NMR Structures and Interactions of Antimicrobial Peptides with Lipopolysaccharide: Connecting Structures to Functions

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Abstract: Antimicrobial peptides (AMPs) establish the first line of host defense mechanism against invading microorganisms including bacteria, viruses, fungi and parasites. In recent years, emergence and spread of antibiotic resistance bacterial pathogens have dawn considerable interest in investigations of AMPs. The ability of AMPs to exert lethality against multiple drug-resistant (MDR) bacteria has incited promising avenues for antibiotic development. As a mode of action, most AMPs perturb the membrane organization of bacterial cells. The outer membrane lipopolysaccharide (LPS) of Gram- negative bacteria establishes a superior permeability barrier, in contrast to the peptidoglycan layer of Gram-positive bacteria. Due to LPS barrier, development of antibiotics for drug resistant Gram- negative bacteria are more complicated, with only fewer compounds in the pipeline. Recent studies have demonstrated that LPS actively regulate mode of action of AMPs on the lethality of Gram-negative bacteria. LPS, also known as endotoxin, is the primary agent for septic shock syndromes in intensive care unit killing over 120,000 people in the USA. Currently, anti-sepsis therapies are greatly lacking. Therefore, LPS has been considered as a target for the development of antimicrobial and antisepsis drugs. In recent and past few years, 3-D structures and interactions of a number of AMPs have been determined in complex with LPS micelles. These studies have generated molecular insights towards mode of action and synergistic activity of AMPs in the outer membrane. In this review, atomic resolution structures and interactions of potent AMPs with LPS are discussed providing novel insights of their mode of action.

Keywords: antimicrobial peptides, Lipopolysaccharide (LPS), Outer membrane, NMR.

1. INTRODUCTION*

Drug resistant bacterial pathogens are of significant threat to the public health around the globe [1-4]. There is an urgent need to develop new antibiotics; however, the pipeline for producing new drugs has been highly reduced over past 30 years [5, 6]. The US Food and Drug Administration (FDA) had approved 20 new antibiotics between 1980 and 1984, but only three new antibiotics were approved in recent years [5, 6]. The lack of new antibiotics is a reflection of reduced productivity of drugs in the pharmaceutical industry. As existing drugs are becoming old, finding new drugs turns out to be difficult [3, 5, 6]. Most importantly, the growing number of resistant bacterial strains indicates that new antibiotics should function with a different mode of action. It is now well documented methicillin resistant Gram-positive *Staphylococcus aureus* (MRSA) infections are difficult to treat [7]. Infectious diseases caused by Gram-negative bacteria are even more major threat in human health. Notably, the spread of multidrug-resistant so called 'ESKAPE' Gram-negative pathognes i.e. *Enterococcus*, *Staphylococcus aureus*, *Klebsiella*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter*, is an enormous challenge [8]. Gram-negative bacterial strains with enhanced resistance to all available antibiotics have been detected [9, 10]. The American Centers for Disease Control has estimated over 2 million cases of infections and 23,000 deaths caused by drug resistant bacteria in the USA annually [11]. As many as 730,000 infections and over 3,400 deaths per year are reported to be caused by Gram-negative bacteria in the US alone. Many drugs against Gram negative bacteria show limited efficacy due to the outer membrane barrier [12-14]. In order to treat bacterial infections, development of novel antibiotics with different mode of action is highly critical. Cationic Antimicrobial Peptides (AMPs) are vital components of innate immunity of host defence system [15-17]. AMPs are multifunctional molecules demonstrating direct killing of broad range of bacteria including multiple drug resistance strains [15-17]. AMPs are found to demonstrate anti-viral, anti-fungal and anti-parasitic activity [18,19]. Some AMPs have been known to kill

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XXX-XXX/14 $58.00+.00

cancer cells [20, 21]. Reports have also suggested signalling functions of AMPs that would modulate functions of innate immune cells [22, 23]. As a mode of action, most AMPs cause lysis of bacterial cells by destabilizing integrity of membranes [24-27]. AMPs remain bactericidal over the course of evolution plausibly indicating difficulty of bacteria to change the membrane compositions and structures. Now, it has been well conceived that the broad spectrum antibacterial activity in conjunction with shorter size and cell selectivity of AMPs could be employed to develop novel antibiotics [28-31]. Pronounced interests have been noted for structure-activity (SAR) correlations of AMPs, designing novel AMPs and various antimicrobial applications of AMPs containing organic scaffolds [28-31]. SAR studies of AMPs often used model membrane systems e.g. detergent micelles, bicelles, liposomes of different compositions to obtain conformational insights for understanding of mode of action [24-27]. 3-D structures, by use of solution state NMR methods, of several AMPs have been determined in detergent micelles (SDS and DPC) as membrane mimics [24-27]. In solid state NMR, AMPs are reconstituted into phospholipid bilayers to gain conformations of peptides and structural perturbations of lipid bilayers [32-35]. Studies of AMPs with liposomes or vesicles are performed to understand peptide/lipid interactions and membrane pore formation [24-27]. By and large, these studies have generated much of the mechanistic insights of mode of action of AMPs e.g. barrel stave, torodial pore, and carpet model [24-27]. Based on the composition, these membrane model systems may mimic either the plasma membrane of bacterial or eukaryotic cells. Observations of lack of correlations between structure and activity of AMPs have potentially indicated alternate mechanisms or interactions of AMPs with more number of targets. Some AMPs are known to interact with intra-cellular molecules e.g. nucleic acids and proteins [36-38]. Interactions with outer-membrane components would like to influence mode of action and mechanisms of AMPs [39-41].

2. LPS OUTER-MEMBRANE BARRIER AND ENDOTOXIN

Bacterial cells are protected from antibacterial substances employing additional membrane components exposed to the external environment. Gram-positive bacteria contain a thick peptidoglycan layer whereas Gram-negative bacteria are surrounded by an asymmetric outer-membrane. The outer leaflet of the outer-membrane is predominantly consisted of a specialized lipid called lipopolysaccharide (LPS). By contrast to peptidoglycan, LPS establishes a permeability barrier limiting access to antibiotics, antibacterial drugs and other molecules [12-14]. Chemically, the covalent structure of LPS molecule follows the connectivity of: lipid A- a core-oligosaccharide-repetitive highly variable polysaccharide or O-antigen. The lipid A unit of LPS is characterized by bis-phosphorylated glucosamine containing six to eight C12 or C14 type fatty acyl chains (Fig. 1). The acyl chains of lipid A anchor with acyl chains of the phospholipids of the inner leaflet of the outer membrane. Polyanionic LPS molecules in the outer membrane are well ordered because of packing among acyl chains and bridging of phosphate groups by divalent cations e.g. Ca and Mg [42-45]. The hydrophilic O-antigen and core sugar groups also contribute to permeability by maintaining a steric shield [42-45]. The permeability barrier imposed by the outer-membrane LPS is responsible for the intrinsic resistance of Gram-negative bacteria against hydrophobic antibiotics. A number of compounds e.g. EDTA, polymyxin B nonapeptide, is known to interact with LPS causing permeabilization of the outer membrane [13, 14]. Outer membrane permeabilizing agents can enhance antibiotic drug uptakes leading to an improvement of Gram-negative activity [46-48]. As LPS in the outer-membrane protects live bacteria, LPS from dead bacteria is highly toxic to humans and other animals [40, 42]. LPS, as known as endotoxin, is a potent stimulator of innate immunity for humans [49-51]. Released LPS from lysed bacteria, often after antibiotic treatment, into blood stream is recognized by the TLR4 transmembrane receptor protein of immune cells e.g. macrophages. Binding of LPS with the receptor causes activation of a signaling cascade that eventually produces interleukins and tumor necrosis factors [49-51]. Over production of these molecules may result in tissue and organ damage of the host, condition termed as septic shock or sepsis. In the absence of any therapeutic modality, annually 120,000 people are estimated to be deceased due to the septic shock syndromes [52, 53]. There have been constant searches for effective drugs to prevent sepsis related fatality. Molecules that would bind and neutralize endotoxin are highly sort after [40, 54, 55].

