the cell wall into the target cytoplasmic membrane. Therefore, the lower ability to bind and disrupt the LPS leaflet may be the reason the diastereomer is less efficient in crossing the LPS outer layer to reach/break the bacterial plasma membrane compared to Esc(1-21).

In addition to the an atomic resolution structure of the two peptides when complexed with LPS and a plausible explanation for their different ability to detoxify LPS, our findings have also provided the important information that may assist the future development of novel antimicrobial agents with improved anti-endotoxin activity.
1.3.4 References:


Chapter 1.4

Indolicidin Targets Duplex DNA: Structural and Mechanistic Insights through a Combination of Spectroscopy and Microscopy

This chapter was adapted from the following publication: A. Ghosh, R. K. Kar, J. Jana, A. Saha, B. Jana, J. Krishnamoorthy, D. Kumar, S. Ghosh, S. Chatterjee, A. Bhunia. (2014) ChemMedChem 9, 2052 – 2058.
1.4.1 Introduction:

Indolicidin, a 13 residue Anti-Microbial Peptide (AMP) rich in tryptophan and proline (ILPWKWPWWR-NH₂) (hereafter referred as IR13) (Scheme 1A) is a promising precursor molecule for drug design due to its activity against a wide variety of Gram positive, Gram negative bacteria, fungi, and protozoa, etc.\(^1\) Structurally, indolicidin is one of the smallest AMP’s which is natively disordered in aqueous solution, whereas, in the presence of lipid micelles environments such as DPC and SDS, it adopts a wedge-shaped conformation.\(^2\) A recent study by Shaw and co-workers has demonstrated that indolicidin must interact with the cell membrane before the membrane permeabilization or the latter inhibition of DNA synthesis.\(^3\) It is well known that the mode of action of indolicidin is through inhibition of the bacterial replication and transcription. However, the question remains unanswered on how indolicidin binds to DNA and perturbs the transcription process.\(^4\) To address this key question, the results from several low-resolution techniques such as micropatterned surface chemistry microscopy, spectroscopic techniques such as fluorescence, CD in conjunction with high-resolution NMR spectroscopy were combined. Compiling these results, the structural details of the interaction of indolicidin with DNA at an atomic resolution were elucidated.

In this study, a sequence GG28 where both G-C and A-T base pairs are present (Scheme 1B) was chosen, so as to compare the preferential interaction of such base pairs with IR13. Complementarily, the sequence context propensity of IR13 to recognize different base pairs of DNA duplex structure was also studied by varying the sequence motif PWWP in IR13 (Scheme 1A), since, the PWWP motif is previously known to recognize DNA and form turn structure.\(^5,6\)

1.4.2 Results and discussion

1.4.2.1 Interaction of duplex DNA (GG28) with indolicidin (IR13) by Fluorescence and UV spectroscopy

As DNA duplex and Trp residues in the peptide fluoresce in the same spectral region, FITC labeling of the peptide helps to identify and differentiate the fluorescence emission that comes exclusively from the peptide for studying the DNA-peptide interaction.

\[\text{(A)}\]

\[
\begin{align*}
\text{IR13} & : \text{ILPWKWPWWP WRR-NH}_2 \\
\text{IR13AA} & : \text{ILPWKWPAAP WRR-NH}_2 \\
\text{IR13FF} & : \text{ILPWKWPFTFP WRR-NH}_2 \\
\text{IR13HH} & : \text{ILPWKWPHTFP WRR-NH}_2 \\
\end{align*}
\]

\[\text{(B)}\]

\[
\begin{align*}
5’-d \ (G & \ C \ C \ G \ C \ T \ G \ C \ A \ C \ G \ G) -3’ \\
3’-d \ (C & \ G \ G \ T \ A \ C \ G \ A \ T \ G \ C \ C) -5’ \\
28 & 27 26 25 24 23 22 21 20 19 18 17 16 15
\end{align*}
\]

GG28
Scheme 1. (A) Sequence of Indolicidin (IR13) and its analog (IR13AA, IR13FF, and IR13HH) in which Trp8 and Trp9 are replaced by Ala-Ala, Phe-Phe, and His-His residues, respectively. (B) The sequence of GC-rich duplex DNA (GG28).

The emission maxima for FITC conjugated IR13 was found at ~ 533 nm. Titration of the GG28 into FITC labeled IR13 peptide solution showed an enhancement in the fluorescence emission intensity for FITC (Figure 1A). This enhancement is due to aggregation of IR13 in the aqueous medium. The characteristic behavior of IR13 in the aggregated form was previously demonstrated by dynamic light scattering (DLS) in the aqueous medium. Also, the one-dimensional proton NMR spectrum of IR13 also confirms the aggregation of the peptide in the aqueous solution through line broadening of up to 15-18 Hz. The occurrence of 7 peaks for five indole NH protons was due to cis-trans isomerism of X-P unit (X= any amino acid) indicating the population of several different conformational states could be undergoing slow exchange on the NMR time scale.

Figure 1. Different fluorescence experiments showing binding affinity and mode of IR13 towards GG28. (A) An increase in the intensity of the fluorescence profile of FITC-labelled IR13 (10 μM) as a function of increasing titrated concentration of duplex DNA, GG28 (up to 25 μM). (B) Graph showing the fluorescence intensity of EtBr (1μM) with GG28 (5μM) and titrated with varying concentrations of IR13 ranging from 2μM to 64 μM. (C) The fluorescence spectrum of DAPI mixed with GG28 with varying concentration of IR13 to confirm whether IR13 binds to the minor groove of duplex DNA.

The titration of the GG28 into peptide yielded a biphasic curve with a classical hyperbolic profile at lower concentrations and a sigmoidal profile at the higher concentration. The estimated dissociation constant (K_D) for the initial binding profile was 3.3±0.2 μM, which signifies an initial non-cooperative form of binding for the DNA to the peptide (Figure 1A). However, further addition of GG28 lead to cooperative binding of DNA to FITC-labelled IR13 (showing a sigmoidal fitting) with an estimated K_D value of about 11.2±0.1 μM (Figure 1A). A previous surface plasmon resonance (SPR) based study of the DNA-IR13 interaction has shown that K_D of complexation was ~ 40 μM, which is closer to the product of both the K_D values estimated in our study (i.e. 3.3 × 11.2=36.9 μM), indicating a combination of both non co-operative and co-operative form of binding for DNA to IR13.[8]

Next to our approach, we used Ethidium Bromide (EtBr) as an extrinsic fluorescence probe and studied its fluorescence properties to recognize the architecture of DNA/peptide complex.[9] EtBr being a carcinogenic dye is well-known to intercalate between the adjacent base pairs of double stranded DNA which are characterized by an enhancement of its fluorescence emission intensity.[9] We found a marked enhancement of EtBr fluorescence emission intensity when GG28 was added to it, which is attributed to its usual interactive binding phenomenon (Figure 1B). On successive addition of IR13 to the sample containing GG28 and EtBr fluorescence, emission intensity did not change significantly. This observation demonstrates the non-intercalative binding behavior of IR13 to GG28 as it is clearly evident that IR13 did not compete with EtBr for intercalative binding to duplex DNA GG28.
Furthermore, to observe groove binding phenomenon we performed the experiment with a minor groove binding fluorescent dye DAPI (4',6-diamidino-2-phenylindole), which binds selectively at the A-T rich minor groove of Duplex DNA.\textsuperscript{[10]} Figure 1C shows the fluorescence emission intensity did not change significantly at lower concentrations of IR13. This specifies that DAPI has higher binding affinity to GG28 as compared to the binding affinity of IR13 with GG28. However, successive addition of IR13 fluorescence emission intensity decreased in a significant manner.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{Relative UV absorbance (~632 nm) enhancement of methyl green with a concomitant increase of IR13 from the GG28-methyl green complex, indicating IR13 binds to the major groove of duplex DNA due to its competition with methyl green.}
\end{figure}

UV spectroscopy of GG28 and IR13 complex was also performed in the presence of methyl green, which preferentially binds to the major groove of duplex DNA.\textsuperscript{[11]} A decrease in the UV absorbance peak for methylene green at around 632 nm was observed in Figure 2 when IR13 bound to GG28. On successive addition of IR13 to the sample containing GG28 and methyl green, the UV intensity of methyl green at 632 nm increases significantly. This result demonstrates that IR13 removes the dye, methyl green from the major groove binding site of duplex DNA (Figure 2). In contrast, a higher concentration of IR13 is required to compete with DAPI, which interacts with the minor groove of duplex DNA (Figure 2). Collectively, UV and fluorescence spectroscopy confirms that the IR13 binds to the major groove of duplex DNA, which is later confirmed by NMR and MD studies.