3. ATOMIC RESOLUTION STRUCTURES AND INTERACTIONS OF AMPs WITH LPS

Understanding mode of action of AMPs on bacterial cells is critical for the further development of non-toxic therapeutics. Atomic resolution structures of AMPs are eminently required to dissect mechanism of actions and structure based generation of new antibiotics [16, 17, 25]. Mode of action of AMPs appears to be quite complex with multiple targets in bacterial cells and different pathways [16, 35 ,37, 39]. Structure-activity relationship studies of AMPs often demonstrate poor correlations due to the lack of 3-D structures in appropriate membrane systems [16, 35]. LPS of the outer membrane of Gram negative bacteria is critically involved in interactions with cationic AMPs [39-41]. AMPs e.g. temporins, K/L designed peptides, are known to be inactivated by LPS [39, 56-58]. Higher MIC values of AMPs for Gram- negative bacteria compared to Gram positive are attributed to protection mechanism rendered by LPS outer membrane [39, 56, 57]. Broad spectrum activity of AMPs requires interactions with LPS and disruption of permeability barrier to gain access into the bacterial cell [17, 39, 40]. Structural elucidation and residue specific interactions of AMPs with the LPS outer membrane would glean knowledge of mechanism of action that will lead to the synthesis of potent antibacterial and antiendotoxin molecules [17, 40]. NMR spectroscopy has been largely utilized to determine 3-D structures and mapping of interactions of a number of potent AMPs with LPS. LPS micelles bound 3-D

structures of AMPs are obtained by transferred nuclear Overhauser effect spectroscopy (tr-NOESY) method [59-61], whereas, saturation transfer difference NMR or STD-NMR [62,63] method has been used to map residue specific interactions of AMPs with LPS micelles.

3.1 NMR studies of polymyxins with LPS

Polymyxins are a group of peptide antibiotics, 10 amino acids long, produced by Gram-positive bacteria *Bacillus polymyxa* [64,65]. Polymyxins are characterized by a cyclic structure with an octanoyl lipid chain at the N-terminus. The amphipathic cyclic structures of polymyxins are conferred by seven cationic di-amino-butyric acid (Dab), a derivative of amino acid Lys, polar residue Thr and nonpolar residues D-Phe/D-Lue along with the acyl tail at the N-terminus [64,65]. Polymyxins kill Gram-negative bacteria by binding to the outer membrane LPS. Among plymyxins, polymyxin B (PMB) and polymyxin E (PME) or colistins are most studied for Gram negative activity and endotoxin neutralization [66-69]. PMB is considered as the 'gold standard' of endotoxin neutralizing agent and acts as a reference molecule for Gram-negative activity assays [66-69]. Unfortunately, nephrotoxicities of polymyxins have prevented systemic applications in humans. Due to the occurrence of drug resistant Gram-negative pathogens, toxicity of polymyxins and several derivatives of polymyxins have been recently re-examined [70-72]. These studies showed promises for the development of non-toxic analogs of polymyxins [70-72]. In order to understand mechanism of endotoxin neutralization and outer membrane interactions, NMR studies, using tr-NOESY method, were performed for PMB and PME in solutions containing LPS micelles. The nona-peptide analog of polymyxin B (PMN) was the first molecule to be examined by tr-NOESY in LPS micelles [73]. The cyclic structure of PMN lacks the N-terminal acyl chain and residue Dab1. PMN is devoid of endotoxin neutralization and antibacterial activity but binds to LPS and permeabilizes the outer membrane [73]. LPS-bound 3-D structure of PMN demonstrated ordered and compact conformations compared to the structure in free solution. LPS-bound structure of PMN is defined by a four residue type II' β-turn centered at the residues D-F6 and L7 and a three residue γ-turn centered at residue T10. Based on the LPS bound 3-D structure of PMN, a molecular model was generated by computational docking for the complex of PMB and lipid A [73]. The model suggested multiple salt-bridges and/or hydrogen bonding among two phosphate groups of lipid A moiety with cationic Dab residues in the cyclic domain of PMB. In particular, the PMB/LPS complex could be maintained through salt bridges and/or hydrogen bonds involving residues Dab3/Dab5 and Dab8/Dab9 with the phosphate groups at positions C-1 and C-4' on lipid A, respectively. The fatty acyl chains of lipid A appears to be packing with the C8 acyl chain and also with the sidechains of residues of D-F6 and L7 of PMB. Later NMR and molecular modelling studies of full-length PMB and PME in LPS showed similar conformations and lipid A interactions [74]. Further, a high resolution NMR investigation, employing isotopically enriched samples, in DPC-lipid A mixed micelles, was carried out for polymyxins [75]. More recently, LPS binding epitopes of PMB were mapped using STD-NMR method [76]. This study revealed that the N-terminal acyl chain and sidechains of D-F6 and L7 are in intimate contact with LPS whereas the cyclic backbone has been observed to be loosely associated with LPS micelles [76]. Collectively, these structural studies have established key elements of the recognition of LPS by polymyxins and mechanisms of outer membrane permeabilization. Recent works have utilized some of these critical structural features of polymyxins/LPS interactions for the further development of polymyxin based novel antibacterial drugs [70-72].

3.2 NMR STUDEIS OF HELICAL AMPs WITH LPS

Based on secondary structures, AMPs are classified into three major groups: helical, β-sheets/β-hairpins and extended. 3-D structures of helical AMPs e.g. melittin, MSI-594, pardaxin, temporins, fowlicidin have been reported in complex with LPS micelles.

3.2.1 3-D structure of melittin and fowlicidin in LPS micelles

Melittin is a bee venom derived 26-residue peptide (GIGAVLKVLTTGLPALISWIKRKRQQ-amide) with high hemolytic and moderate antibacterial activity [77]. Melittin has been extensively used over years as a model system highlighting basic biophysical understanding of membrane-protein interactions [78, 79]. A tetrameric helical structure of melittin has been known in solid state, determined by x-ray, whereas monomeric helical structures have been determined in DPC micelles and in solutions containing helix stabilizing co-solvents [80-82]. Helical structures of melittin obtained under these conditions show a kink or bend at TTGLP sequence. Melittin based peptide analogs with deletion of the TTGLP motif forms a rather straight monomeric helical conformation in DPC micelles and retain antibacterial activity with lowered hemolysis [83]. Melittin demonstrates interactions with LPS possibly as a mode of bacterial cell killing and endotoxin neutralization [84, 85]. NMR structure of melittin in LPS micelles revealed a partially folded helical structure encompassing residues A15-R24 at the C-terminus; whereas most of the N-terminal residues are found to be in extended conformations [86]. A C-terminal peptide fragment of melittin (LPALISWIKRKRQQ) binds to LPS and responsible for outer membrane permeabilization. The antibacterial activity of the C-terminal fragment of melittin has also been reported [87]. The 3-D structure of melittin in LPS micelles has pointed out: (i) the C-terminal cationic region of melittin is more critical for outer membrane permeabilization and antimicrobial activity, (ii) structure stabilized in LPS might demonstrate significant differences in comparison to structures determined in membrane mimic environments e.g. DPC, SDS or in co-solutes.

Fowlicidins are highly potent, 26-residue long, broad spectrum AMPs of cathelilcidin family from chicken [88,89]. Fowlicidin-1 forms a tetrameric helical structure in DPC micelles [90]. Shorter fragments of fowlicidin-1 also retain antimicrobial activity. These fragments interact with LPS and adopt monomeric helical structures in LPS micelles [91]. Although, atomic resolution structure of full length fowlicidin is yet to be determined. LPS-bound structures of

fragments of fowlicidin-1 indicated plausible mode of outer membrane interactions and bactericidal activity.

3.2.2 Structures of MSI-594 and mutant MSI-594F5A in LPS micelles

Genaera and Adis have developed a series of potent hybrid AMPs combining sequences of naturally occurring maganin 2 and melitttin [92]. One of these peptides MSI-594 (GIGKFLKKAKKGIGAVLKVLTTGL-amide) was investigated for 3-D structure and structure activity correlations in LPS micelles [93]. MSI-594, while unstructured in free solution, assume folded unique helical hairpin structure in complex with LPS micelles. The helical hairpin structure of MSI-594 is defined by helix 1 (residues I2-K10)-loop (K11-G12)-helix 2 (residues I13-L24) topology. A number of hydrophobic residues, e.g. I2, F5, A9, of the helix 1 demonstrate long-range packing interactions with hydrophobic residues I13, L17 and L20 of the helix 2. The aromatic residue F5 shows multiple packing interactions with other non-polar residues and appears to be critical for maintaining the hairpin fold of MSI-594 in LPS. The compact tertiary structure of MSI-594 displays all the cationic residues onto one surface that is largely exposed for LPS interactions (Fig. 2). It may be noteworthy that MSI-594 adopts 'open' helical conformations, without any tertiary packing, in complex with DPC detergent micelles [94]. In an attempt to correlate helical hairpin structure of MSI-594 with antimicrobial activity and outer membrane disruption, a mutant peptide MSI594F5A was made harbouring a replacement of the key residue F5 to Ala [95]. Interestingly, MSI-594F5A peptide retains antibacterial activity against Gram-positive bacteria; however, the mutant peptide remains significantly inactivity toward Gram-negative bacteria [95]. Further, MSI-594F5A delineated, in comparison to the native peptide, significantly lower membrane permeabilization and membrane depolarization of *E. coli* cells. Biophysical characterization, using ITC and optical spectroscopy methods, indicated impaired LPS binding and lower perturbation of LPS aggregates by the mutant peptide. These findings established reduced ability of the MSI-594F5A peptide to overcome LPS mediated barrier. 3-D structure of MSI-594F5A in complex with LPS micelles discloses an open 'V' shaped kinked helical structure opposed to the well packed helical hairpin structure of the MSI-594 (Fig. 3A). 3-D structures of MSI-594 and MSI-594F5A in complex with LPS have been correlated with their antibacterial activity and LPS-outer membrane recognition. The helical hairpin structure of the native MSI-594 displays an exposed cationic surface which is largely uniform and directed. In addition, sidechains of basic residues K4/K18 and K7/K18 across the two helices are in optimal distances of ~13-14 Å that would allow formation of strong ionic and/or hydrogen bonding with the two phosphate groups of lipid A of LPS (Fig 2). By contrast, cationic charges are observed to be rather disorientated or diffused in the 'V shaped helical structure of the mutant MSI-594FA (Fig. 3B). Notably, in the non-compact helical structure of MSI-594F5A positively charged sidechains of residues K4, K7 and K18 remain far from the required distance for binding to the di-phosphate groups of lipid A. Such structural differences are plausibly key elements contributing lower LPS binding affinity and outer membrane permeabilization of MSI-594F5A. These studies have underscored importance of LPS bound structure of a potent AMP with its antibacterial activity.

3.2.3 3-D structure and localization of pardaxin in LPS micelles

Pardaxins (Pa1, Pa2, Pa3 and Pa4) are host defense peptides isolated from sole fishes [96]. These peptides are characterized by multiple biological effects ranging from neurotoxins to antimicrobial activities [96, 97]. Pardaxins are investigated as model systems for pore forming membrane active peptides [97]. Due to potent antibacterial activity and low hemolytic activity, pardaxins have drawn considerable attention for antimicrobial drug development [98]. Structural and biophysical studies of pardaxins in model membranes indicated oligomeric forms that may induce barrel stave pore in membrane [97]. 3-D structure of Pa4 (GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE-amide) has been reported in DPC micelles showing a long C-terminal helix and a more flexible N-terminal helix/turn conformations [99]. Despite a net low positive charge, only two Lys residues, Pa4 and other pardaxins contain antibacterial activity. Specifically, pardaxins permeabilizes highly negatively charged LPS outer membrane exerting lethal effect against Gram-negative bacteria. In order to understand, mechanism of action of pardaxins on the outer membrane, 3-D structure and interactions of Pa4 were probed in *E. coli* LPS using a variety of techniques [100]. Pa4 efficiently permeabilizes outer membrane of *E. coli* cells as revealed from 1-*N*-phenylnaphthylamine (NPN) dye uptake assays. Using ITC, energetics of interactions Pa4 with LPS have been determined. LPS-Pa4 complex formation was found to be entropy driven (at 298 K) with ΔG ~ -6.82 Kcal/mol and K_d of 8.5 μM. ^{31}P NMR, FITC-LPS fluorescence and DLS studies revealed that binding of Pa4 to LPS micelles cause structural perturbations of LPS and disaggregation to small size micelles. 3-D structure of Pa4 was obtained in complex with LPS micelles by NMR method. Pa4 assumes helix (residue L5-S12)-loop (residues P13-F15)-helix (residues K16-Ser28) structure resembling a 'horseshoe' type fold (Fig. 4A). The LPS bound structure of Pa4 is distinctly different from the structure obtained in solution of DPC micelles. LPS induced structure of Pa4 demonstrates packing interactions among non-polar residues in which aromatic sidechain of residue F15, from the loop region, formed an interface between the two helices. In particular, residue F15 delineates van der Waals' packing interactions with sidechains of residues I9, I10 of the N-terminal helix and sidechains of residues L18 and L19 of the C-terminal helix (Fig. 4B). Strikingly, in the 3-D structure the sidechain NH_3 groups of cationic residues K8 and K16 are placed within a distance of ~ 13 Å, compatible with the inter-phosphate distance of the lipid A of LPS. Residues of Pa4 in contact with LPS micelles were deduced by two-dimensional STD-TOCSY experiments. STD data demonstrated that Pa4 is highly embedded with LPS micelles, namely aromatic residues F2, F3 and F15 and several non-polar residues e.g. L5, I6, I9, I10, L14 are in close contact with LPS micelles. Two Lys residues, K8 and

K16 of Pa4 are also found to be in close proximity with LPS micelles. 3-D structure of Pa4 and its interactions with LPS micelles have provided mechanistic insights into outer membrane permeabilization. The horseshoe shaped helix-loop-helix structure of Pa4 may be inserted into the outer membrane through intimate packing of non-polar residues with acyl chains of LPS. The distance compatibility of the sidechains of K8 and K16 with the negatively charged phosphate of lipid A might initiate the attachment of Pa4 onto the LPS outer membrane. Outer membrane interactions could be further enhanced by potential hydrogen bonds among polar residues e.g. S11, S12 and T17 of Pa4 with the hydrophilic sugar residues of LPS. Insertion of Pa4 into outer membrane might disrupt higher order packing interactions among LPS molecules leading to a permeable outer membrane.