\subsection{1.4.2.2 GG28 retain its native B-form after binding with IR13 probed by circular dichroism}
In the near UV CD spectral region (210-320 nm), the signal originated principally from the DNA component and changed herewith reveal the conformational changes of DNA secondary structure influenced by the peptide binding.\textsuperscript{[12]} The observed spectrum of GG28 consists of a positive maximum at ~ 275 nm due to polynucleotide base stacking and a negative maximum at 255 nm, which emanates from the dissymmetric helical structure (Figure 3).\textsuperscript{[12]} The nature of CD spectrum depicts the characteristics of a right-handed B-DNA in aqueous phosphate buffer solution of pH ~ 7.2. As indicated in Figure 3, there is a diminutive enhanced intensity in the spectrum at 255 nm attributed to the successive addition of IR13. On the contrary, a decrease in intensity of the peak at ~ 275 nm, hypothesized to non-intercalative binding of IR13 to GG28 was observed.
Figure 3. Secondary structure elucidated by CD spectroscopy for the interaction of GG28 and IR13. CD spectra of GG28 (10 μM) titrated with IR13 concentration ranging from 5 to 20 μM.

Therefore, the differences in peak intensity at 255 and 275 nm suggest a perturbation of base pairing due to peptide binding. As conceived from a recent study it was reported that such changes at 255 or 275 nm indicate the perturbation of base-stacking and base dehydration. According to the data points, as obtained from CD spectrum, the secondary structure of DNA does not change significantly even after binding to the peptide, thereby retaining its native B-form.

1.4.2.3 Sequential assignments of non-exchangeable sugar proton for control GG28 and IR13–GG28 complex from NMR

Figure 4 gives an overview of the sequential assignment for the non-exchangeable aromatic base protons (purine H8 and pyrimidine H6) with ribose protons (H1', H2', H2'', H3', H4'). All the NOE cross peaks from the two-dimensional 1H-1H NOESY spectrum corresponds to that of a right-handed B-DNA helix. Each base aromatic proton (H8/H6) exhibits strong NOE cross peaks to its own 5'-flanking H1' sugar protons, as shown from G1 to G14 for the single and its complementary strand. The sequential assignment was performed using through space connectivity between adjacent bases in each strand. In a combination with aromatic (H8/H6) protons of bases (n) with the H1'-ribose protons of the preceding residue (n-1) in the 2D 1H-1H NOESY spectra (Figure 4A and 4B) assignment was traced without interruption. The H5 and H6 protons of cytosine showed a similar type of strong intra-residual NOE cross peaks in the absence as well as in the presence of the peptide; IR13 are labeled with a superscript “pound-sign” (Figure 4A and B).

The aromatic-aromatic connectivities between base H5 protons (n) with the base H8 protons of the preceding residue (n-1) are grouped together in the “gray areas”. Analyzing the NOEs qualitatively, we found the general pattern as similar to that of right-handed B-type DNA; that is \( \text{NOE}_{H2'} (i)-H8/H6 (i) >> \text{NOE}_{H2''} '(i-1)-H8/H6 (i) > \text{NOE}_{H2'} (i-1)-H8/H6 (i) \). All the NOEs including sequential, intra-residue and aromatic-aromatic (H8-H5) are almost comparable in nature between GG28 (Figure 4A) and its complex (Figure 4B), demonstrating normal base stacking for both duplexes. Most resonances have similar intensities as well as similar chemical shifts between control and complex DNA. The NOE contacts for H1'-H2'/H2" in both the DNA structures (free/peptide bound) are found to be isochronous (Figure 4C), suggesting that the sugar backbone conformation in both the B-type DNAs remain almost the same.
Figure 4. Analyzes the sugar portion of NOESY spectra of GG28 in the free and complexed state. Two-dimensional $^1$H-$^1$H NOESY NMR spectra (500 MHz, 298K, 100 ms mixing time) showing the sequential assignments of the base H6/H8 protons with H1'-ribose protons of (A) control DNA duplex, GG28; (B) GG28-IR13 complex (GG28: IR13= 1:1). Solid and dashed lines indicate the assignments of both strands. The H8/H5 NOEs are located in the gray areas whereas H6/H5 NOEs are marked with symbols. (C) Selected region of two-dimensional $^1$H-$^1$H NOESY spectra of GG28 (purple) and in complex with IR13 (orange) at 500 MHz, 25°C and at 100 ms mixing time. Both the spectra are isochronous, indicating that the backbone conformations of GG28 are not changed in the presence of IR13.
This clears our understanding that sugar moieties play no key interaction as prevailed for complex formation with IR13. In summary overall sugar region of NOESY spectra of control DNA and complex DNA were almost similar regarding (i) chemical shift, (ii) intensity, (iii) resolution, thereby depicting homogeneous structural features as in both cases.

1.4.2.4 IR13 binds selectively to A-T base pairing without adopting any characteristic secondary structure

The hydrogen-bonded guanine and thymine imino protons region of the $^1$H-$^1$H 2D NOESY spectrum correspond to a most downfield region (12-14 ppm). In this region A-T and G-C cross peaks originate from either H3 (Thymine) with H61 and H62 (Adenine) or H1 (Guanine) with H41 and H42 (Cytosine) connectivity. The imino protons of the eight guanines and four thymine residues are distinctly observed except terminal G1 and G14 resonances. This may be attributed to the fact that continuous exchange phenomenon with solvent water molecules might cause the disappearance of G1 and G14 imino protons. Among the hydrogen bonded A-T and G-C base pairs out of two amino protons, the hydrogen-bonded proton got downfield chemical shift as compared to their non-hydrogen bonded proton. However, all the cross peaks demonstrate canonical Watson-Crick type bonding for all the base pairs of GG28. Conversely, we found a marked change in the chemical shift value observed for the thymine-imino proton regions in GG28-IR13 complex compared to that of the free DNA (Figure 5A). Chemical shift perturbations were detected significantly in the $^1$H-$^1$H 2D NOESY spectrum for some cross peaks in A-T region such as T9-A20, T19-A10, T24-A5 and T6-A23 of GG28. These cross peaks were found to be perturbed by addition of IR13 (Figure 5) and shows as a chemical shift towards up field. On the other hand, no significant chemical shift perturbation was observed in the imino proton regions of guanine residues in the presence of IR13. This suggests the possibility that IR13 might selectively hamper weak A-T base pairing, compared to that of strong G-C base pairing of GG28. This result reflects that probably the base-pair breathing dynamics in A-T regions is more compared to that of G-C in the presence of IR13. All the thymine of GG28 are highly dynamic in the presence of IR13 while the guanines are rigid in the complex system, could be due to stable G-C base pairing. Furthermore, $^1$H-$^1$H 2D NOESY spectrum of IR13 in the context of duplex DNA did not account for enhanced numbers of NOE cross peaks (data not shown). This also implicates that IR13 does not adopt any particular secondary structure in the presence of duplex DNA.

Figure 5. NMR spectroscopy study indicates interaction of A-T base pairs of DNA with IR13. (A) One-dimensional NMR spectra of the imino region of GG28 in the absence (Black color) and the presence of IR13 (GG28: IR13=1:1) (Red color). (B) 2D $^1$H-$^1$H NOESY spectra of T-imino and G-imino base pairs of GG28 in the absence (Red color) and presence (blue color) of IR13.
These interesting results motivated us to look further into the atomic level interaction of IR13 with GG28 using NMR spectroscopy. To understand the base pairing stability of duplex DNA imino proton, the melting study of DNA (control DNA and DNA-peptide complex) was performed. The chemical shift based melting profile showed that the Thymine N3-imino protons are more disordered than the Guanine N1-imino protons of free GG28. Interestingly, there is a significant reduction in the melting profiles of Thymine imino and Guanine imino protons of GG28-IR13. Relatively, the Thymine imino protons are more affected than the Guanine imino protons in the melting profile (Figure 5B). A slow rise in temperature from 15 to 55°C for the free DNA showed that the imino protons of the Thymidine residues, T6, T9, T19 and T24 were readily denatured compared to that of all the Guanines, except G16 and G27, which were present in the terminal part of the duplex DNA (Figure 5B). However, the same imino protons of Thymidines in the complex form showed a greater stabilization over melting indicating the general stabilization of the core region of DNA in the presence of IR13.