3.2.4 3-D structures and synergistic interactions of temporins in LPS micelles

Temporins are a family of short AMPs (8 to 13 residues long) found in skin secretion of European frogs [101]. Interestingly, primary structures of temporins contain only few cationic residues and more non-polar residues. Due to shorter length and low cytotoxicity, temporins are attractive targets for antibacterial drug development [102, 103]. However, many temporins are poorly active against Gram-negative bacteria [102, 103]. LPS has been found to be important for regulation and activity of temporins [56, 57]. Low Gram-negative activity of temprions and other AMPs have been attributed to their self-associations or aggregations in complex with LPS [56, 57]. Interactions and aggregations of temporin-1 Ta or TA (FLPLIGRVLSGIL-amide) and temporin-1 Tb or TB (LLPIVGNLLKSLL-amide), in LPS outer membrane are thoroughly investigated [56, 57]. It has been proposed that aggregated states of temporins may be unable to translocate to inner membrane limiting cell killing activity. By contrast, temporin-1 Tl or TL (FVQWFSKFLGRIL-amide), lacking self-association in LPS, exhibits broad spectrum antibacterial activity [56, 57]. Moreover, TL imparts a synergistic mechanism in *E. coli* 0111:B4 LPS, containing long sugar chains, either with TB and TA alleviating LPS induced self-association of peptides [56, 57]. Atomic resolution structures and interactions of TL, TL+TB in *E. coli* 0111:B4 LPS have been determined by NMR spectroscopy and other techniques [104]. In complex with LPS, TL adopts a novel dimeric anti-parallel helical structure. The amphipathic dimeric structure of TL demarcates a cationic face and a hydrophobic face constituted by basic residues and aromatic, nonpolar residues from both the helical sub-units. STD-NMR studies have demonstrated that cationic and aromatic/non-polar faces residues of TL dimer are in contact with LPS micelles. These cationic residues of dimeric TL appears to be involved in ionic and/or hydrogen bonding with the di-phosphate groups of lipid A of LPS as suggested by ^{31}P NMR experiments. On the other hand, the non-polar face of the dimeric TL may be involved in packing interactions with the acyl chains of LPS. Atomic-resolution structure of TB in *E. coli* 0111:B4 LPS could not be determined because of existence of multiple oligomeric states. However, in the presence of TL, oligomeric states of TB are destabilized and a monomeric helical structure of TB has been deduced in LPS micelles. STD and ^{31}P NMR showed that TL+TB combined delineates enhanced interactions and perturbation of LPS micelles compared to independent TL and TB peptides. In another NMR study, TA peptide also exhibited oligomeric forms in *E. coli* 0111:B4 LPS micelles employing N and C-termini residues [105]. An oligomeric model structure of TA had been proposed whereby N and C-termini of monomeric helical structures of TA may be critical for self-associations in LPS [105]. NMR investigations of TL, TB and TL+TB and TA in *E. coli* 0111:B4 LPS micelles provide atomic-resolution insights into mechanism of outer membrane disruption and synergistic activity of temporins [104,105]. TB_KKG6A (KKLLPIVANLLKSLL-amide) a variant of TB was designed for board spectrum antibacterial activity [106,107]. TB_KKG6A was found to be antibacterial against a number of Gram-negative and Gram-positive strains and also demonstrated anti-inflammatory activity. TB_KKG6A assumed a monomeric kinked helical structure in LPS and also in the presence of live *E. coli* cells [106, 107].

3.2.5 3-D structures and interactions of β-hairpin AMPs with LPS micelles

Disulfide (S-S) stabilized β-hairpin structures represent an important category of AMPs [15, 25]. By contrast to helical AMPs, β-hairpin AMPs are folded in aqueous solution even in the absence of membrane mimics. Tachyplesins and protegrins serve as representative model systems of β-hairpin AMPs which have been extensively investigated both in terms of functions, analogs and conformations [108, 109]. Structural studies on tachyplesins and protegrins either in detergent micelles or in model lipid bilayers demonstrated preservation of the β-hairpin structure [110, 111]. An earlier study, based on far UV CD experiments, reported that β-hairpin conformations tachyplesin I may be stabilized upon interactions with LPS micelles [112]. A recent NMR study demonstrates that the β-hairpin structures of tachyplesin I become more ordered and compact in complex with LPS micelles [113]. A docked model, of LPS and tachyplesin I, has further revealed interactions within the complex [113]. Although disulfide bonds are known to be important for stabilizing β-hairpin conformations and antimicrobial activity, surprisingly, a cysteine deleted analog of tachyplesin I, or CDT (KWFRVYRGIYRRR-amide) was found to contain antimicrobial activity and less haemolytic compared to the parent peptide [114]. CDT efficiently disrupted outer membrane of *E. coli* cells and neutralized LPS toxicity *in vitro* [114 115]. CDT appeares to be a flexible peptide in free solutions as judged by CD and NMR spectroscopic methods [114,155]. CD and IR studies showed β-sheet type conformations upon binding to LPS micelles or liposome containing LPS. LPS micelles bound structure of CDT has been solved and interactions were mapped using STD-NMR [115]. 3-D structure of CDT in LPS micelles showed a β-hairpin like fold. The β-hairpin structure of CDT has been sustained by two anti-parallel β-strands of residues W2-V5 and Y10-R13 and a type II' β-turn centred at residues R7 and G8 (Fig. 5A). The β-hairpin structure of CDT demonstrates potential inter-strand sidechain packing interactions among non-polar residues and cation-π interactions between residues W41 and R11 (Fig. 5B).

Further, STD-NMR indicated that sidechains of most of the residues including cationic residues R4, R7, R11 and R13 and aromatic residues of CDT are in close proximity with LPS micelles. A model structure of CDT-LPS discloses potential ionic interactions between guanidinium groups of residues R7 and R13 with di-phosphate groups of the lipid A moiety whereas residues R11 and W4 forming cation-π interactions could be buried within acyl chains of LPS. Mechanistically, molecular interactions between CDT-LPS and the compact β-hairpin conformation of CDT might be critical for outer membrane perturbation and translocation to the plasma membrane.

A cysteine deleted analog of protegrin-1 or CDP-1 (RGGRLYRRRFVVGR-amide) has been investigated for antibacterial activity, membrane permeabilization and structures-interactions with LPS [116]. CDP-1 exhibits bactericidal activity against a range of Gram-negative and Gram-positive strains. RR11 (RLYRRRFVVGR-amide) and LR10 (LYRRRFVVGR-amide) truncated variants of CDP-1, at the N-terminal residues, shows lower antibacterial potency. LR10 peptide has been found to be the least active in antibacterial assays. The efficacy of membrane permeabilization of *E. coli* cells follows the order: CDP-1>RR11>LR10. ITC was used to determine binding affinity of CDP-1 and analogs with LPS micelles. CDP-1interactes with LPS micelles with higher affinity, K_d 0.35 μM, as compared to RR11, K_d ~ 0.96 μM and LR10 K_d ~ 3.0 μM. Perturbation of higher order structure of LPS by peptides also follows the same trend. Atomic resolution structures of CDP-1, RR11 and LR10 peptides have been determined in complex with LPS micelles. LPS-peptides structures reveal that CDP-1 and RR11 adopt β-hairpin like structures whereas LR10 assumes extended β-strand type conformations. However, the β-hairpin structure of CDP-1 delineates more amphipathic in comparison to that of RR11. In CDP-1 β-hairpin, cationic sidechains of residues R4, R7, R8, R9 and R14 are situated into one face of the structure whereas aromatic and aliphatic sidechains of residues Y6, F10, V11 and V12 are congregated into the opposite face (Fig. 6A and Fig. 6B). By contrast, β-hairpin structure of RR1 has been found to be less amphipathic. Activity and LPS bound structures of CDP-1, RR11 and LR10 peptides show correlations. An amphipathic β-hairpin structure turns out to be essential for membrane disruption and antimicrobial activity. Linear CDT and CDP-1 peptides indicate that disulfide bonds may not be essential for bactericidal activity. However, β-hairpin structure is the key element for efficient membrane disruption and bacterial cell killing. On the other hand, a compact β-structure has been determined for an 11-residue peptide fragment, LF11, derived from human antimicrobial protein lactoferrin in complex with LPS micelles [117]. The LPS-bound conformation of LF11 observed to be more compact in comparison to conformations in SDS and DPC micelles. In another study, 3-D structure of the V3 loop peptide of gp120 of HIV was determined in LPS micelles by tr-NOESY method [118]. V3 loop peptide adopted β-turn like conformations in LPS [118].

3.2.6 3-D structures and interactions of designed β-boomerang peptides with LPS micelles

A set of 12-residue peptides have been designed to interact with LPS for antiendotoxic and antimicrobial activity [119,120]. These peptides contain a centrally located four cationic residues K^5RKR^8 and four non-polar residues at the N-and C-termini. The first generation peptide YI12 (or YW12): YVLWKRKRMIFI shows relatively weak LPS neutralization potency, however, NMR structure of this peptide in LPS micelles displays a novel fold resembling a 'boomerang' [119]. The 3-D structure of YI12 is defined by the presence of multiple β-turns and a long-range packing between residues W4 and M9. All of hydrophobic and aromatic residues and the centrally placed basic residues have been found to be segregated into an amphipathic structure. A docked structure of LPS and β-boomerang structure of YI12 elucidates potential ionic and/or hydrogen bonds involving cationic sidechains of residues $K^5R^6K^7$ with the negatively charged phosphate groups of lipid A of LPS. The acyl chains of LPS are in close proximity with the aromatic and aliphatic sidechains of residues of YI12 at the N- and C-termini. The β-boomerang structure of YI12 peptide in LPS micelles appears to be sustained by the long-range inter-sidechain packing interactions between residues W4 and M9. In order to correlate antimicrobial and antiendotoxic activity with β-boomerang structure, three peptides have been synthesized, YI12WF:YVLWKRKRFIFI-amide, YI12WY:YVLWKRKRYIFI-amide and YI12WW:YVLWKRKRWIFI-amide where residue M9 was replaced by aromatic residue F, Y, W. Three more peptides were also prepared YI12FF:YVLFKRKRFIFI-amide, YI12LL:YVLLKRKRLIFI-amide, YI12AA:YVLAKRKRAIFI-amide to underscore roles of aromatic and aliphatic packing in β-boomerang structure and activity. Y12WF, YI12WY and YI12WW peptides demonstrate efficient endotoxin neutralization (≥80% neutralization of LPS at 1 EU and 3 EU/mL doses) [120]. Other peptides including YI12FF either show weak or no detectable inhibition of LPS. YI12WF, YI12WY and YI12WW peptides also exhibit activity against tested Gram-negative and Gram-positive organisms, whereas other peptides are devoid of antimicrobial activity [120]. Designed peptides interacted with LPS micelles, however, only active peptides, YI12WF, YI12WY and YI12WW, delineated disaggregation and perturbation of LPS micellar structures. Atomic-resolution structures of active peptides, YI12WF, YI12WY and YI12WW, in LPS complex clearly demonstrate preservation of the β-boomerang fold with persistent long-range aromatic packing between W4 and F, Y and W at the 9th position [120]. By contrast, the LPS-bound structure of the inactive peptide YI12AA showed alternate conformations which are loosely packed and more open. Interestingly, a truncated octa-peptide (GG8: GWKRKTFG-amide) assume folded β-boomerang motif or structured LPS binding motif in LPS micelles even in the absence of N- and C-termini residues [120]. This structured LPS binding motif has been recently been utilized to generate broad spectrum hybrid antimicrobial peptides functioning through LPS outer membrane [121, 122]. Plausible role of the central positive charged residues in forming β-boomerang structure in LPS micelles has also been investigated. Peptides containing KRKR motif either at

the N- or C-termini are found to interact weakly with LPS and lack antimicrobial and endotoxin neutralization activity (our unpublished results). Another peptide YW12D: YVKLWRMIKFIR-amide has been designed by reshuffling YI12 sequence mimicking a tripeptide sequence motif aromatic/hydrophobic-basic-hydrophobic observed in LPS binding proteins [123]. The YW12D peptide demonstrated endotoxin neutralizing potency and assumed a bifacial amphipathic structure for LPS recognition [123]. Altogether, these studies have pointed out important structural and sequence attributes that could correlate the β-boomerang structure with antimicrobial and antiendotoxic activity.

4. LESSONS FROM 3-D STRUCTURES AND LPS-AMP INTERACTIONS

Structures and interactions of AMPs with LPS provide mechanistic insights into outer-membrane permeabilization, synergistic activity and endotoxin neutralization. 3-D structures of potent AMPs in complex with LPS demonstrated compact structures in terms of sidechain and backbone folding. Helical hairpin structures of MSI-594 and pardaxin showed inter sidechain packing interactions. The dimeric helical structure of 13-residue long temporin L also delineated close packing among aromatic sidechains. The β-hairpin structures of CDP, CDT and β-boomerang structures of design peptides were stabilized by extensive sidechain/sidechain packing. 3-D structures of AMPs in LPS also showed an optimal distance of nearly 13-15 Å among the sidechains of cationic residues which is geometrically compatible for interactions of with the two phosphate groups of the lipid A of LPS. Interactions studies of LPS-AMPs by STD-NMR revealed that most of the residues of AMPs may be intimately associated with LPS. Compact conformations, binding of cationic sidechains to lipid A and extensive association of AMPs in complex with LPS are likely to be the key elements for outer-membrane permeabilization and translocation to the inner membrane and cytosol. These structural elements of AMPs may be involved in neutralization of endotoxin. However, not all AMPs can neutralize LPS toxicity, therefore, additional factors are likely to be involved in endotoxin neutralization.

5. CONCLUSIONS AND FUTURE OUTLOOK

Outer-membrane LPS is actively related to the mode of action of AMPs. Atomic-resolution structures and interactions of AMPs in complex with LPS are important for the development of potent antimicrobial and antiendotoxic therapeutics. This review summarizes successful applications of NMR methods, tr-NOESY and STD-NMR, in determining 3-D structures and mapping of amino acid residues in contact with LPS for different structural classes of AMPs. Current studies imply that AMPs structures in LPS micelles are critical for membrane permeabilization and translocation to the cell interior. These studies have also elucidated vital structure-activity correlations of AMPs with LPS outer membrane. It may be noted that LPS provides a native-like environment for AMPs compared to synthetic lipids and detergents frequently used to mimic membranes. Therefore, 3-D structures of AMPs obtained in LPS might find a better correlation with mechanisms of action. Although more structural studies of high-resolution of AMPs with LPS would be highly essential to establish a firm basis of structure-activity relationship. Towards this, $^{15}N/^{13}C$ labelled AMPs may be prepared for heteronuclear NMR studies. Several fusion protein systems are known for expression and isotope labelling of AMPs and membrane active peptides in *E. coli* cells [124, 125]. In addition, use of isotope labelled LPS and LPS derivatives in lipid bilayers for solution state and solid state NMR would enhance structural resolution of LPS-AMP complexes.