A series of T$_1$, T$_2$ and T$_{1p}$ experiments for the imino protons of free GG28 and its complex with IR13 was performed to understand the dynamics of GG28 at an atomic level. A remarkable decrease in the R$_1$ profile of T9 and T19 compared to that of the Guanine bases, except for the
terminal bases G27 and G16 was observed, which are highly dynamic due to its location at the end (Figure 6). Since all the A-T base pairs (T19, T9, T6 and T24) were located in the central region of GG28, we were not able to compare the R1 profile of T9, T19 with a reference ‘T’ which is located away from the central region. The decreased dynamics of T9 and T19 in the complex form is a consequence of the strong hydrophobic interactions between the aromatic rings of Pro3-T9 or Trp8-T9 and Trp11-T19 (Figure 6). Due to the signal overlap of imino protons of T6 and T24 of GG28, we did not consider the R1 values of these residues to explain the actual dynamics (Figure 6). Overall, the average R1 of the imino protons had decreased by a factor of 1.35 compared to that of the free DNA, suggesting that the complex formation has attenuated the high-frequency motions. In contrast, the R2 of the complex has increased by a factor of 1.4 compared to that of the free DNA (Figure 6) indicating that the peptide in its bound state has an increased overall correlation time. Furthermore, from the on-resonance T1ρ measurements at spinlock field strength of 3 KHz and 6 KHz for the bound GG28, we could estimate a Rex value of 8 Hz.[14, 15] The presence of residual Rex indicates that the GG28 and IR13 dynamically interact with each other on the NMR time scale of fast to the intermediate regime (µs-ms).

1.4.2.5 Structural features of GG28 and GG28-IR13 complex from NMR and molecular dynamics
A total of 355 NOEs such as 90 strong, 113 medium and 152 weak, 62 Watson-Crick hydrogen bond and a total of 192 torsional angles including α, β, γ, δ, ε, ξ was used for the structure calculation (Table 1 and 2).

Figure 7. (A) An ensemble of GG28 structures derived from NMR constraint steered MD simulation. (B) An ensemble of GG28-IR13 complex structures derived MD simulation.
The stereospecific assignment of sugar protons of a duplex DNA of this size is practically impossible due to severe signal overlap to get the $^1$H-$^1$H coupling constant. However, we obtained the correlation between 3’-5’ phosphorous with H5’/H5’’ and H3’ that lead us to conclude that the $\gamma$ and $\xi$ dihedral exists in $+$ and trans conformations, respectively (data not shown). The simulated annealing in a vacuum with Generalized Born Model of DNA duplex with NMR-derived restraints was used to obtain an ensemble of 8 stable conformations of B-type DNA structures with an RMSD of 0.7 Å (Figure 7A). In general, the NMR-derived ensemble structure represents the average of the structure of a large number of molecules accumulated over an observed period of experimental time. The three-dimensional structure of IR13 (PDB accession code: 1G8C)[22] was then docked onto the solution structure of GG28 using the program Hex,[16] followed by MD simulation for 50 ns (Figure 7B).

Table 1. Distance Restraints obtained from two-dimensional NOESY experiments, used for three-dimensional structure calculation of GG28 in solution.

<table>
<thead>
<tr>
<th>NOE contacts</th>
<th>Strong (1.8-3.0) Å</th>
<th>Medium (2.5-4.0) Å</th>
<th>Weak (3.5-5.0) Å</th>
<th>Watson-Crick (1.8-3.0) Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic-Aromatic</td>
<td>12</td>
<td>11</td>
<td>26</td>
<td>62</td>
</tr>
<tr>
<td>Aromatic-Anomeric</td>
<td>78</td>
<td>78</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>Exchangeable contacts</td>
<td>0</td>
<td>24</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Total NOE</td>
<td>90</td>
<td>113</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>Total Distance Constraints</td>
<td></td>
<td></td>
<td></td>
<td>417</td>
</tr>
</tbody>
</table>

Table 2. Torsion angles and pseudo phase angle restraints used for three-dimensional structure calculation of GG28 in solution.

<table>
<thead>
<tr>
<th>Torsion angle</th>
<th>NO</th>
<th>LOWER LIMIT(°)</th>
<th>UPPER LIMIT(°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma$</td>
<td>28</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>$\delta$</td>
<td>28</td>
<td>110</td>
<td>170</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>27</td>
<td>140</td>
<td>220</td>
</tr>
<tr>
<td>$\zeta$</td>
<td>27</td>
<td>260</td>
<td>340</td>
</tr>
</tbody>
</table>
Figure 8. Representation of GG28-IR13 complex from different snapshots of MD simulation time scale.

Representation of IR13 orientation over GG28 of the simulation time course is shown in Figure 8 where the B-form helicity of the duplex DNA is found to be well conserved in the complex system. The average RMSD of all the DNA residues from 50 ns MD suggests a stable GG28-IR13 complex (Figure 9). Alignment of free and IR13 bound structure yields 3.0 Å RMSD because of the shift in the backbone dihedral in the IR13 complexed DNA compared to that of the control. Amber-ff99SB force-field based MD simulation predicted the RMSD for all the residues in the free state to be comparable or higher to that of the nucleobases in the complex state except for A5 in free duplex DNA.[17] The structural perturbation of G14, C15, G16, C17 and G18 are much more in GG28, which indicates that the IR13 binds to the major groove of the DNA duplex (Figure 9).
1.4.2.6 **Trp 8 and Trp 9 are key residues for binding of IR13 with GG28 probed by thermal melting studies by CD**

Additional evidence for the GG28-IR13 binding phenomenon was obtained from thermal denaturation studies by CD spectroscopy. It is well known that the DNA double-helical structure is quite stable due to hydrogen bonding and base stacking interactions. However, with an increase in temperature, the double helix dissociates into single strands due to breakage of hydrogen bonding between base pairs and stacking interactions.\(^\text{[12]}\) In this context, the melting temperature \(T_m\) can be defined as the temperature, at which a half amount of a double-stranded DNA sample is dissociated into single-stranded DNA.\(^\text{[12, 18]}\) A change of \(T_m\) may be observed if a molecule binds to DNA.\(^\text{[18]}\) Thus, the thermal behavior of GG28 in the presence of IR13 provides useful information on the conformational changes and the strength of the DNA-peptide complexes. The melting temperature \(T_m\) of the control DNA, GG28 was found to be \(52.2 \pm 0.7\) °C (Figure 10) whereas the melting temperature \(T_m\) of the GG28-IR13 complex has been found to be \(63.2 \pm 0.7\) °C (Figure 10), which marks a marked difference of \(11\) °C as compared to that of control. This result signifies that GG28 is getting stabilized over melting in IR13 bound condition than its free state. The peptide wraps up the DNA structure and thus it cannot unwind easily (Figure 7B and 8).

In another case, GC28 \([d(GCGCATATTAATGC)]_2\), a DNA duplex, with differential sequence identity was also investigated to verify whether IR13 stabilizes DNA duplexes in the context of their sequence. The \(T_m\) measurement using CD for the GC28 complexed with IR13 was \(50.6\) °C, whereas, the \(T_m\) for the native GC28 was \(40.1\) °C (data not shown). This data reflects that the sequence context of GC28 does not affect its binding to IR13, significantly. It is noteworthy to mention that the PWWP motif infact recognizes the DNA and hence investigate the sequence context effect of PWWP motif on DNA binding, we replaced the Trp-Trp (extended aromatic system) dipeptide to Phe-Phe, His-His and Ala-Ala, consequently decreasing their surface area and hydrophobicity. We premised that increasing hydrophobicity as well as the surface area of the side chains of “xx” in the core of PxxP motif (where x= methyl, imidazole, benzyl, indolyl) could stabilize the DNA structure (Figure 10 and inset).
Figure 10. The interaction of IR13 and its mutants with DNA. CD is melting curve of GG28 in the absence (black color) as well as in the presence of IR13 (red color), IR13AA (green color), IR13FF (violet color) and IR13HH (purple color). The inset shows that T_m of GG28 is increased in the complex form with increasing surface area of xx motif of PxxP of IR13.