CONFLICT OF INTEREST

Author confirms that the content of this article has no conflict of interest.

ACKNOWLEDGEMENTS

Funding from the Ministry of Education (MOE), RG11/12 is gratefully acknowledged. I thank Dr. Mukesh Mahajan for helping with the figure preparation.

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FIGURE CAPTIONS

Figure 1: Structure of lipopolysaccharide (pdb:3FXI) showing acyl chains as white sticks, phosphate groups as red balls, sugar moieties are labelled as GlcN, Kdo and Hep.

Figure 2: Atomic resolution structure of MSI-594 (pdb: 2k98) docked onto LPS showing ionic and/or hydrogen bonding interactions for cationic residues of MSI-594 with the phosphate groups of lipid A moiety of LPS. Proximity of the sidechains of non-polar residues I2, F5, V16 and L20 with the acyl chains of LPS indicated plausible packing interactions.

Figure 3: Ribbon representation of the 3-D structure of MSI-594F5A (pdb: 2L36) showing secondary conformation and sidechain disposition (panel A) and orientation of the sidechains of cationic residues in the V shaped curved helical structure (panel B).

Figure 4: Ribbon representation of the 3-D structure of pardaxin (pdb:2KNS) showing secondary conformation and sidechain disposition (panel A) and packing of the

sidechains of non-polar residues in the helical hairpin structure (panel B).

Figure 5: LPS-bound structure of CDT peptide (pdb: 2LM8) showing β-hairpin backbone folding (panel A). In the 3-D structure of CDT the cationic sidechains of R13 and R7 are separated by a distance of 15Å which is geometrically compatible to the inter-phosphate distance, 13Å of lipid A of LPS (panel B)

Figure 6: LPS-bound structure of CDP peptide (pdb: 2MQ5) showing β-hairpin backbone folding (panel A). (panel B) Amphipathic disposition of the sidechains of cationic and non-polar residues of CDP.

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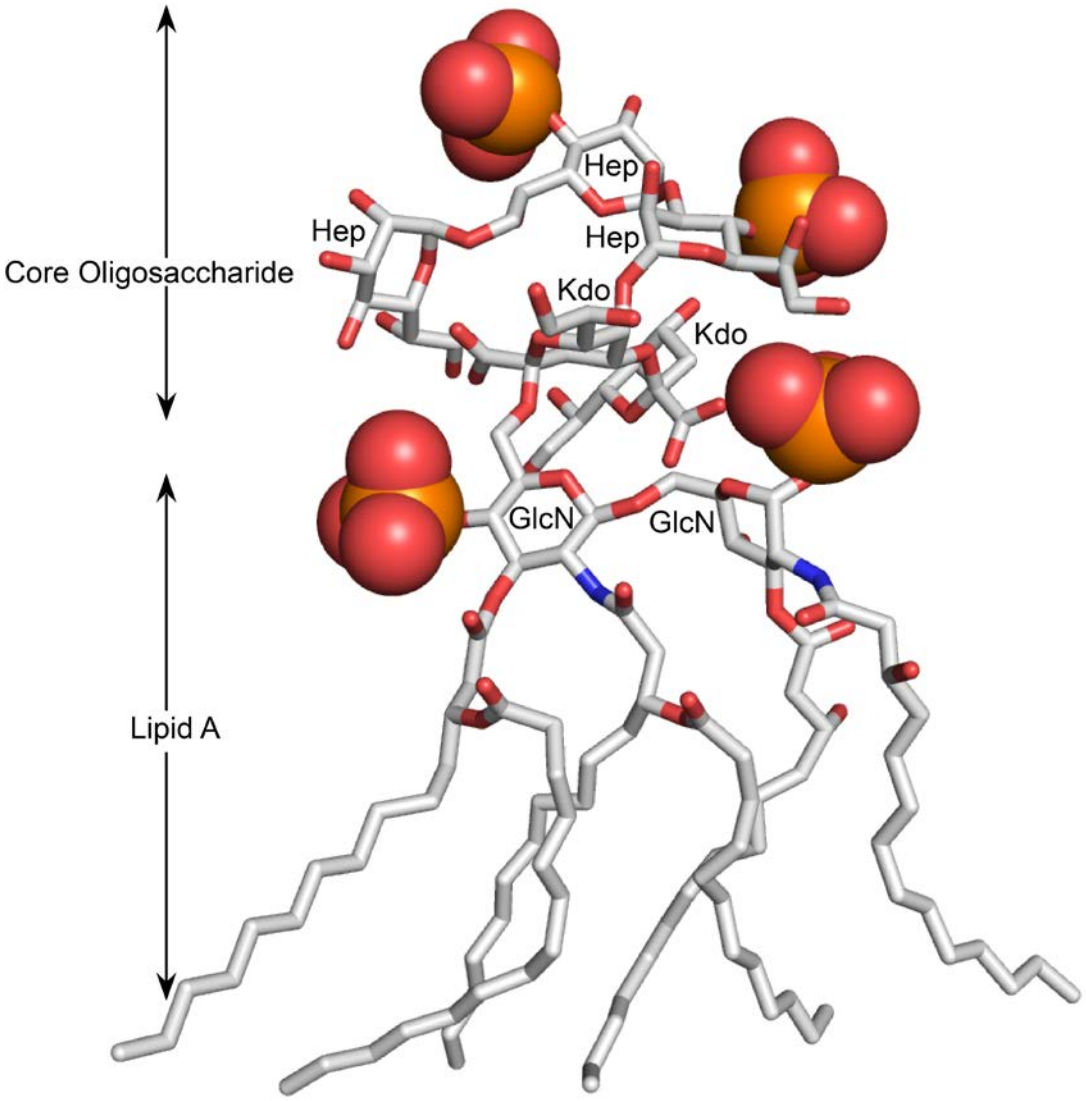