The analysis of Van’t Hoff’s Isotherm (using CD melting data) of free DNA and its complex with IR13 or its mutant analogs shows that the complexation of IR13 to DNA increases the ΔΔG from -9.1 kcal mol\(^{-1}\) to -10.8 kcal mol\(^{-1}\). Additionally, the magnitude of the enthalpy and entropy of the GG28-IR13 complex increases from -22.0 kcal mol\(^{-1}\) to -31.4 kcal mol\(^{-1}\) (for enthalpic change) and -43.4 to -69.2 cal. mol\(^{-1}\) K\(^{-1}\) (for entropy change), respectively, favoring the complex formation thermodynamically (Table 3). A similar scenario was observed in the duplex DNA GC28 bound to IR13. Taken together, the increasing order of the thermodynamic stability of different complexes was found to be GG28-IR13 >> GG28-IR13HH ≥ GG28-IR13FF > GG28-IR13AA > GG28.

Table 3. Thermodynamics parameters derived using van’t Hoff equation from CD melting curve.

<table>
<thead>
<tr>
<th>DNA</th>
<th>DNA+ Peptide</th>
<th>T_m (°C)</th>
<th>T_m (K)</th>
<th>ΔH (kcal.mol(^{-1}))</th>
<th>ΔS (cal.mol(^{-1})K(^{-1}))</th>
<th>ΔG (kcal.mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG28</td>
<td>GG28</td>
<td>52.2 ± 0.7</td>
<td>325.2</td>
<td>-22.0</td>
<td>-43.4</td>
<td>-9.1</td>
</tr>
<tr>
<td></td>
<td>GG28 + IR13</td>
<td>63.2 ± 0.7</td>
<td>336.2</td>
<td>-31.4</td>
<td>-69.2</td>
<td>-10.8</td>
</tr>
<tr>
<td></td>
<td>GG28+IR13AA</td>
<td>54.5 ± 1.1</td>
<td>327.5</td>
<td>-23.3</td>
<td>-47.1</td>
<td>-9.3</td>
</tr>
<tr>
<td></td>
<td>GG28+IR13HH</td>
<td>56.4 ± 0.8</td>
<td>329.4</td>
<td>-24.0</td>
<td>-48.6</td>
<td>-9.5</td>
</tr>
<tr>
<td></td>
<td>GG28+IR13FF</td>
<td>55.5 ± 1.0</td>
<td>328.5</td>
<td>-23.6</td>
<td>-47.6</td>
<td>-9.4</td>
</tr>
<tr>
<td>GC28</td>
<td>GC28</td>
<td>40.1 ± 1.2</td>
<td>313.1</td>
<td>-21.7</td>
<td>-45.0</td>
<td>-8.3</td>
</tr>
<tr>
<td></td>
<td>GC28+IR13</td>
<td>50.6 ± 0.7</td>
<td>323.6</td>
<td>-24.4</td>
<td>-51.1</td>
<td>-9.2</td>
</tr>
</tbody>
</table>
It is interesting to find in the molecular dynamics simulation that the residues, Trp8, and Trp9 of IR13 assist the DNA to be non-solvated (Figure 11). A structural representation of such phenomenon is shown in supplemental Figure 12, where hydrophobic regions created by IR13 residues are displayed. The blue “hydrophilic mesh” which represents the solvation prone region for the nucleotide is comparable for free as well as complex DNA. The hydrophobic region as created because of Trp9, Pro10, Arg12 and Lys5 can be seen clearly from figure 12. The desolvated snapshot thus generated is believed to be the reason, which avoids the perturbation of base pairing by the solvent.

![Diagram of hydrophobic region induced by IR13 residues responsible for desolvation of nucleobases.](image)

**Figure 11. A diagrammatic comparison of the hydrophobic region induced by IR13 residues responsible for desolvation of nucleobases.** The hydrophilic/hydrophobic maps were calculated using hpp map in Schrödinger, LLC. The nucleotides of interest were selected and placed on a grid of 3Å for the calculation of solvation effect. The hydrophilic regions are shown by blue mesh, and hydrophobic maps are shown by yellow contours. Corresponding residues of IR13 responsible for desolvation are represented in ball and stick model. (A) Hydrophobic/hydrophilic map (hpp) in the region of DNA base: G3, C4, and A5. (B) Hydrophobic region induced by the presence of Trp9. (C) HPP map in the region of T6: A23, G7: C22 and G21. (D) Hydrophobic region induced by the presence of Pro10 and Arg12. (E) HPP map in the region of T6, G7 and C8. (F) Hydrophobic region induced by the presence of Lys5 and Trp4. These images were produced using Schrödinger (Maestro) LLC.

### 1.4.2.7 Colocalization and cellular uptake study by Fluorescence microscopy

To study further on how IR13 interacts with GG28 we decided to use our recently developed micropatterned surface technique using fluorescence microscopy. Here, we have attached two fluorophores, Biotin, and Rhodamine at the 5'-end of each of the two single stranded GG28 (Scheme 1B). The tagged duplex GG28 was immobilized onto the neutravidin loaded biotin micropatterned surface through biotin-neutravidin-biotin interaction (Figure 12) for 10 minutes. The unbound and excess FITC-IR13 were removed by repeated washing with the buffer. Microscopic images at 561 and 488 nm wavelength reveal red and green color micropattern for GG28 and IR13, respectively (Figure 12A and B), whereas, the co-localization of both is identified...
by the yellow color pattern (Figure 12C) indicating the interaction of IR13 with the immobilized GG28 present onto the square blocked micropatterned surface.

![Figure 12. Interaction of IR13 and DNA on biotin micropatterned surface.](image)

Figure 12. Interaction of IR13 and DNA on biotin micropatterned surface. (A) Red colored, micropatterned surface indicates the immobilization of Biotin-rhodamine-DNA on biotin micropatterned surface through biotin-neutavidin-biotin interaction. (B) Green colored, micropatterned surface indicates the binding of the FITC-lebelled-IR13 peptide on micropatterned surface. (C) The yellow colored merged image confirms that FITC-IR13 peptide specifically binds to biotin-rhodamine labeled DNA immobilized biotin micropatterned surface. Scale bars correspond to 10 µm.

To check whether IR13 binds to the micropatterned surface directly or through DNA, we performed a control experiment, where the avidin-Rhodamine dye was incubated on to the micropattern and subsequently loaded with FITC-IR13 to observe under the inverted microscope. The microscopic images showed the binding of the avidin-Rhodamine dye onto the micropattern but, not the FITC-IR13 peptide (not shown). Above results clearly indicate that the FITC-IR13 peptide specifically binds to the micropattern only through DNA. These results from the micropatterned surface chemistry validated this assumption by the fact that the mutant of FITC-IR13, containing non-aromatic dipeptide Ala-Ala instead of Trp-Trp named as FITC-IR13AA, did not show any green micropatterned surface (Figure 13A-C). This clearly proves that the bulkier and hydrophobic Trp-Trp in IR13 has a significant role in binding with GG28.

![Figure 13. Interaction of IR13AA and DNA on biotin micropatterned surface.](image)

Figure 13. Interaction of IR13AA and DNA on biotin micropatterned surface. (A) Red colored, micropatterned surface indicates that Biotin-rhodamine-DNA is immobilized on biotin micropatterned surface through biotin-neutavidin-biotin interaction. (B) The absence of green colored, micropatterned surface indicates that FITC-lebelled-IR13AA peptide did not bind to micropatterned surface. (C) Merged image of (A) and (B) also indicates that FITC-IR13AA peptide did not bind to immobilized Biotin-Rhodamine labeled DNA on micropatterned surface. The scale bars correspond to 10 µm.

Further, we tested the cellular uptake of IR13 by A549 lung cancer cells. Surprisingly, we observed that the FITC-IR13 could localize not only in the cytoplasm (Figure 14A) but also in the...
nucleus of A549 cells (Figure 14B). The combined images of FITC tagged IR13 (green), and DAPI stained nucleus (blue) (Figure 14C), clearly indicates that IR13 diffuses into the nucleus of the cancer cell and directly interact with the DNA.

Figure 14. Uptake of the FITC-IR13 peptide into the lung cancer cell (A549 cell line). (A) Green colored image of cells indicates the uptake of the FITC-IR13 peptide into the cell. (B) The blue colored image indicates the nucleus stained with DAPI. (C) Merged image indicates that FITC-IR13 peptide penetrates into the nucleus of the cell. The scale bar corresponds to 10 µm.

1.4.3 Conclusion:

In conclusion, we have shown that IR13 binds to the duplex DNA and wrapped it so that it cannot unwind easily.

This leads to the subsequent inhibition of DNA replication and the transcription process (Figure 16). We also found that the **PWWW** motif of indolicidin is crucial for the binding of IR13 to GG28 by various spectroscopic and microscopic techniques. We hope that the high-resolution structure of indolicidin-DNA presented here will open up new possibilities to modify IR13 to enhance its antibacterial potency, concomitantly, with diminished cytotoxic effects.
1.4.4 References:

Chapter 1.5

Structural elucidation of a designed anti-endotoxic peptide, KR7 with lipopolysaccharide

The content of this chapter will be included in the following reference: A. Ghosh, P. Dutta, S. S. Das, A. Bhunia. (2016), Manuscript in preparation.
1.5.1 Introduction:

The lipopolysaccharide (LPS) molecules that exist in the outer leaflet of the outer membrane of Gram-negative bacteria are representative of microbe-associated molecular patterns and possess diverse biological activities.\(^1\) LPS is a glycolipid which contains conserved lipid A (glucosamine residues with fatty acid chain), core oligosaccharide unit composed of 10–15 sugar residues, and the O-antigen part (polysaccharide chain composed of consecutive units of 1–8 sugar residues).\(^2\) LPS presents itself as an impermeable obstacle in bacterial membrane, protecting them from antibacterial agents.\(^2\) The disruption of the outer membrane by the AMP occurs by a "self-promoted" uptake mechanism that involves an electrostatic interaction between phosphate groups of LPS and cationic residues of peptides after displacing bivalent cations.\(^3\) It is also noteworthy to mention that LPS or endotoxin is responsible for the septic shock syndrome which is one of the major cause of mortality in the world.\(^1, 3\) During this infection, the LPS through its immune-modulatory effects causes excessive release of inflammatory cytokines (i.e., TNF-α) by a cascade of signaling pathway leading to multiple tissues and organ damage and finally death. Therefore, the development of anti-endotoxin drug has been a major challenge to the pharmaceutical and medical industries since to-date, no therapeutic is available to combat sepsis-mediated lethality. In this scenario peptide having LPS binding, and neutralizing activity can be an attractive solution which can effectively bind LPS and neutralize its endotoxicity. Antimicrobial peptides (AMPs) are 10 to 40 amino acid long cationic peptides having activity against a wide range of microbes such as bacteria, fungi, and protozoa.\(^4, 5\) There are several reports of proline-rich peptides, which kill bacteria through the non-lytic mechanism and interact with intracellular targets like DNA, RNA, etc.\(^6, 7\) This mode of action implies that nonlytic AMPs have the capacity to be internalized by cells and are therefore acts as cell-penetrating peptides (CPPs). Indolicidin (ILPWKWPWWPWRR-NH₂) is the smallest known naturally occurring linear antimicrobial peptides having a unique composition consisting of 39% tryptophan and 23% proline. Due to the distribution of 3 proline and 5 tryptophan residues throughout the indolicidin sequence, it assumes a structure distinct from the well-described α-helical and β-structured peptides.\(^8\) Furthermore it is shown that PWWP motif of indolicidin bind to duplex DNA and inhibit protein synthesis which is responsible for its biological activity.\(^7\) Recently it is also shown that indolicidin has high affinity against bacterial lipopolysaccharide (LPS), and proline residues have immense important in LPS recognition and subsequent biological activity.\(^3, 9\) In this work, we have designed a 7-residue cationic peptide from indolicidin and studied its binding interaction with LPS through a series of low resolution and high resolution spectroscopic and microscopic techniques. Preliminary biological experiments show it has remarkable LPS neutralizing and anti-endotoxic activity. This study shed light on potential aspects of LPS-neutralizing peptides which are of immense importance in designing antiseptic drugs.

1.5.2 Results and discussion:

1.5.2.1 Designing of peptides

The amino acid sequence of KR7 consist of PWWP motif flanked by one Lys and two Arg residues at the N- and C- termini respectively (Figure 1). This sequence is marginally modified from Indolicidin (IR13), which is known as one of the smallest linear cationic antimicrobial peptide in cathelicidin family having the highest percentage of Trp (39%) and Pro (23%).\(^8\) Similar to Indolicidin another 12 residues cationic peptide omiganan which has demonstrated efficacy in phase III clinical trials as a topical gel for preclusion of catheter-associated infections enclosed
same domain. Interestingly, PWWWP motif is also present in tritrpticin, a cathelicidin AMP identified through screening a porcine bone marrow cDNA library.\[^{10}\] Puroindoline series of AMPs include this XWWX/ XWWXWWX (where X=Lys) motif which has promising antimicrobial potency against different microorganisms. From the structure-activity relationship Trp and Pro modulated hydrophobic interactions with the phospholipid membrane play an essential role as manifest from in vitro as well as in vivo experiments. From stereo-selective mechanism of action of AMP at least one of the hydrophobic WPW motif is necessary for biological activity. Recently our group also established that DNA is an alternative target for certain AMP to understand their mechanism of action.\[^{7}\] We have found that PWWP has immense importance in DNA binding as well as LPS binding.\[^{7, 9}\] Also, PWWP motif has already been present in some eukaryotic protein that is well known for DNA binding. Therefore, we have designed this 7 residue peptide to understand the detailed role of PWWP motif in LPS binding and subsequent neutralization with the aid of vast biophysical and molecular biology techniques. The positively charged residues such as Lys5, Arg12, and Arg13 are responsible for favorable interaction with negatively charged phosphate groups of lipid A moiety of LPS by a salt bridge and hydrogen bonding interactions.

| Indolicidin : | ILPWKWPWWP WRR-NH₂ |
| Omiganan : | IL RWPPWP WRRK-NH₂ |
| Tritriptcin : | VR RFPPWWP FLRR |
| KR7 : | KPPWWP RR-NH₂ |

Figure 1. Amino acid sequences of KR7 and its parent fragments, i.e., Indolicidin, Omiganan, Tritriptcin.

1.5.2.2 Outer Membrane permeabilization assays through NPN dye uptake

NPN uptake assay indicates permeabilization of the outer membrane facilitated by the cationic AMPs/CPPs, as designated ‘self-promoted’ uptake process.\[^{11, 12}\] NPN molecules fluoresce weekly in an aqueous environment due to self-quenching. When NPN was mixed with intact *E. coli* cells, it emitted a weak fluorescence at ~415 nm, as it was unable to penetrate the outer membrane permeability barrier. However, additions of KR7 peptide caused a remarkable enhancement in the relative fluorescence intensity of NPN in a dose-dependent manner along with ~ 7 nm blue shift (Figure 2A and B).
Figure 2. Permeabilization of the outer membrane of the E. Coli cell by KR7. (A) Fluorescence emission spectra for NPN dye in the presence of e.coli cell and KR7 showing 7 nm blue shift. (B) The plot shows the extent of permeabilisation of E. coli BL21 in the form of the percentage increase of fluorescence of NPN dye plotted against increasing concentrations of KR7.

The blue shift can be attributed to the uptake of NPN into the hydrophobic milieu of perturbed cell membranes. The intensity enhancement is due to decrease in solvent mediated non-radiative decay channels since the dye inserts deeply into the cell due to outer membrane permeabilization by KR7. These results indicate that KR7 is capable of disrupting the outer membrane efficiently.