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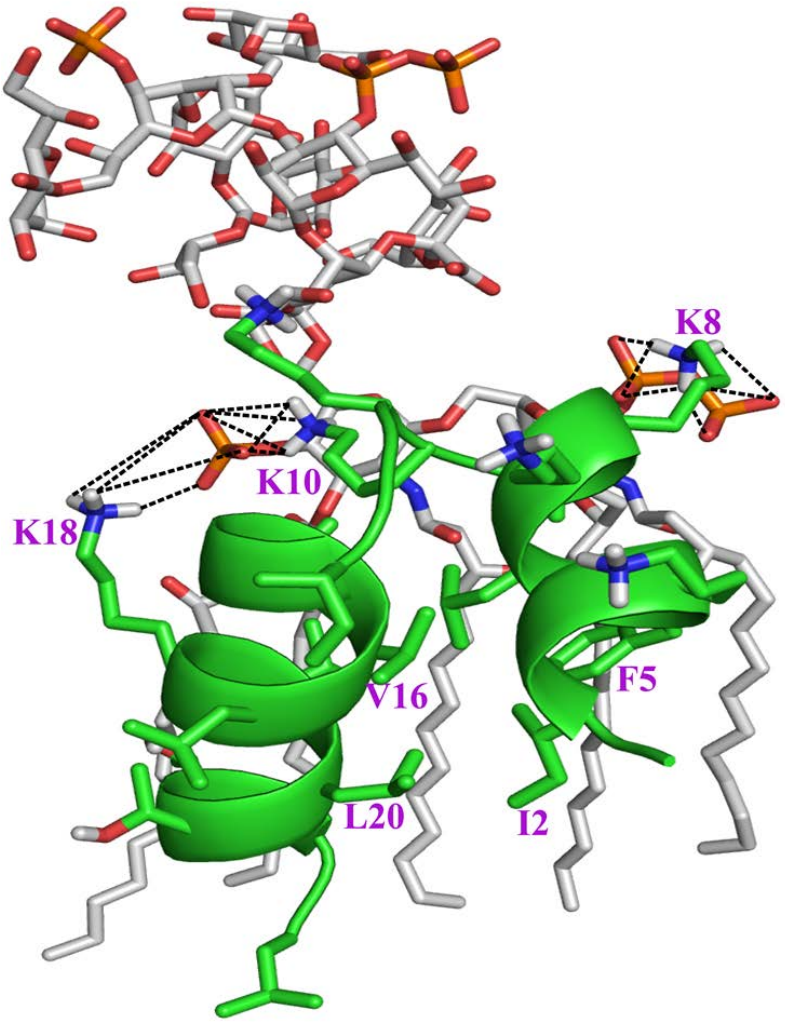

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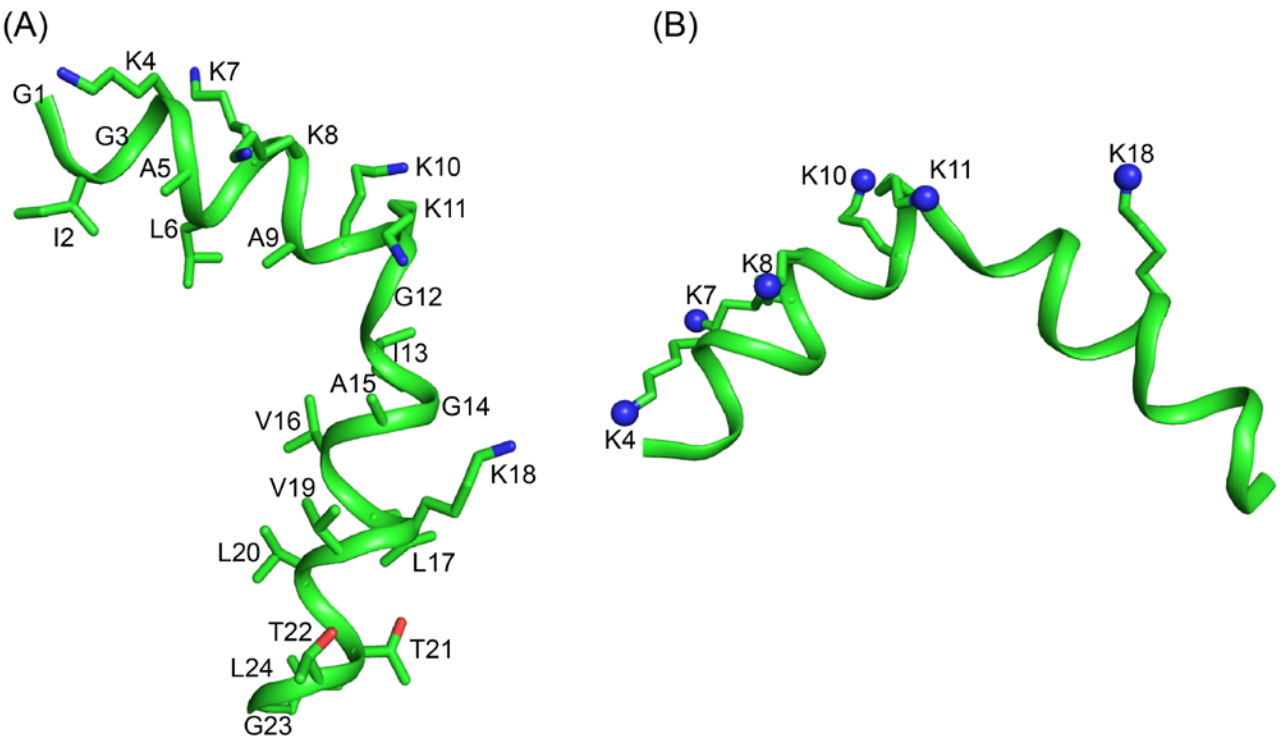

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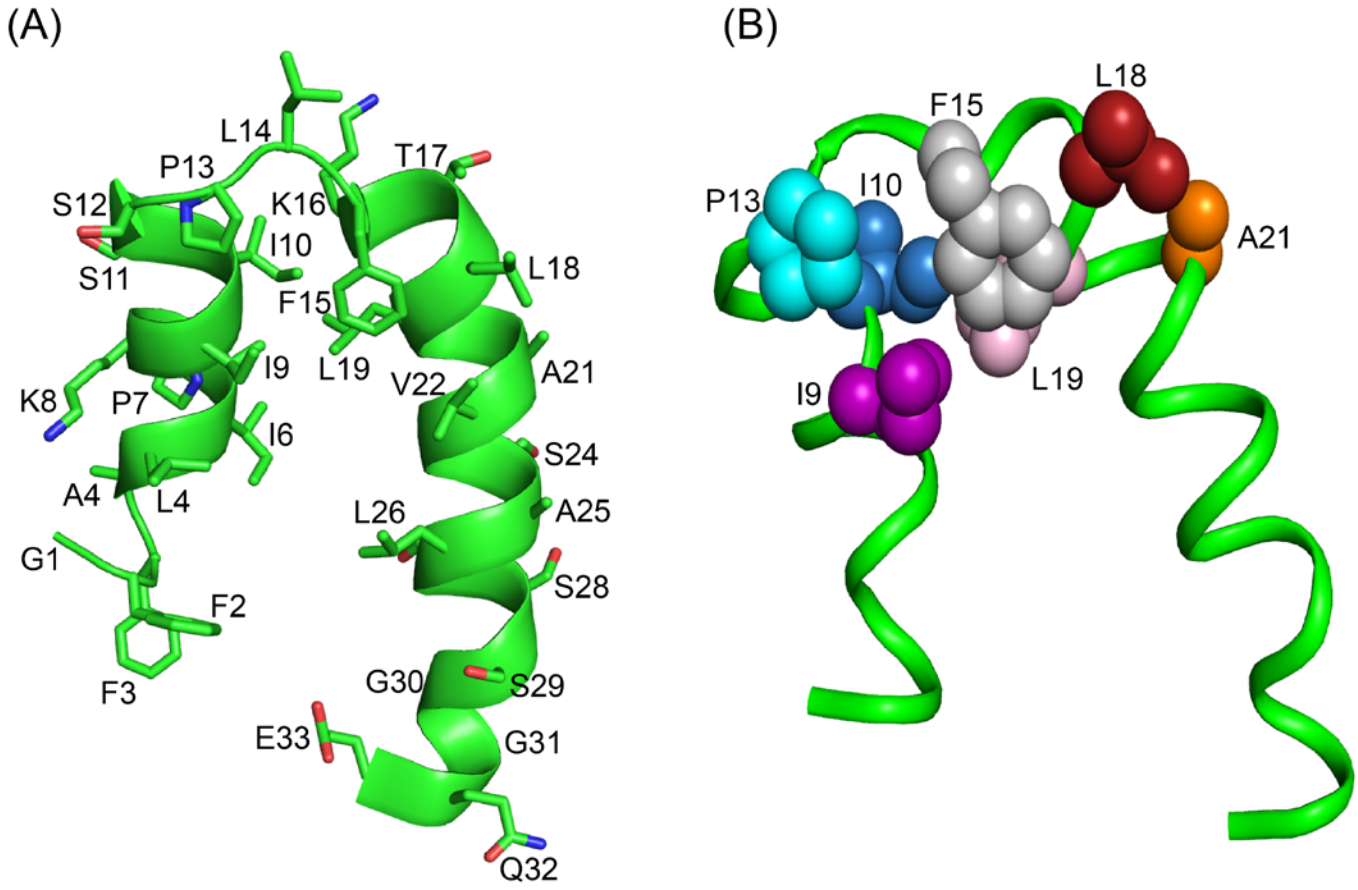

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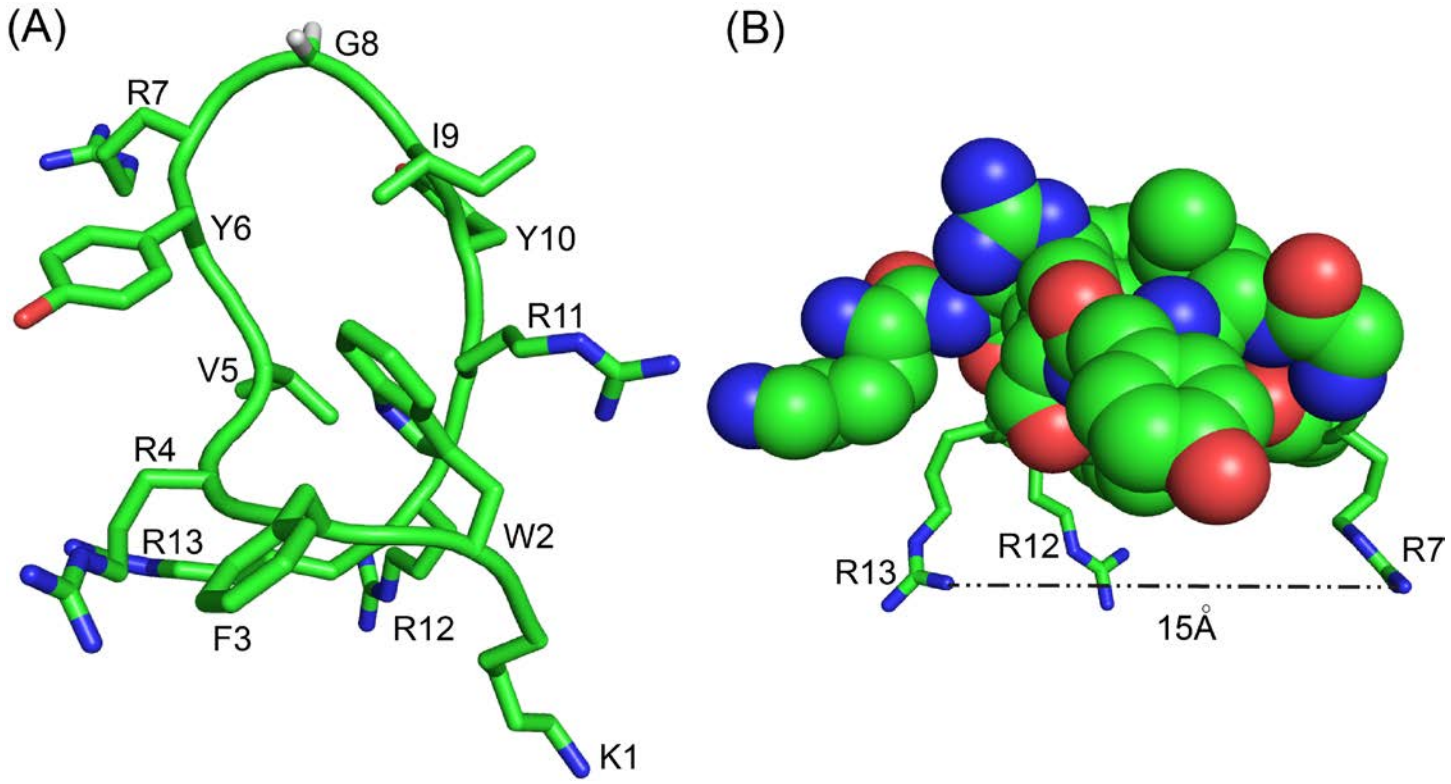

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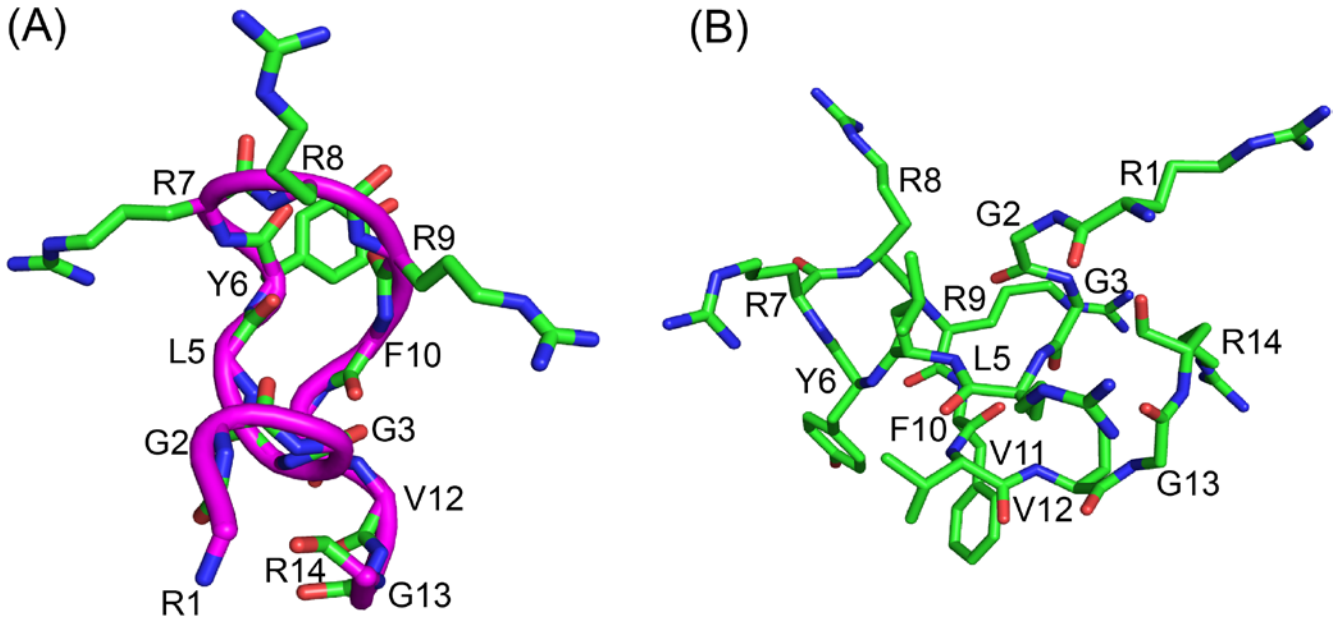

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