1.5.2.3 Interaction study using Fluorescence Spectroscopy and ITC

As KR7 contains two adjacent tryptophan residues, the fluorescence property of these two residues is highly dependent on the polarity of local microenvironment. Figure 3A represents steady-state intrinsic tryptophan fluorescence studies of KR7 in the presence of LPS micelles. The inclusion of LPS at increasing molar ratios into KR7 solution produced an increase of Fluorescence emission intensity of tryptophan and a significant blue shift (12.5 nm) of emission maxima (λ max) from ~352 nm to ~339.5 nm. These observations depicted the incorporation of KR7 into the hydrophobic pocket of LPS which is manifested by moderate K D value of ~ 8.5 µM (Figure 3B). The increase in fluorescence intensity accounts for the increased quantum yield due to a diminution in non-radiative decay pathways due to binding of the fluorophore in the hydrophobic province of the LPS.

![Figure 3A](image)

Figure 3. Different fluorescence experiments showing binding affinity of KR7 towards LPS. (A) Intrinsic tryptophan fluorescence emission spectra of KR7 in the presence of LPS at a molar ratio of 1: 6. (B) Difference in wavelength vs. LPS concentration plot is demonstrating the equilibrium dissociation constant (K D) of the peptides in LPS bound state. All fluorescence experiments were performed in 10mM sodium phosphate buffer (pH 7.2) at 298 K.

Also to access the degree of the solvent coverage of Trp residues in KR7 neutral quencher acrylamide was used. The trend in K SV values suggests KR7 experienced a larger extent of quenching in their free states compared to LPS bound state (Figure 4A). This indicates the exposure of tryptophan residues is greatly diminished in LPS bound state compared to the free state (Figure 4A). Overall the blue shift and quenching data suggests that Trp residue is well surrounded inside the non-polar milieu of LPS micelles.
Furthermore, the position of tryptophan residues of KR7 from the center of the LPS bi-layer was found to be around 8.25 Å from parallax analysis method. This data along with the blue shift and acrylamide quenching data suggests that KR7 is well embedded in the LPS micelle. Both tryptophan residues play an anchoring role in its insertion by forming van der Waals interaction with the acyl chain of LPS. Taken altogether the results is in good concurrence to the point that the tryptophan takes an inherent preference for the interfacial area of the membrane.

![Figure 4. Probing solvent exposure and restricted dynamics of KR7 in the LPS micelle using acrylamide quenching, fluorescence anisotropy, and REES experiments.](image)

1.5.2.4 Dynamics of Peptides bound to LPS Micelle from Fluorescence Anisotropy and REES experiment

Fluorescence anisotropy measurement to study the effect of the macromolecular LPS moiety on KR7 was performed. As the tumbling and rotation of free KR7 in the solution is very fast, the rotational diffusion rate will overcome the emission rate of the excited state. Therefore, the emitted light will be maximum depolarized, and the anisotropy will average to zero which is evident from very low anisotropy value of 0.0095. The anisotropy value of KR7 increased to 0.075, ~8-fold enhancement, in the presence of LPS micelle. Binding of KR7 to LPS restricted its mobility ensuing in an 8-fold enhancement of anisotropy of KR7 in the bound form. (Figure 4B).

A stepwise increment in excitation wavelengths ranging from 280 to 308 nm, does not show any appreciable change in the fluorescence emission maxima (~4 nm) for KR7 in free states (Figure 4C), suggesting a mobile aqueous environment surrounding the tryptophan residue. In contrast, the emission maxima are shifted from 339 nm (excitation at 280 nm) to 359 nm (excitation at 308 nm) for LPS bound state which corresponds to REES of ~20 nm for KR7.

1.5.2.5 Isothermal titration calorimetry (ITC) to reveal the thermodynamic parameters in the binding process

The LPS peptide interactions are characterized by an exothermic heat released and enthalpy-driven course, as specified by down word trends of the ITC thermogram and consequent negative integrated heats (Figure 5). The enthalpy (ΔH) of association between KR7 and LPS has been determined to be ~ -15.5 kcal mol⁻¹ with an equilibrium association constant (K_a) of 0.07 µM⁻¹ and a stoichiometry (N) of ~0.8 obtained. This clearly demonstrates that the binding reactions are driven primarily by enthalpy contributions and the dominant interactions are indicative of non-covalent forces like π-π stacking, cation-π interaction, and electrostatic interaction.
Figure 5. Isothermal titration calorimetric (ITC) profiles of KR7 in LPS. The upper panel shows the exothermic heat of reaction vs. time (minute) upon interaction with LPS. The lower panel shows enthalpy change per mole of peptide injection vs. molar ratio (peptide: LPS). The inset shows different thermodynamic parameters as obtained from ITC. All peptides and LPS were dissolved in 10 mM phosphate buffer, pH 7.2.

1.5.2.6 Circular dichroism

Far-UV CD spectrum of KR7 is depicted by a large negative maximum around 200 nm and a very weak positive shoulder band around 228 nm due to cotton effect associated with Aromatic side chain of Trp (W) residues (Figure 6). This spectral pattern can be attributed to the random coil unordered backbone conformation. However, the low ellipticity of the peak compared to the values reported for random coil conformations suggested that subpopulations of the ordered conformations such as turns, poly-L-proline helices existing in the solution by the previous literature. No major secondary structure change was observed in the LPS bound complex as evident from the CD spectra. It shows a shift of negative maxima from 200 nm to 208 nm which
can be correlated as the turn type conformation of KR7 in LPS bound complex (Figure 6). The disappearance of the positive shoulder has been attributed to the change in orientation of the Trp residue about the native peptide.

Figure 6. The secondary structure of KR7 in LPS. CD spectra of KR7 (blue) and in the presence of LPS at 1:2 molar ratio (red). CD experiments were performed in 10 mM phosphate buffer, pH 7.2 at 298 K.

1.5.2.7 Dynamic light scattering (DLS) shows the disaggregation of LPS
As a consequence of fluorescence and thermodynamic depiction of the association of KR7 with LPS; we inspected the effect of KR7 upon the size of LPS by dynamic light scattering (DLS) measurements. The capability to disturb micelle structure of LPS by AMPs could be interrelated with the anti-endotoxic activities.

Figure 7. Structural deformation of the LPS micelle by peptides revealed by DLS. Bar diagrams showing hydrodynamic diameter (nm) versus intensity (AU) of scattered light for LPS in the absence of KR7 (A) and the presence of KR7 (B) at a 1:1 molar ratio in 10 mM sodium phosphate buffer (pH 7.2) at 298 K.
LPS forms soluble high molecular weight aggregates in aqueous solution due to its inhomogeneous structural scaffold which is shown by broad size distribution pattern in DLS (Figure 7A) with mean hydrodynamic diameter ~540 nm. However, in the presence of KR7 peptide, the LPS aggregates severely disintegrates to smaller molecular weight aggregates with a concomitant decrease in mean hydrodynamic diameter of ~80 nm (Figure 7B). Also, the polydispersity index factor resultant from DLS experiments is a sign to probe the homogeneity of size distribution that may range from values 0 (mono-disperse) to 1 (poly-disperse). The high polydispersity index (80%) shown for LPS in the absence of KR7 indicating a high degree of polydispersity in aggregation pattern. Interestingly in the presence of KR7, a drastically reduced polydispersity index of 0.31 is observed which is identical to a monodisperse solution.

1.5.2.8 Tunneling electron microscopy (TEM) depicts the change in supramolecular morphology of LPS in the presence of KR7

Transmission electron microscopy images of LPS alone and LPS-KR7 complex shows the influence of the peptide on the morphology of LPS. LPS in aqueous solution aggregates to form an elongated ribbon-like network (Figure 8).\cite{17} By contrast in the presence of KR7, this assembly was completely fragmented into smaller amorphous aggregates with thread-like filamentous morphologies (Figure 8). Also, few small dense spherical assemblies of LPS were also observed from the TEM data. Also, similar morphological changes of LPS in the presence of the different potent LPS neutralizing peptides have been observed.\cite{18-20} This observation supports that LPS neutralization by KR7 includes strong binding to LPS with high affinity, and subsequently fragmentation LPS aggregates into smaller entities that are not available to LBP.

![Figure 8. Morphology of LPS in absence and presence of KR7 depicted by Tunneling electron microscopy (TEM). (A) ribbon like aggregates of LPS alone and (B) Perturbation of LPS network by KR7 at 1:1 molar ratio.](image)

1.5.2.9 $^{31}$P NMR and paramagnetic relaxation experiments

$^{31}$P NMR experiments established interactions of positively charged residues of KR7 with LPS phosphate head groups. Figure 9A shows an overlay of $^{31}$P NMR spectra of LPS alone and after the addition of KR7 at different molar ratios up to 1:6. $^{31}$P NMR spectra of LPS shows two intense well-resolved peaks which are significantly affected by the addition of KR7 with increasing molar ratios. With the help of previously assigned spectrum, we assumed that the upfield peak at -1.30 ppm corresponds to the diphosphate groups of LPS, and the downfield-shifted peak at 0.00 ppm belongs to the monophosphate group of LPS, attached to the O4 of GlcN I.\cite{21} The addition of KR7
shows significant line broadening of the up fielded $^{31}$P resonance in concentration dependent manner. It is worth mentioning that the diphosphate groups of LPS afford a probable interaction site for the cationic AMP due to its higher negative charge density. Also, the downfield peak at 0.0 ppm undergoes less perturbation in the presence of KR7. Such broadening of resonances has been recognized to the chemical or conformational exchange processes between KR7-LPS complex happening at the ms to µs time scale. Therefore, phosphate head groups of LPS experience severe structural disordering upon interaction with Lys and Arg side chains of KR7 that essentially result in dissociation of larger LPS aggregates into small sizes.

The permeability of LPS LUVs in the presence of KR7 has been examined using paramagnetic quencher Mn$^{2+}$. The paramagnetic ion partially quenches the intensity of $^{31}$P resonances of LPS phosphate head groups in its vicinity at 1:10 molar ratio (Figure 9B).

![Figure 9. Structural perturbation of the LPS micelle by KR7 revealed by $^{31}$P-NMR.](image)

This outcome is anticipated for an intact membrane, as Mn$^{2+}$ can invade the outer leaflet but does not have admittance to the inner leaflet in the absence of pores. The addition of Mn$^{2+}$ in 1:10 molar ratio of the KR7-LPS complex (1:1) has little effect on $^{31}$P resonance. This confirms LPS inner leaflet remains intact after the addition of KR7 at the lower molar ratio. But at higher KR7: LPS molar ratios, e.g., 1:2 and 1:4 the observed $^{31}$P signal intensity decreased to a larger extent (Figure 9). This result indicated that the integrity of the bilayer has been lost due to pore formation by KR7. Subsequently, Mn$^{2+}$ can penetrate into the inner leaflet. At a saturating KR7 molar concentration both peaks of LPS completely disappear in the presence of Mn$^{2+}$ indicating robust
disruption of LPS bilayer integrity. Therefore, such observation obviously demonstrates the extensive dissociation of LPS micellar aggregation into smaller fragments in the presence of KR7.

1.5.2.10 NMR Studies of Peptides in free form and LPS Micelle
The lack of sufficient intra and intermolecular NOE cross peak between the backbone and side chain proton resonances clearly confirms KR7 has highly dynamic conformation in solution (Figure 10A). Also, aromatic ring protons of Trp residues showed a very few aromatic cross peaks with neighboring amino acids (Figure 10C). Therefore, NOESY footprinting data of KR7 suggests that KR7 adopts an unstructured conformation, which is also confirmed by CD data.

Figure 10. Selected region of NOESY spectra for KR7 in free and LPS bound form. (Upper panel) the aromatic region of NOESY spectra of KR7 in free (A) and in the presence of LPS (B). (Lower panel) Indole region of tryptophan residues in free (C) and LPS bound form (D). The NOESY experiments were carried out in aqueous solutions (pH 4.5) using a Bruker AVANCE III 500 MHz spectrometer at 298 K.

Figure 11 showed indole NεH and amide and aromatic regions of One-dimensional 1H NMR spectra of KR7 alone and titrated with increasing concentrations of LPS up to 6.67 μM. Interestingly, the addition of low concentration of LPS (~1-7 μM) to KR7 (~ 1 mM) exhibited concentration dependent linewidth broadening of amide, aromatic proton resonances without causing any chemical shift perturbation. The extent of broadening was assessed to be within 5–8 Hz. Also, the most downfield indole ring protons of two (NεH) tryptophan shows multiple peaks
due to the presence of cis-trans isomerism for two consecutive XPY moiety (X, Y are any non-proline amino acids) which also showed an extensive line broadening effect (Figure 11). This result is a clear evidence of the peptide undergoing conformational exchanges between the free and LPS bound form, at a fast to intermediate NMR time scale regime.\textsuperscript{[22]} This preliminary data encouraged us to observe the conformations of LPS-bound peptides using \textit{transferred} Nuclear Overhauser effect spectroscopy (\textit{tr}NOESY) experiments.\textsuperscript{[21, 23]}

Two-dimensional TOCSY and NOESY spectrum were used for the complete Sequence-specific resonance assignment for KR7. Interestingly in the presence of LPS, there is severe differentiation in the NOESY spectra, in the terms of number and intensity of NOE cross-peaks (Figure 10B and 10D). This type of spectral pattern confirms the formation of folded conformation in the presence of LPS. However, intermolecular \textit{tr}NOE between KR7 and LPS could not be detected due to the considerably lower concentration of LPS present in the sample for \textit{tr}NOE experiment.

Due to the presence of two proline residues at KR7 sequential assignment of C\textalpha{}H/HN (\textit{i} to \textit{i}+1) was interrupted. Also due to the special structural feature of two proline residues KR7 adopted several conformational states owing to cis-trans isomerism of X-P bonds (X is any non-proline amino acids). Among them, we could able to identify two major conformations from NOESY. However, a subpopulation of other minor conformations could not be ruled out completely [ peak marked by a star (+) sign] due to the scarcity of sufficient peaks (Figure 12). The peaks for the 2\textsuperscript{nd} conformation are marked with a (’) sign. Except for Proline preceding residues bonds we get all sequential (i to \textit{i}+1) connectivity between neighboring residue. We got only a few side chain NOE connectives (i to \textit{i}+2) among K1’-W3’, P2’-W4’, W3’-P5’, W3’-R7’ and P2-W4 in the amide proton region (Figure 12A). This precludes formation of regular alpha helical and beta sheet structures and higher dynamics present in the structure of KR7 in LPS micelle. Interestingly, among two

![Interaction of peptides with LPS using NMR. One-dimensional amide proton resonance NMR spectra of KR7.](image)
proline residues P2 always resides in “trans” conformation as evident from the P2δ/K1α and P2′δ/K1′α cross peaks while P5 can have both cis and trans conformation as evident from W4 α/P5α and W4 α/P5′δ cross peaks respectively (Figure 12B). It also noteworthy to mention that indole ring protons of W3 and W4 residues showed ample number of NOE peaks with alkyl side chains of K1, P2, P5 and R6 residues.

![Figure 12. Analyzes of trNOESY spectra of KR7 in the LPS micelle. (A) Fingerprint region of two-dimensional 1H–1H trNOESY spectra of KR7. (B) Ha region of KR7 is showing α-δ and α-α connectivities for two major conformations.](image)

**1.5.2.11 Structural description of the LPS-bound structure of KR7 peptide**

The three-dimensional structures of KR7 in LPS micelle was determined solely from trNOESY spectra derived distance restraints. For calculation of P2(trans)-P5(trans) conformation [named as conformation I, marked by (’) sign] 11 intra-residue 5 sequential and 9 medium range while for calculation of P2(trans)-P5(cis) conformation II 16 intra-residue 25 sequential and 13 medium range NOESY derived distance restraints were used (Table 1). Figure 13A shows the superposition of all heavy atoms and backbone atoms (Cα, N, and C’) of the 20 lowest energy structures of both conformations of KR7. From the backbone and heavy atom rmsd it is clear that LPS bound conformation of KR7 is well-defined in both conformations. The central PWWP motif is conserved with slight fluctuation in the terminals due to the presence of flexible alkyl side chains of lysine and arginine residues in the conformations. It is noteworthy to mention the backbone of LPS bound KR7 does not implement any common secondary structures such as the alpha-helix or beta-sheet nature rather they are making turn conformation. In both the conformations K1, R6 and R7 making a unique orientation such that the distance between the side chain NH2 groups becomes with 5-16 Å (Figure 13B). Therefore, this interresidue distance is comparable to the separation between monophosphate and diphosphate groups of lipid A of LPS moiety. Therefore, these hydrophilic hub establishes strong polar interactions with LPS phosphate head groups facilitating the stabilization of KR7 in LPS micelles.
Figure 13. NMR-derived three-dimensional structures of KR7 in the LPS micelle calculated by CYANA 2.1. (A) superposition of backbone atoms (N, C\(^\alpha\), and C) of the twenty lowest energy structures of two conformations of KR7 bound to the LPS micelle. (B) cartoon representation of 2 conformations of LPS-bound KR7 describing side-chain positioning and backbone topology. (C) Overlaid structures of two conformations of KR7 marked by blue (P2\(^{\text{trans}}\) \(\rightarrow\) P5\(^{\text{trans}}\)) and red (P2\(^{\text{trans}}\) \(\rightarrow\) P5\(^{\text{cis}}\)) (D) PWWP motif of two conformations showing W4-P5 trans orientation for one conformation (blue) and W4-P5 (cis) orientation for other conformation (red). These figures were produced using PyMOL.
Also, in both conformations prolines (P2 and P5) and tryptophans (W3 and W4) are near each other. So they are creating some amount of hydrophobic stabilization in the LPS micelles. Apart from this K1 and W4 shows strong cation-pi (π) interaction among them in 2nd conformation. Altogether, the arrangement of lysine and arginine residues along with proline and tryptophan residues gives unique amphipathic structural features in LPS, which in turn stabilize KR7 in LPS micelles.

Table 1. A Summary of Structural Statistics for the 20 Final NMR Structures of KR7 in LPS micelle.

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<th>Conformation II</th>
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<td>Medium-range (2≤</td>
<td>i–j</td>
<td>≤4)</td>
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<td>Long-range (</td>
<td>i–j</td>
<td>≥5)</td>
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<td>(66.7+33.3) =100</td>
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<tr>
<td>% Residues in the generously allowed Region</td>
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<td>0</td>
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1.5.2.12 STD NMR and docking studies show proximity of peptide in LPS micelles

STD-NMR has been comprehensively utilized to investigate chemical groups or residues of ligand that are near to the high molecular weight receptors.[24, 25] Therefore, to understand the localization of KR7 in LPS micelle as well as residue specific binding interaction, STD NMR was performed. Figure 14A and B shows the reference one-dimensional proton NMR spectrum of KR7 in the presence of LPS and the corresponding STD NMR spectrum respectively. The STD NMR spectra of KR7 emerge to almost parallel to their reference spectra signifying that they are intimately linked with LPS. As can be seen, the aromatic indole ring protons of W3 and W4 delineate strongest STD effects indicating their proximity in LPS micelle. This is also in good agreement with fluorescence data where we found that Tryptophans were deeply inserted in the LPS. However, as a result of significant spectral overlap among the benzyl and indole proton resonances
of Tryptophan residues in different conformations of KR7, unambiguous assessment of STD effects was impossible. In the aliphatic region side chain methylene protons of K1, R6 and R7 shows medium STD effects. Apart from this P2 and P5 pyrrolidine ring protons shows little higher STD effects. The two non-degenerate beta protons of W3 and W4 showed moderate STD effect. The Hα of P2, W3 and R6 showed modest STD effect owing to close vicinity with LPS. Collectively, STD-NMR establishes that KR7 makes intimate communications with LPS micelles.

![Figure 14. STD NMR and docking studies of the interaction between KR7 with LPS.](image)

To understand a structural model of LPS and peptide interactions, the NMR-derived, two conformations of KR7, were separately docked on LPS (Figure 14C and D). Interestingly both the KR7-LPS complexes are stabilized by electrostatic interactions among the anionic diphosphate and monophosphate groups of the lipid A domain of LPS and the positively charged side chains of Lys and Arg residues. In Figure 14C, the ammonium side chains of K1 and guanidium side chain of R6 and R7 are in close vicinity of the monophosphate and diphosphate groups of glucosamine unit respectively. On the contrary R6 and R7 making close contacts with the monophase and diphosphate groups of LPS (Figure 14D). Interestingly in both conformations K1, R6, and R7 constitute a hydrophilic triad network with an inter-residue spacing of 9-12 Å, which is geometrically compatible with the inter-phosphate distance of the lipid A moiety (Figure 9B).[20] Also, the two tryptophan residues i.e. W3 and W4 are deeply buried in the acyl chains LPS unit and thereby facilitating hydrophobic interaction among themselves which was assisted by neighboring proline residues. This is also supported by fluorescing measurements where we found the similar depth of insertion of Tryptophan in the LPS bilayer. Therefore, electrostatic interaction in combination with hydrophobic interactions are responsible for the binding of KR7 in LPS moiety, which is in good accordance with STD NMR data.
1.5.2.13 KR7 suppress LPS induced secretion of the pro-inflammatory cytokine TNF-α and IL6.

To determine the lowest dose of KR7 that exhibits anti-endotoxin activity, RAW264.7 cells were stimulated with LPS (10 and 100 ng/ml) for a period of 4 h in complete RPMI 1640 cell culture medium in the absence or presence of KR7, which was added simultaneously at concentrations ranging from 0.5 to 10µg/ml. Tissue culture supernatants were assayed by ELISA for the presence of the pro-inflammatory cytokines, TNF-α, and IL6 (Figure 15A and B). KR7 suppressed LPS induced TNFα and IL6 production in a dose-dependent manner. KR7 was effective at dose ≥ 0.5µg/ml (appx 40% inhibition) and the inhibition was almost 80% at 10µg/ml. The LPS blocking effects of KR7 was more pronounced with the use of a lower dose of LPS (10ng/ml), a dose which is at the lower end of the dose used for TLR signaling but again a considerable higher dose compared to those found in the circulating blood of sepsis patients. In the case of use of a higher dose of LPS(100ng/ml) an equivalent inhibition was found at ≥4µg/ml. These results indicated that physiological concentrations of KR7 exhibit an anti-endotoxin effect on LPS present at low and high concentrations.

![Figure 15. Antiendotoxic property of KR7.](image)

(A) RAW264.7 cells were stimulated with 10 or 100 ng/ml LPS in the presence of increasing concentrations of KR7 (x-axis) for 4 h and checked for TNF-α in the cell supernatant. (B) RAW264.7 cells were stimulated with 10 or 100 ng/ml LPS in the presence of increasing concentrations of KR7 (x-axis) for 6 h and checked for IL6 in the cell supernatant. The results are an average (+/-SD) of three independent experiments.

1.5.3 Conclusion

Certain AMPs have the ability to act as anti-endotoxin molecule. It blocks the LPS mediated signaling by either triggering the pathways which block LPS signaling or by preventing the binding of LPS to LPS binding protein. In this study, a 7 residue peptide fragment (KR7) is chosen from indolicidin. Initially steady state fluorescence study shows that it has high LPS binding affinity, and the two tryptophan residues reside at interfacial region of LPS micellar environment. Then ITC experiments demonstrates that the binding interaction between LPS and KR7 is enthalpy driven, and electrostatic interactions play a vital role in its binding. From the secondary structural point, CD spectroscopy showed that it did not adopt any particular conformation in LPS bound state rather it adopts a turn conformation. High-resolution trNOESY studies have revealed that there exist two major conformations of KR7 in the presence of LPS due to cis-trans isomerism of proline residues. STD and molecular docking studies identifies crucial residues responsible for
binding to the LPS. Lysine and Arginine residues making a hydrophilic network of 10-12 Å distance which is comparable to the inter-phosphate distance of lipid A moiety of LPS. The perturbation of LPS phosphate head groups is further confirmed by DLS and $^{31}$P NMR studies. Preliminary biological experiments show very low concentrations (less than 1µg/ml) of KR7 inhibited TNF-α and IL6 release from LPS-induced cells which shows it has potential anti-endotoxin activity. Detailed studies regarding the effect of KR7 in LPS induced NFκB and MAPK activation signaling pathways and mouse septic shock model are in progress. Therefore, in a nutshell, this study shows LPS binding and atomistic details of structural insights of KR7 in LPS micelles which have significant anti-endotoxin property.
1.5.4 References: