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Review

The Sushi peptides: structural characterization and mode of action against Gram-negative bacteria

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Abstract. The compositional difference in microbial and human cell membranes allows antimicrobial peptides to preferentially bind microbes. Peptides which specifically target lipopolysaccharide (LPS) and palmitoyl-oleoyl-phosphatidylglycerol (POPG) are efficient antibiotics. From the core LPS-binding region of Factor C, two 34-mer Sushi peptides, S1 and S3, were derived. S1 functions as a monomer, while S3 is active as a dimer. Both S1 and S3 display detergent-like properties in disrupting LPS aggregates, with specificity for POPG resulting from electrostatic and hydrophobic forces between the peptides and the bacterial lipids. During interaction with POPG, the S1 transitioned from a random coil to an α-helix, while S3 resumed a mixture of α-helix and β-sheet structures. The unsaturated nature of POPG confers fluidity and enhances insertion of the peptides into the lipid bilayer, causing maximal disruption of the bacterial membrane. These parameters should be considered in designing and developing new generations of peptide antibiotics with LPS-neutralizing capability.

Keywords. Synthetic antimicrobial peptides, Sushi peptides, LPS-binding and disruption, membrane phospholipids.

Introduction

Gram-negative bacteria (GNB) are among the most challenging pathogens to the human host [1–3]. Bacterial infections can originate from exogenous and endogenous sources [4–6], causing excessive release of inflammatory cytokines, leading to multiple organ failure and death. Infection by GNB is the leading cause of sepsis [7, 8]. Since resistance is rising rapidly against traditional chemical antibiotics, new antimicrobials which elicit novel mechanisms of actions are urgently needed. Thus, emphasis has been placed on the development of antimicrobial peptides into efficacious antibiotics. Some of these peptides are derived from proteins which bind or target lipopolysaccharide (LPS), also known as endotoxin, and other anionic microbial phospholipids. Other peptides which are undergoing clinical trials are rationally designed. Some of these include nisin, which has undergone phase I clinical trials [9]; echinomycin, in phase II of study [10]; recombinant bactericidal/permeability-increasing protein (BPI_{21}), in phase III of study, has provided supportive

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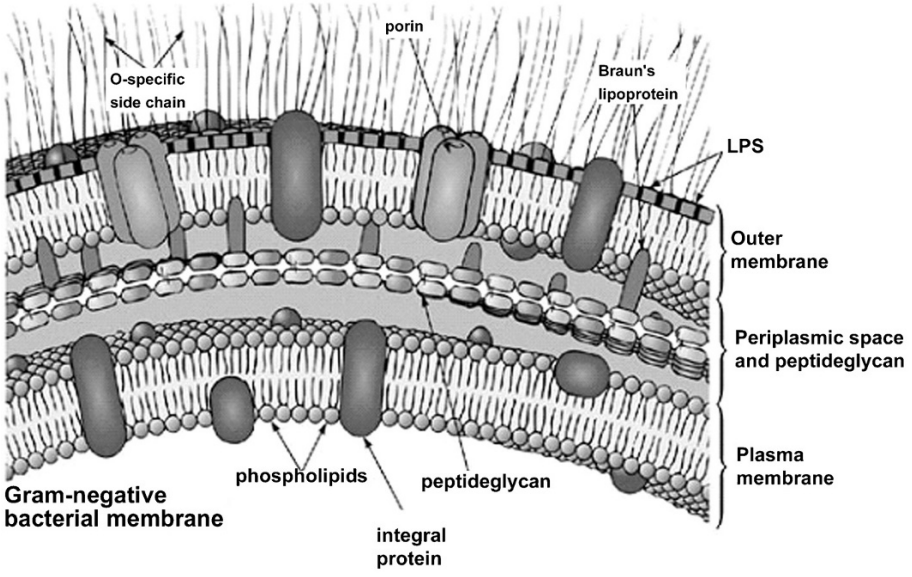


Figure 1. The location of lipopolysaccharide on the outer membrane of Gram-negative bacteria. LPS, lipopolysaccharide. Adapted with some modifications from [15], with permission from McGraw-Hill, NY.

therapies with the ability to dramatically neutralize endotoxicity [9]. These peptides promise to be more powerful antibiotics of the future and to astutely overcome resistance, since they act at the initial stage of Gram-negative pathogen invasion by binding avidly to LPS to (i) incapacitate bacterial cell membrane division and/or survival and (ii) prevent LPS-mediated inflammation and septic shock. Rapid, high-affinity binding to LPS and neutralization of endotoxicity are crucial to preventing potential transformation of bacteria into resistant strains.

Gram-negative bacterial membrane

The GNB membrane, which is a metabolite-based armor, is a very well studied target of peptide antibiotics. Absent in eukaryotic hosts, and unique to the bacteria, these metabolites are collectively known as pathogen-associated molecular patterns (PAMPs). One such PAMP is LPS, also known as pyrogen (Figs. 1, 2), which causes fever when introduced intravenously. Despite extensive research on Gram-negative septicaemia and efforts to develop antibiotics, infection by GNB is still a leading cause of endotoxaemia, accounting for 45–60% of sepsis [7, 8]. In GNB infection-related sepsis, LPS stimulates the host's macrophages to release inflammatory cytokines, which alert the host of pathogen invasion. However, excessive inflammation causes septic shock. Subsequently, multiple organ failure ensues and becomes the main clinical problem and cause of mortality [11]. Persistent exposure to LPS drives the inflammatory response out of control, leading to septic shock that rapidly kills the patient before the bacteria could cause any direct harm [12]. In the United States, a multicentre observational cohort study has projected an estimated 751000 cases of sepsis per annum [13]. In Singapore, septicaemia is the 10th principal cause of death, with approximately 100 casualties each year [14].

Subtle differences in the membrane phospholipids of bacteria and mammalian cells

Phospholipids are important constituents of bacterial and mammalian cell membranes. Interestingly, the composition of bacterial membrane phospholipids is rather different from that of mammalian cells [18–20]. The mammalian cell membrane comprises mainly phosphatidylcholine (~45% PC), phosphatidylethanolamine (~15% PE), phosphatidylserine (~5% PS) and small amounts of other phospholipids, most of which are neutral at physiological pH. In contrast, the *Pseudomonas* membrane harbors substantial amounts of negatively charged phospholipids, such as phosphatidylglycerol (PG), in addition to neutral phospholipids like PE (Table 1) [21, 22]. Figure 3 illustrates the relative composition of the phospholipids in the bacterial and mammalian cell membranes. Bacterial phospholipids such as PG which are negatively charged are similar in structure to lipid A of LPS [23]. Both lipid A and anionic phospholipids have negatively charged head groups and lipid tails. Thus, some anionic phospholipids, for example PG, are thought to be bacterial membrane mimics. Furthermore, purified PG strongly interferes with the interaction between LPS and its receptors on the host cell membrane, thereby inhibiting LPS-induced tumor necrosis factor (TNF)-α production [24–26]. However, whether PG

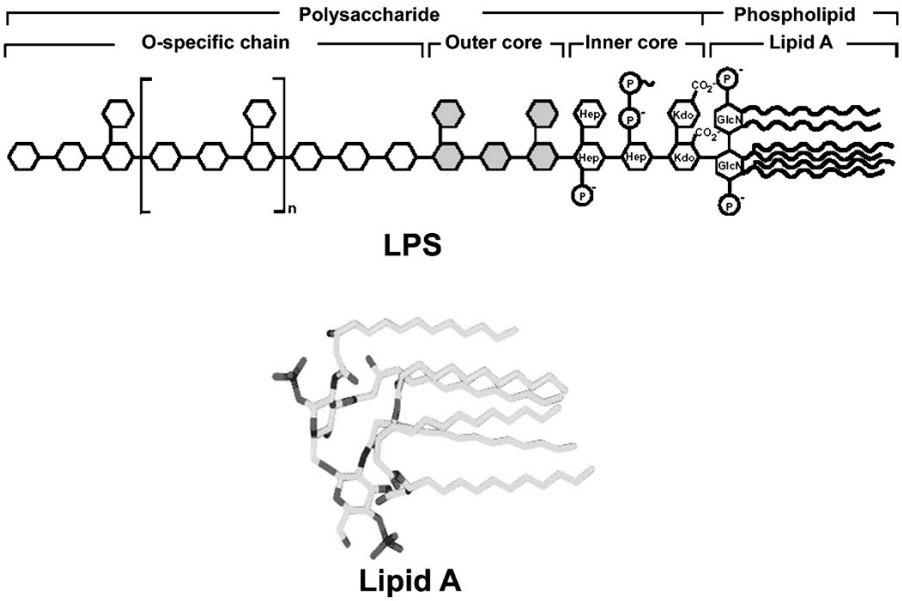
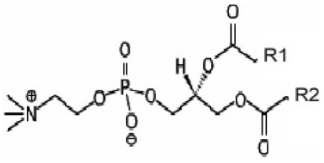
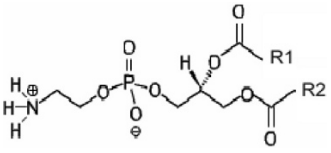
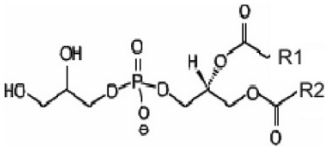
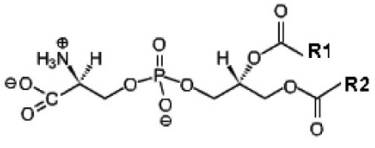


Figure 2. The structure of LPS. LPS consists of an O-specific antigen, a core oligosaccharide and the lipid A moiety. The core oligosaccharide, which varies from one bacterial species to another, is made up of outer and inner sugar regions. Lipid A virtually always includes two glucosamine residues modified by phosphates and a variable number of fatty acid chains [16, 17]. The LPS structure was kindly contributed by Dr Artur J. Ulmer (ajulmer@fz-borstel.de) [16].

Table 1. The characteristics of four kinds of phospholipids [27].

Phospholipids	Full name	Chemical structure*	Head group	Source
PC	phosphatidyl-choline		zwitterionic	mammalian cell membrane
PE	phosphatidyl-ethanolamine		zwitterionic	mammalian cell/bacterial membrane
PG	phosphatidyl-glycerol		anionic	bacterial membrane
PS	phosphatidyl-serine		anionic	mammalian cell membrane

R1 and R2 are fatty acyl chains. Reprinted with permission from Li et al. [27]. Copyright 2007 American Chemical Society.

could be used as an LPS analogue in clinical practice requires further investigation.

The physicochemical features of LPS and its pathophysiological roles

Studies on the molecular biology of Gram-negative septicaemia have so far focused on the chemical structure of LPS [29]. LPS is also referred to as an endotoxin because of its toxic and pyrogenic properties in human and other mammalian hosts. This terminology was introduced in the 19th century to describe the pathophysiological phenomena associated with GNB infection. Endotoxins are uniquely thermostable, fairly insensitive to pH changes, indomitable and ubiquitous chemical molecules. Removing endotoxin requires high heat of 200 °C for at least 2 h,

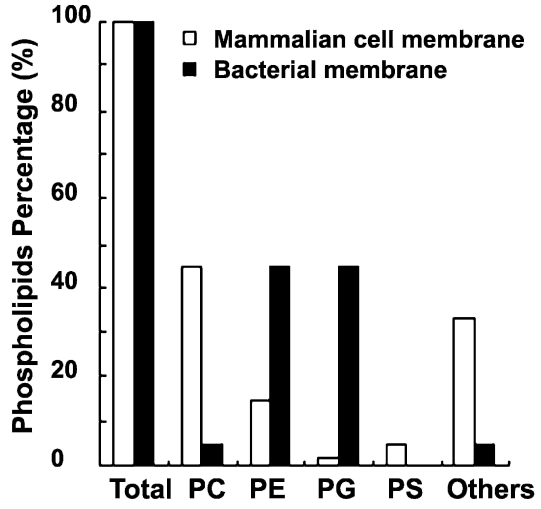


Figure 3. The composition of phospholipids on membranes. White bars represent the mammalian cell membrane, and black bars denote the bacterial (e.g. *Pseudomonas*) membrane. This plot is based on information obtained from the scientific literature [19, 27]. Reprinted with permission from Li et al. [28]. Copyright 2007 American Chemical Society.

or short durations in high concentrations of acids or bases, for example in citric acid, pH 1.0 for 3 min. The envelope of a single *Escherichia coli* contains 2×10^6 LPS molecules, constituting about 20 femtograms [30]. LPS molecules are of great compositional and structural diversity, and yet they share a common architectural principle (Fig. 2).

Generally, LPS comprises three covalently linked domains: (a) the O-specific antigen, made up of a chain of repeating oligosaccharides of three to eight units which are strain-specific and determine the serological identity of the respective bacterium [31]. For this reason, considerable structural diversity is noted among the O-specific antigen chain structures of LPS from different GNBs. Furthermore, the major anti-LPS immune response is directed to the O-chain polysaccharides. However, it is reported that the O-specific antigen of LPS is not necessary for bacterial growth or survival [32]. (b) The core oligosaccharide, with an inner KDO (2-keto-3-deoxy-D-mano-octonate)-heptose region and an outer hexose region, is made up primarily of glucose, galactose and N-acetylglucosamine (GlcNAc) [33, 34]. The hexose molecules in the outer core are more variable than that in the inner core. The KDO sugar is linked directly to the lipid A moiety [35]. (c) Lipid A, which is the minimum moiety of LPS capable of sustaining bacterial growth and survival, is the most conserved moiety of the LPS molecules from diverse strains of GNB. The lipid A moiety is composed of two to three KDO residues [36] attached to a phosphorylated β1,6-linked D-glucosamine disaccharide, carrying variable numbers of asymmetrically placed amide or ester-linked acyl chains. As the bioactive centre, lipid A induces pathophysiological responses in the host. The most convincing evidence comes from studies of free lipid A [37] and synthetic lipid A [38], both of which show full endotoxic activity. The unique structure of lipid A reflects its important roles in the outer membrane assembly, and it ensures resistance to phospholipases.

The Achilles' heel of LPS-binding proteins – provocation of septic shock

Upon infection, the bacteria release LPS as a virulence factor into the bloodstream to trigger the innate immune system. This initiates a series of defenses against the invasive GNB which can result in inflammation and septic shock (Fig. 4). The LPS-binding protein (LBP) binds the lipid A moiety of LPS [40]. The LBP-LPS complex subsequently interacts with CD14 [41], a 55-kDa glycophosphatidylinositol (GPI)-linked receptor protein that is found on the surface of macrophages, monocytes and neutrophils (Fig. 4) [42, 43].

Toll-like receptor 4 (TLR4)-MD-2 complex, localized on the host immune cell membrane [45], senses LPS to induce signal transduction through protein kinases such as p38 and JNK [46], leading to the activation of nuclear factor kappa B (NF-κB), which transactivates expression of numerous pro-inflammatory cytokines, tissue factors, adhesion molecules and inducible nitric oxide synthetase. Amongst the pro-inflammatory cytokines, TNF-α plays a critical role in the LPS-induced inflammatory response [47], which can sensitize the host to an LPS-induced uncontrolled acute inflammatory response encompassing CD14, TLR4, MAPK and NF-κB, resulting in septic shock and multiple organ failure/death (MOF/MODS) (Fig. 4) [48].

LPS also activates the complement cascade, which further fuels the inflammatory response, and the coagulation cascade, which leads to disseminated intravascular coagulation that quickly exhausts the clotting components in the blood, leading to haemorrhage [49]. Thus, at the initial stage of Gram-negative infection, binding of the LPS receptors to LPS triggers an inflammatory response to which an overreaction leads to septic shock and downstream casualties. Accordingly, recent studies are directed towards intervening in this early step with potential LPS-binding drugs to compete against binding of LPS effectors and to attenuate the consequent damage to the host.

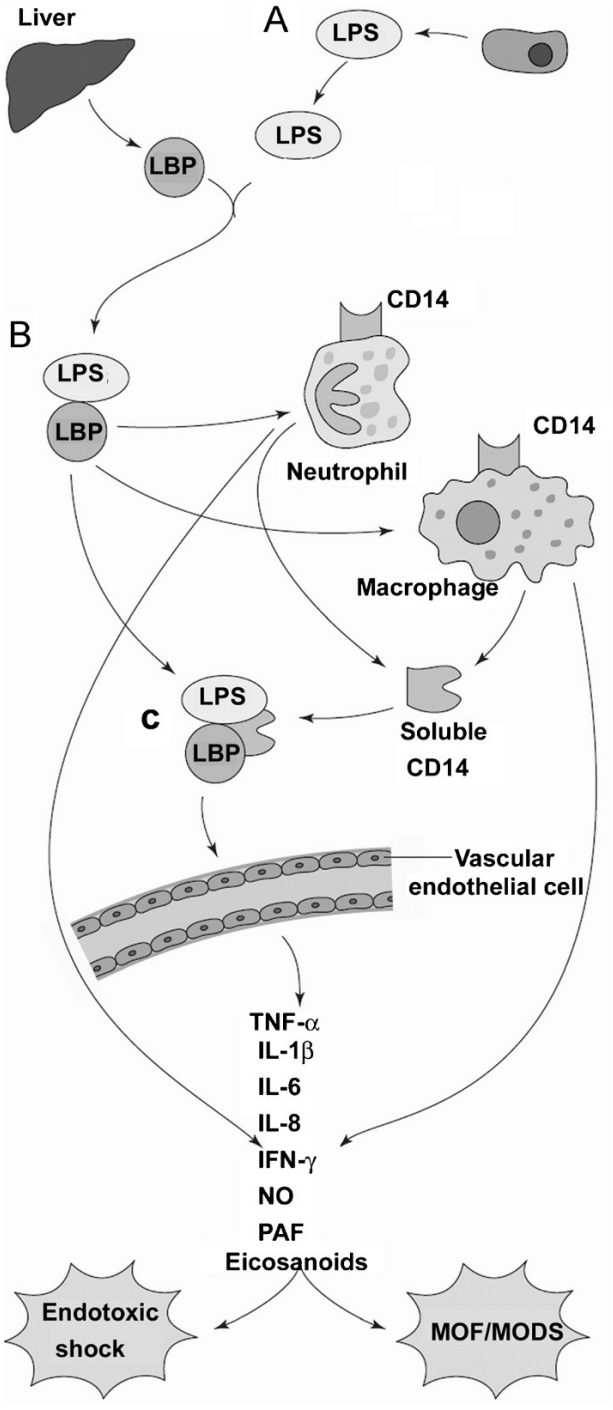


Figure 4. Host cellular activation by lipopolysaccharide. *(A)* In the plasma, LPS is released from the Gram-negative bacteria. *(B)* LPS-binding protein (LBP) transfers LPS to CD14 and facilitates the interactions of LPS with CD14 expressed on the surface of monocytes/macrophages or neutrophils. Endothelial cells and some other types of cells do not express CD14. *(C)* LPS stimulates these cells by binding soluble CD14. IFN-γ, interferon γ; IL-1, interleukin 1; MOF/MODS, multiple organ failure/multiple organ dysfunction syndrome; NO, nitric oxide; PAF, platelet-activating factor; TNF-α, tumor necrosis factor. Adapted from [44] with permission of Elsevier, Copyright Clearance Centre.

LBPs in the hosts

As a countermeasure against GNB invasion, insects and mammalian hosts seem to have evolved numerous proteins and peptides for the purpose of binding and neutralizing LPS [50–52]. It is thus worthwhile to modify and develop these molecules for use against GNB-mediated sepsis. Several LBPs have been isolated from humans, other mammals and invertebrates (Table 2). One of the most well studied LBPs is bactericidal/permeability-increasing (BPI) protein [53]. BPI is a 50–60 kDa membrane-associated protein with remarkable potency and specificity against GNB. BPI can displace LPS from LBP-LPS complex by interacting with the lipid A moiety [54], resulting in inhibition of LPS-mediated pro-inflammatory effects *in vivo*.

Other proteins containing LPS-binding domain(s) include the Limulus anti-LPS factor (LALF), a small basic protein of 101 amino acids, which binds LPS and has a strong antibacterial effect on GNB [62]. Hoess et al. [63] have obtained the crystal structure of LALF. Based on the sequence similarity and other evidence, they suggest that an exposed amphipathic loop, which constitutes a series of alternating positively charged and hydrophobic residues, represents an LPS-binding motif. Another well-recognized human plasma LBP and membrane-bound CD14 (mCD14, a GPI-anchored protein) present at the surface of monocytes are central to the innate immune system. LBP and mCD14 respond strongly to LPS [44, 64]. LBP facilitates binding of LPS to mCD14. Complexes of LPS and CD14 are key intermediates in the cellular response to LPS that provokes septic shock [65].

LBP, CD14, LALF and BPI each bind to one molecule of LPS by recognizing its lipid A moiety [58, 66–68]. These four LBPs possess amphipathic regions deemed to be sites of interaction with amphipathic lipid A. The basic residues are involved in ionic interaction with the phosphate head groups of lipid A.

Smaller antimicrobial fragments with LPS-binding activities that were cleaved from larger proteins *in vivo* have been discovered. For example, lactoferrin (LF) was found to release a smaller antimicrobial fragment called lactoferricin. Similarly, cloning of CAP-18 protein cDNA (complementary DNA) revealed that the identified active smaller antimicrobial fragment corresponded to 37 amino acids at the C-terminus of the precursor which might have been cleaved either after synthesis or on exposure to butyrate [69]. Thus, the derivation of corresponding small LPS-binding peptide or fragments from the larger LPS-binding precursor protein are feasible and more advantageous in binding LPS than the parental native LBPs.

Table 2. Examples of LBPs that have been isolated and characterized.

Protein	Source	Size (kDa)	Properties	References
Bactericidal/ permeabil-ity – increasing protein (BPI)	azurophilic granules of human neutrophils	55	cytotoxicity limited to GNB, blocks signaling by LPS	[55]
CD 14	human monocyte or macrophage	55	LPS recognition protein	[56]
Limulus anti-LPS factor (LALF)	horseshoe crab amoebocyte	12	inhibits endotoxin-induced coagulation in amoebocyte lysate	[57]
LPS-binding protein (LBP)	acute phase serum protein produced by the liver (from human, mice and rabbits)	60	facilitates binding of LPS to CD14 and high-density lipoproteins	[58]
Cationic antimicrobial protein 18 kDa (CAP18)	azurophilic granules of rabbit neutrophils; epithelia of lung	18	contains a 37-aa C-terminal peptide with antimicrobial and anti-LPS activities	[59]
Lactoferrin (Lf)	milk and other secretions; human azurophilic granules of neutrophils	80	a glycoprotein that binds iron and has antimicrobial properties	[60]
Serum amyloid P component (SAP)	a human serum protein	25.5 (sub-unit)	involved in the pathogenesis of amyloidosis; binding and clearance of host- or pathogen-derived cellular debris at sites of inflammation	[61]

Table 3. Sequences of peptides derived from LBPs.

Derived peptide	LBPs	Sequence	References
BPI82-108	BPI protein	NANCKISGKWKAQKRFLKMSGNFDCSI	[80, 81]
BU3	BPI protein	HIKELQVKWKAQKRFLKMSIIVKLNDGRELSLD	[82]
CAP18106-137	cationic antimicrobial protein	GLRKRLRKFRNKIKEKLKKIGQKIQGLLPKLA	[76, 83]
CAP3720-44	cationic antimicrobial protein	NQGRHFCGGALIHARFVMTAASCFQ	[84]
LALF28-54	limulus anti-LPS factor	DHECHYRIKPTFRRLKWKYKGKFWCPS	[77, 80]
LBP82-108	LBP	DSSIRVQGRWKVRKSFFKLQGSFDVSV	[80]
LBP91-105	LBP	WKVRKSFFKLQGSFD	[85]
LF-33	lactoferrin	GRRRRSVQWCAVSQPEATKCFQWQRNMRKVRGP	[86]
MBI-27	Cecropin–Melittin hybrids	KWKLFKKIGIGAVLKVLTTGLPALIS	[87]
MBI-28	Cecropin–Melittin hybrids	KWKLFKKIGIGAVLKVLTTGLPALKLTK	[87]
PMB	polymyxin B	mo-K′TK′K′K′fLK′K′T	[88]
SAP	serum amyloid P component	EKPLQNFTLCFRA	[71]

mo, methyl octanoate; K′, diaminobutyric acid; f, D-Phe.

The derivation of synthetic antimicrobial peptides from LBPs

Recently, intense efforts have been made to derive small antimicrobial peptides from LBPs, mainly because small peptides are less immunogenic and are easily produced by chemical synthesis, and their structures are more readily determined and modified. Thus, small peptides have been derived from naturally occurring proteins such as cationic antimicrobial protein, LBP and serum amyloid P component (Table 3) [70–74].

Many peptides have been derived from the BPI protein and extensively studied for their anti-LPS and antimicrobial activities. Some of these peptides are currently undergoing clinical trials [75]. Multiple patents and companies such as XOMA Corporation (Berkeley, USA) exist to protect the intellectual property of the peptides. Moreover, hybrid peptides of BPI and LBP have also been synthesized and examined for their therapeutic value [68]. The CAP18-derived peptide contains 37 amino acids of the C-terminal LPS-binding domain of rabbit CAP18 protein, which was found to harbor antibacterial activity against various Gram-negative and Gram-positive bacteria [76]. Another peptide derived from the active domain of LALF [27, 29–54] was also found to exhibit antimicrobial activity similar to that of

LALF itself and could potentially be useful as a therapeutic agent [77]. These peptides, derived from parts of LBPs with or without modification, have been analyzed for their high antimicrobial potency. The antimicrobial properties are reportedly related to the positively charged basic amino acids on the putative LPS-binding sites of these antimicrobial peptides [78, 79].

A variety of synthetic antimicrobial peptides have also been rationally designed. These are based on the identified LPS-binding domains of selected LPS-binding peptides and proteins. Using computer-aided molecular modeling and predictions based on amino acid motifs, Frecer et al. [39] have rationally designed a series of 19-amino acid peptides, referred to as V-peptides, that contain LPS-binding sites harboring 'basic-hydrophobic-basic/polar-hydrophobic-basic residues [BH(P)HB]'. Through chemical synthesis of these V-peptides and validation of their antimicrobial potency against various Gram-negative and -positive bacteria versus parameters such as hydrophobicity, lipophilicity, amphipathicity, solubility, hemolytic activity and LPS-binding affinity, Frecer et al. [89] have shown that such rational *in silico* designed synthetic peptides, which are then tested and validated empirically, can give rise to very powerful antimicrobial peptides compared with other synthetic and natural peptides [17].

Because these peptides are derived from or based on LBPs, their biological activities are assumed to be related to their high affinity for LPS binding. In aqueous solution, binding of antimicrobial cationic peptides to LPS appears to involve a dual-step process: (a) preliminary interaction of the cationic amino acids (e.g. lysine, arginine) with the anionic groups of lipid A from LPS (phosphate head groups), followed by (b) stabilization of the resulting molecular complex through hydrophobic interactions between the fatty acyl chains of lipid A and hydrophobic amino acids (e.g. phenylalanine and leucine) as well as alkyl groups of lysine or arginine [90, 91].

Rationale for deriving Sushi peptides from the horseshoe crab

The evolutionary success of horseshoe crabs over 500 million years attests to the organism's strong immune defense mechanisms. It has relied solely on its innate immunity to thrive in microbiologically challenging habitats where myriads of pathogens are found. The horseshoe crab harbors many proteins and peptides that participate in several frontline defense mechanisms to stave off infection: (a) complement cascade involving C3 [92, 93]; (b) melanization reaction in the phenoloxidase-induced production of toxic compounds from a protein like hemocyanin [94, 95]; (c) an antimicrobial peptide defense involving tachyplesin [96]; and (d) an extremely sensitive blood coagulation cascade initiated by an LPS-sensitive protein, Factor C [97, 98]. Amongst the proteins participating in the above-mentioned innate immune response pathways, Factor C, a serine protease, functions as a frontline defense molecule to fight against the invading GNB and LPS.

Factor C: a horseshoe crab serine protease with multiple high-affinity LPS-binding sites

The horseshoe crab hemolymph mainly contains one category of blood cells called amoebocytes which are extremely sensitive to LPS. During Gram-negative infection, the amoebocytes release granular components into the plasma to participate in self-defense via blood coagulation [99], which incapacitates the invading microbe. The amoebocytes contain two kinds of secretory granules: large and small. Studies on these granules suggest that coagulation factors such as Factor C are localized in the large granules, whereas antimicrobial peptides such as tachyplesin are located exclusively in the small granules (Fig. 5) [100]. In the past decade, the molecular mechanisms of the coagulation cascade have been established [101, 102] (Fig. 6). This cascade is based on three serine protease zymogens – Factor C, Factor B, proclotting enzyme and one clottable protein, coagulogen [103]. Because it occurs at the initial step of the coagulation cascade, Factor C functions as a very sensitive and specific biosensor that is capable of detecting picogram to nanogram levels of LPS [104]. LPS from GNB induces amoebocytes to degranulate, thus initiating blood coagulation, which is an important defense mechanism used by horseshoe crabs to trap invading GNB [105]. No homologues of Factor C have been found in mammals, although the C-terminal serine protease domain has substantial similarities (36.7%) with human α-thrombin [106].

The *Limulus* amoebocyte lysate (LAL) test has been used for decades as a tool to detect trace levels of LPS in solution and in medical devices for parenteral use [108]. However, the dwindling population of horseshoe crabs has spurred the search for alternative sources of Factor C. Recent attempts to genetically engineer Factor C have included the use of *Escherichia coli* [109], yeast [110] and mammalian cells [111] as recombinant hosts. These efforts have yielded recombinant Factor C (rFC) capable of binding LPS. Further cloning, molecular manipulatios and expression in baculoviral systems produced an rFC with a remarkable sensitivity of 0.001 EU per millilitre of endotoxin [107, 112]. The biologically functional rFC acts as a biosensor for endotoxin. The rFC remains as a zymogen until it encounters trace levels of endotoxin,

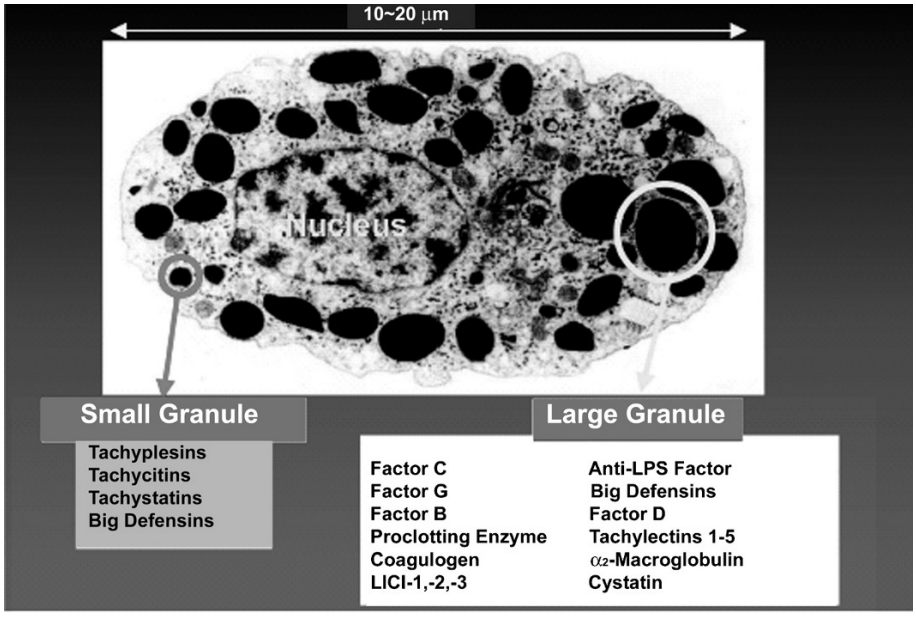


Figure 5. The amoebocytes of the horseshoe crab. Some small antimicrobial molecules, such as antimicrobial peptides that have been identified, are in small granules, while Factor C and other defense molecules are localized in large granules. Adapted from [99] with permission of the editor-in-chief, *Journal of Biochemistry and Molecular Biology.*

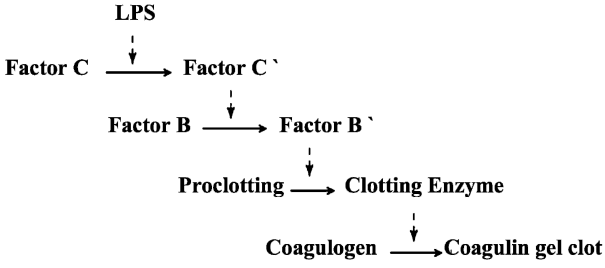


Figure 6. The coagulation cascade in the horseshoe crab amoebocyte lysate. In the presence of LPS, Factor C is autocatalytically activated to an active form, Factor C′. Factor C′ activates the proenzyme Factor B into Factor B′, which in turn activates proclotting to active clotting enzyme. Clotting enzyme then converts coagulogen into coagulin gel clot, which traps the invading bacteria. Adapted from [98, 101, 107] with permission of Elsevier, Copyright Clearance Centre.

whereupon it undergoes limited proteolysis [99]. The altered conformation of the molecule induces full-blown C-terminal serine protease activity, thus indicating the presence of endotoxin in a test sample [107, 113]. New microfluorimetric or microcolorimetric assays integrated into the rFC assay have enabled high-throughput screens of LPS in multiple samples. Currently, the rFC has been used in an assay to sensitively and specifically detect LPS [107]. The rFC has also been incorporated into the PyroGene kit (Lonza, Inc.). Since rFC has extremely high affinity for LPS, it could be a potential LPS-neutralizing 'prototype drug', and it was therefore logical to derive LPS-binding peptides from the Factor C molecule.

Deriving Sushi peptides from Factor C

The LPS-binding region of Factor C from the horseshoe crab, *Carcinoscorpius rotundicauda,* has been systematically characterized. *C. rotundicauda* Factor C (CrFC) is a 132-kDa glycoprotein that consists of a heavy chain of 80 kDa and a light chain of 52 kDa. Near the N-terminus of the heavy chain are several repeats of Sushi domains of ~60 amino acids each, containing several disulfide bonds which hold the Sushi domain in a unique folding structure resembling a sushi, hence its name. The Sushi domains are also known as short consensus repeats (SCRs) or complement control protein (CCP) domains [113]. By expressing and analyzing the N- and C-terminal fragments of the CrFC protein, Ding et al. [98] revealed that the major LPS-binding site(s) in Factor C is located at the N-terminal region. Tan et al. [114] subcloned the N-terminal fragment (CrFCES) and demonstrated that it binds LPS with high affinity of 10^{-12} M. Those small fragments in CrFCES were further subcloned and expressed as recombinant Sushi domains [115]. Subsequently, Wang et al. [112] demonstrated that the Sushi 1 and Sushi 3 domains are the major LPS-binding regions [52, 116]. Although the LPS-binding sites of Factor C have been discovered, defining the precise amino acid residues in the Sushi domains responsible for interacting with LPS would provide insight into the structure-activity relationship as well as the mechanisms of LPS binding.

Based on understanding of the amino acid sequence in the Sushi domains, and comparison of LPS-binding motifs of several other LBPs, Frecer et al. [39] used computer-aided molecular modeling to show that a predominance of lysine and arginine residues occurs

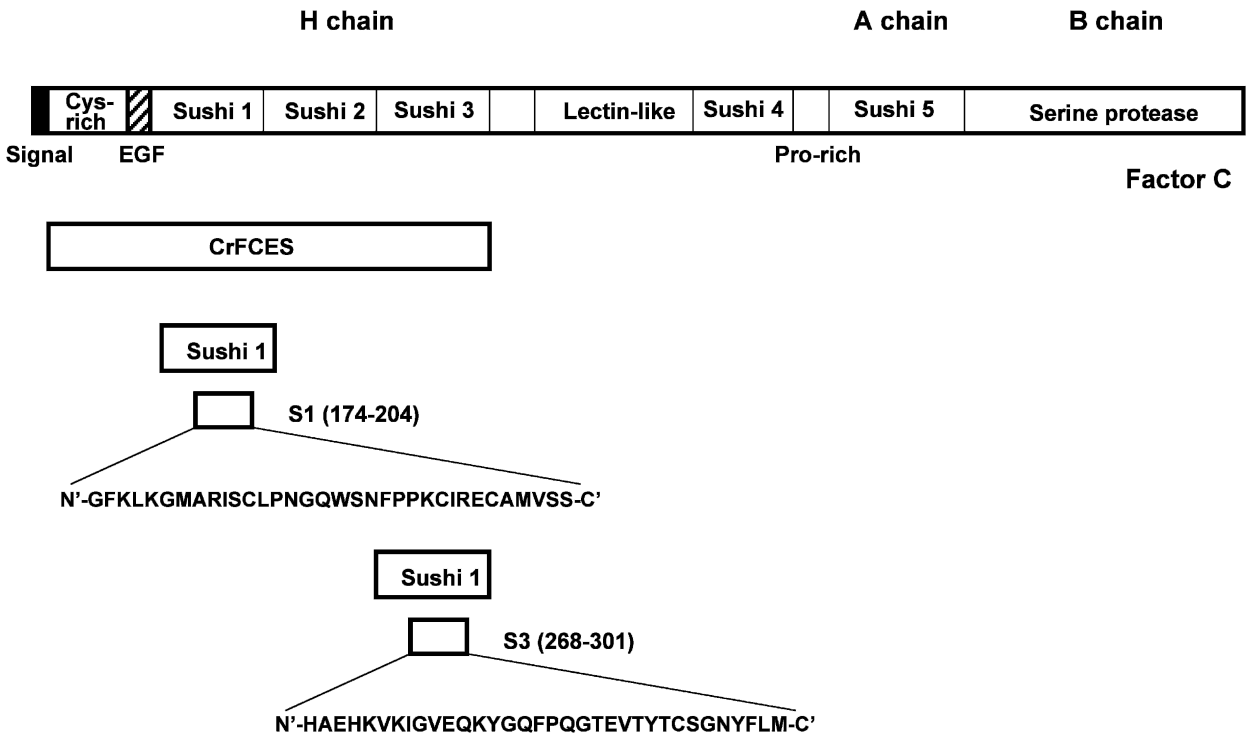


Figure 7. Factor C and its derived Sushi 1 and Sushi 3 peptides. The truncated fragments of the CrFC protein (CrFCES), Sushi 1 domain, Sushi 3 domain, and the S1 and S3 peptides are illustrated as open boxes. Reprinted from Tan et al. [115] with permission of the managing editor, FASEB Office of Publications.

in alternation with hydrophobic residues, BHB(P)HB (B = basic; H = hydrophobic; P = polar). Thus, two 34-amino acid sequences containing BHB(P)HB residues were found within the Sushi 1 and Sushi 3 domains of Factor C. Interestingly, following such a rational approach, Pristovsek and Kidric [52] have further tested various synthetic peptides containing BHB(P)HB motifs and showed LPS-binding and -neutralizing capabilities similar to those of Sushi peptides.

The LPS binding regions of Sushi 1 and Sushi 3 domains reside within these two 34-mer peptides, henceforth referred to as S1 and S3 peptides (Fig. 7). Furthermore, Sushi peptides have been demonstrated to neutralize LPS [115] and, specifically, kill GNBs, particularly that of an opportunistic pathogen, *Pseudomonas aeruginosa* [117], while exhibiting minimal cytotoxicity to human monocytes (THP-1) [115]. This specificity has been thought to be related to the anti-LPS ability and selectivity of the Sushi peptides [117].

The structure of Sushi peptides compared with other synthetic peptides

Based on their secondary structures, antimicrobial peptides are commonly classified into three groups: Group I comprises linear peptides with an α-helical structure, for example, magainin [118] and alamethicin [119]. Group II comprises peptides containing a β-sheet structure and one or more intermolecular disulphide (S–S) bonds, for example, bactenecin [120] and lactoferricin [121]. Group III encompasses other peptides which cannot be included into the two former groups. These are cyclic peptides like trichogin G IV [122], nisin [123] and RTD-1 [124]. Our results demonstrate that the S1 tends towards an α-helical structure. S3, which exhibits a mixture of secondary structures containing β-sheets upon interaction with LPS, has a propensity to dimerize via an intermolecular disulphide bond, which is probably responsible for its high affinity for LPS. Thus, we classified the S1 and S3 peptides into group I and group II peptides, respectively.

S1 and other α-helical peptides

It has been shown that in an aqueous environment, many short peptides have no defined structure. For example, typical amphipathic α-helical peptides such as magainins and cecropins are unstructured in aqueous solution [125]. In contrast, under the same conditions small β-sheet peptides, such as tachyplesin, protegrin and lactoferricin B, are already in a more or less defined amphipathic sheet structure [126]. In view

of the presence of LPS-binding Sushi domain 1 (Sushi 1) at the N-terminal region of the Factor C molecule, the secondary structure of the chemically synthesized 34-mer S1 peptide (N-GFKLKGMAR-ISCLPNGQWSNFPPKCIRECAMVSS-C) was examined. Since S1 peptide contains three cysteine residues, it could possibly form multimers. Furthermore, the structure of S1 peptide could be stabilized by the intermolecular disulphide bond. Thus, the structure-activity relationship of S1 was studied using biophysical methods. It was shown that under physiological conditions, S1 peptide was monomeric with a molecular mass of 4 kDa, and it adopted a random structure in aqueous solution [28]. This is similar to the secondary structure reported for magainin and other monomeric peptides [127–129]. With a relatively high net positive charge (pI of 9.63), S1 monomers may encounter charge repulsion and hence the lack of intermolecular disulfide bond formation. Our work has also suggested that the Trp residue of S1 peptide was partitioned into the hydrophobic acyl chains of lipid A in LPS, like other Trp residue-containing peptides [130, 131]. Since lipid A is the bioactive moiety of LPS, the binding of S1 peptide to lipid A could compete against other LBPs and peptides (e.g. LBP and CD14), and therefore reduce their interaction with LPS in the host. Thus, Li et al. [132] reasoned that this will effectively reduce the probability of LPS-mediated triggering of inflammation. Indeed, the S1 peptide protects human monocytes from endotoxicity exerted by the *Pseudomonas aeruginosa* LPS, showing a substantially reduced level of cytokine production, which is otherwise a hallmark of inflammatory response.

S3 and other β-sheet peptides

Turning to the family of β-sheet peptides, the Sushi 3 (S3) peptide (N-HAEHKVKIGVEQKYGQFPQG-TEVTYTCSGNYFLM-C), which is another Factor C-derived peptide, was investigated. Our work consistently showed that the interaction and activity of the Sushi peptides are related to the peptide structure, particularly when S3 acts as a dimer. The secondary structures confer stability to the S3 peptides for its prolonged and persistent interaction with LPS aggregates prior to their disruption. S1 peptide is random in an aqueous environment and exhibits a characteristic α-helical structure in the presence of anionic phospholipids. In contrast, the distribution of secondary structures of S3 remains unchanged in a physiological buffer, but the content of α-helicity increases slightly in the presence of POPG (palmitoyl-oleoyl-phosphatidylglycerol), producing a mixture of α-helix and β-strand structures [133]. The reason for this difference lies in the intrinsic secondary structures of S1 and S3 peptides, where S3 is probably stabilized by the intermolecular disulfide bond (Fig. 8). This is consistently observed with other peptides such as MSI-78 and MSI-594 [134]. It has been reported that for some peptides, multimers are more active than their corresponding monomers [135–137]. Thus, the single cysteine residue in S3 enables its intermolecular dimerization and probably affects its propensity to interact with the lipid A moiety of LPS.

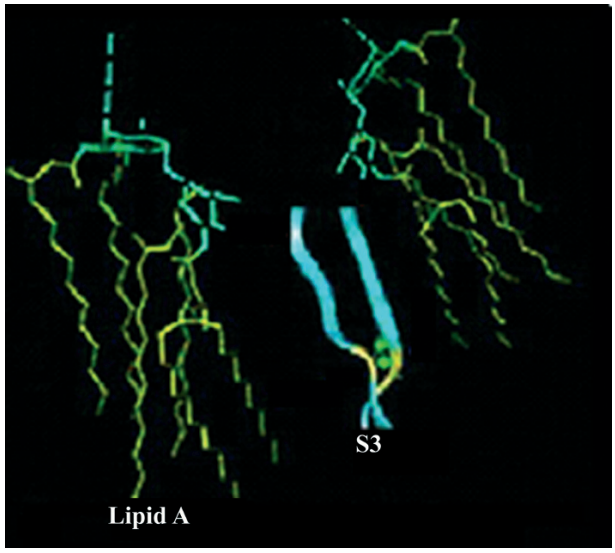


Figure 8. S3 functions as a dimer. Dimerization of S3 peptide occurs through an intermolecular disulfide bond. S3 dimer interacts with lipid A of LPS molecules. Adapted from Li et al. [132] with permission from the ASBMB (American Society for Biochemistry and Molecular Biology).

The disulfide bond stabilizes the secondary structure of the S3 dimer, in agreement with the report of Tam et al. [138]. Presumably, this structure is important for the interaction between S3 dimer and LPS, during which the S3 dimer forms a β-sheet structure to provide better shielding of the hydrophobic acyl chains of LPS (Fig. 8). This may explain why the S3 dimer displays a stronger LPS-neutralizing capability than its monomeric counterpart. This possibility was clearly demonstrated and corroborated by the S3-C27S mutant, which as a monomer exhibits a complete structural transformation, remaining as a random coiled structure even when incubated with LPS [133], with a marked decrease in binding of LPS and a lack of suppression of LPS-induced toxicity [133].

The S3 dimer peptide attains a 'detergent-like' activity after reaching a threshold concentration, consistent with the report by Bechinger for other peptides [125]. *In vivo*, antimicrobial peptides are thought to disrupt bacterial membranes. Peptide multimerization seems to be essential to their mode of membrane disruption.

Some multimeric peptides are found to be more active than their monomeric counterparts. Noncovalent dimerization has also been described for human β-defensin 3, which has been linked to enhanced antibacterial activity compared with the counterpart of human β-defensin 1 and human β-defensin 2, which remain monomeric in aqueous solution [139]. Accordingly, the synthetic and natural dimerized peptide analogue showed enhanced antimicrobial activity compared with that of the monomeric peptide, indicating that dimerization of the β-sheet peptides may potentiate peptide antimicrobial activity [140, 141]. This was also observed by Li et al. [142], who reported that the recombinant tetramer of S3 (rS3-4mer) displays an enhanced inhibitory effect against LPS-induced activities.

Given that S1 and S3 peptides are derived from different LPS-binding domains of the Factor C molecule [115], and that S1 and S3 resume unique structural properties in the presence of LPS [28, 133], it appears that, *in vivo*, Factor C probably harbors versatile modes of interaction against GNB where the α-helical and β-sheet structures of the Sushi 1 and 3 domains, respectively, bind LPS and conceivably cooperate to confer an extremely sensitive LPS-biosensor against Gram-negative infection [107, 143, 144].

The affinity of the Sushi peptides for LPS and lipid A

The affinity of Sushi peptides for LPS and lipid A was tested [115] by surface plasma resonance (SPR) analysis in real-time biointeraction between the Sushi peptides and LPS and lipid A. Within the native Factor C molecule, the Sushi 1 and 3 domains together exhibit K_D values of 10^{-10} to 10^{-12} M for lipid A. When subcloned separately, the recombinant Sushi 1 and 3 domains, each ~60 amino acids long, still showed very high affinity for lipid A at K_D values of 10^{-9} and 10^{-10} M, respectively. The synthetic 34-mer Sushi peptides, S1 and S3, displayed K_D values of 10^{-6} and 10^{-7} M, respectively [115]. Other peptides derived from the LBPs, for example, LBP_{197}, also showed a significant decrease in affinity for LPS [145] . Tan et al. [115] demonstrated that S1 and S3 peptides are able to bind LPS with varying LPS-neutralizing potencies. Both S1 and S3 inhibit LPS-induced LAL (limulus amoebocyte lysate) activity. S1 was shown to bind LPS with positive cooperativity between at least two molecules of S1 and one molecule of LPS (Hill's coefficient = 2.42). On the other hand, a Hill's plot for S3 gave a coefficient of 0.99, indicating simple and non-cooperative binding to LPS. SPR studies also showed that S3 has a 10-fold stronger affinity for LPS than S1, possibly due to the propensity of S3 to dimerize and form a stable structure in aqueous phase [115].

The mechanisms of action of Sushi peptides and other synthetic peptides

The investigation of the mechanisms of action of many synthetic peptides, of either the α-helical or β-sheet type, has been an area of intensive research focus in the recent quest for new peptide antibiotics. Only by understanding the specificity and selectivity of such peptides and how they interplay with various physicochemical forces against the bacterial membrane components instead of interacting with the host membrane molecules can one clearly define a potential prototype peptide candidate that may be worth pursuing for further development into an antibiotic drug. Studies have indicated that the mechanisms of action of the synthetic peptide HP(2-9)-MA(1-12) [146] and alamethicin are associated with the bacterial membrane [147], but the specificity of these peptides to it remains unclear.

The function of Sushi peptides is intricately regulated by its sequence and structural properties. Some of these features, described below, illustrate how various biochemical forces contribute to the specificity of interaction between a synthetic peptide and the bacterial membrane components, culminating in perturbation of the microbial invader's armor while sparing the host cell membrane.

Electrostatic interactions are crucial at the initial stage of bacterial membrane recognition

The action of Sushi peptides is directed specifically to the chemo-landscape on the GNB. Being more well endowed with anionic phospholipids, such as POPG, the head group of this phospholipid confers greater negative charge to the bacterial membrane (Table 1 and Fig. 3). This electrostatic difference contributes subtly to the specificity of the Sushi peptides for LPS. The cationic residues in the S1 and S3 peptides conceivably target the anionic microenvironment formulated by (i) the anionic bisphosphorylated sugar head groups of LPS [39] as well as (ii) the POPG. Thus, the initial electrostatic interaction is crucial in determining the specificity of the Sushi peptides for the bacterial membrane preferentially over the mammalian host cell membrane. Generally, charge–charge interactions between the cationic peptides and the anionic membrane are deemed to be the most critical binding force, explaining why the increase in the net positive charge of the peptides could enhance binding to the anionic lipids [138, 148]. This possibility is corroborated by observations that muta-

tions of the N-terminii of the respective Sushi peptides by introducing two extra lysine residues resulted in an increase in LPS-neutralizing activity [115]. Recent findings suggest that the initial charge interaction is responsible for the selective binding between the peptides and the bacterial membrane [28]. Moreover, this electrostatic difference between the phospholipids accounts partly for the charge difference between the mammalian and GNB cell membranes, ultimately contributing to the specificity of the antimicrobial peptides, which preferentially bind to the exposed anionic surface of bacterial membranes but not to the zwitterionic amphiphiles present in the extracellular monolayer of mammalian plasma membranes. This could spare mammalian host cells from any undesired peptide-induced plasma membrane injury. Thus, the initial electrostatic interaction determines the specificity and selectivity of the Sushi peptides for the bacterial membrane [117].

Hydrophobic forces reinforce and maintain the strength of interaction between Sushi peptides and the bacterial membrane

Hydrophobic forces contribute significantly to the binding interaction between the peptides and bacterial LPS [149, 150]. The microenvironment of the peptide is transformed from a hydrophilic to a hydrophobic state, thus engaging the hydrophobic region of the peptide into an interactive bond with the acyl chains of the anionic bacterial lipids (Fig. 9) [48].

Using NMR (nuclear magnetic resonance), Raman and fluorescence measurements, Bechinger and Matsuzaki et al. [125, 151] have indicated that, initially, the cationic amphipathic peptides would bind parallel to the lipid layer. Therefore, it may be envisaged that owing to the simultaneous electrostatic and hydrophobic forces, the binding of Sushi and other peptides with anionic lipids is highly salt-tolerant [117, 152]. Indeed, Yau et al. [117] have demonstrated that the Sushi peptides maintain their high affinity for LPS and lipid A even at high salt concentrations of 0.3 M NaCl, although the osmolarity of body fluids ranges from 0.12 to 0.15 M in a normal individual. The Sushi peptides were found to maintain their antibacterial function over the salt concentrations tested (0.05–0.3 M NaCl), albeit transitioning from bactericidal to bacteriostatic activity. This indicates the potential applications of Sushi peptides in antibacterial action in controlling the proliferation of *P. aeruginosa* in a high salt environment, similar to the lung fluids of cystic fibrosis patients, where most antibiotics are inaccessible or unsuitable [153, 154]. Hence, Sushi peptides can be developed for topical and aerosol applications.

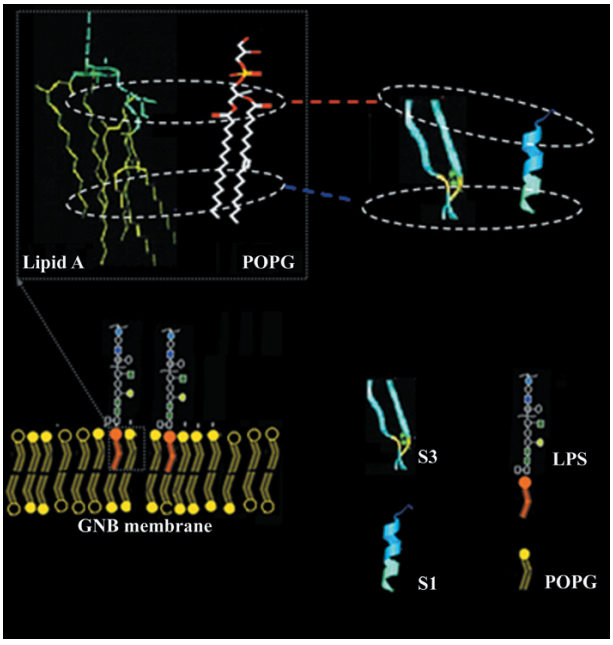


Figure 9. Sushi peptides S1 and S3 interact with bacterial membrane lipids. The positively charged amino acids at the N-termini of S1 and S3 contribute to electrostatic interaction with the diphosphoryl head groups of the LPS/POPG; the C-termini are more hydrophobic, and probably interact with the hydrophobic acyl chains of the LPS/POPG, forming a stable molecular complex. Taken together, both the electrostatic (red dashed line) and hydrophobic interactions (blue dashed line) are important in binding of Sushi peptides to bacterial lipids, which contributes to the specific binding of Sushi peptides to GNB membrane lipids. This figure is reprinted from Li et al. [48] with permission of Portland Press, London, UK.

In a high salt environment, when the electrostatic interaction is weakened, the hydrophobic interaction becomes the dominant force that maintains the affinity of the Sushi peptides for the anionic lipids. This confirms that hydrophobic interaction between Sushi peptides and LPS is indispensable for the specificity and reinforcement of the antibacterial action.

The unsaturated state of the microbial lipid tails enhances penetration of the peptide into the bacterial membrane

Work from our lab [27, 48] and others [155] has demonstrated that the unsaturated state of the lipid tail of POPG probably enhances the interaction between the peptide and POPG, which suggests that the bacterial phospholipid confers fluidity to the microenvironment, and possibly augments the insertion of such a peptide into the membrane bilayer. The assessment of the effects of different temperatures on the real-time binding of S1 peptide to POPG showed that at 37 °C, where the unsaturated environment of the membrane is anticipated to be more fluid than at

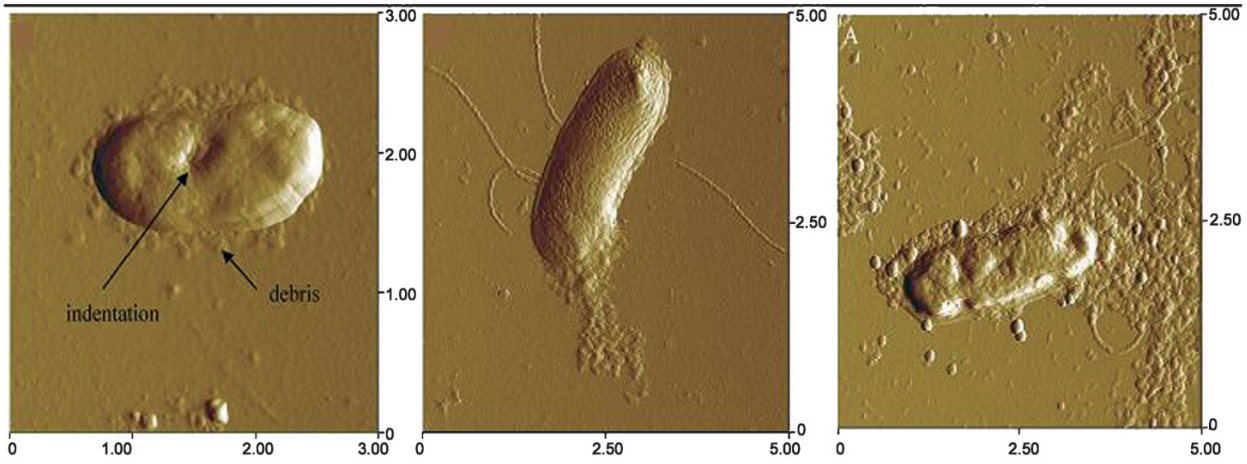


Figure 10. Atomic force microscopy study of the antimicrobial action of Sushi peptides. Treatment of *E. coli* with S3 (0.25–5 μM) for 5–30 min shows disruption of the bacterial membrane and leakage of periplasmic and cytoplasmic fluid as well as disintegration of the bacterium. The figures are taken from Li et al. [156] with permission of Elsevier, Copyright Clearance Centre.

25 °C, the peptide binds more readily to the lipid [27]. This result lends credence to the above postulate that the unsaturated nature of the bacterial phospholipids enhances the penetration of the peptide into the GNB membrane, resulting in membrane perturbation and antimicrobial assault by the peptide. This finding should be taken into consideration and exploited for the future design and development of novel LPS-neutralizing drug peptides. For example, a cocktail formulation consisting of peptides and phospholipids might enhance antibacterial activity to facilitate greater specificity and initial penetration of the peptides into the anionic unsaturated LPS and the membrane bilayer of the GNB.

Conclusion

The era of developing synthetic peptides such as Sushi peptides, derived from LBPs into a new generation of potentially non-resistant antibiotics, is upon us, and no effort should be spared towards realizing that goal. These peptides exhibit specificity for bacterial membrane lipids, suggesting their preference for GNB. Recent atomic force microscopy (AFM) study of the antimicrobial action of Sushi peptides on GNB showed that the Sushi peptides evoke comparable mechanisms of action against different strains of GNB, indicating that the Sushi peptides appear to act in three stages: disruption of the bacterial outer membrane, permeabilization of the inner membrane and disintegration of both membranes [156]. Figure 10 shows AFM micrographs of the antimicrobial activity of the Sushi peptide S3 against *E. coli.*

The biological activities of the antimicrobial peptides are generally thought to be related to their high affinity for LPS, for example, the specificity of binding of the Sushi peptides to bacterial membrane is a consequence of the electrostatic and hydrophobic forces. Furthermore, the unsaturated nature of POPG at the vicinity of the LPS confers fluidity to the lipid bilayer and enhances the interaction between the peptides and the bacterial membrane lipids. Thus, although the biochemical nature of the phospholipids may be one of the ultimate determinants of the binding specificity of a peptide to the bacteria, the structural propensity of the peptides in the micro-environment of anionic phospholipids could strongly influence the function of the molecule, which in turn determines its specificity for the lipids in the bacterial membrane.

In view of the dual preference of the Sushi peptides for LPS and POPG on the GNB membrane, we envisage that POPG could be applied as a potential adjuvant to improve the accessibility and anti-LPS activity of the Sushi peptides and other cationic antimicrobial peptides. Future work may focus on further clarification of the mechanisms of antimicrobial action of the Sushi peptides in preventing septic shock, extending the recent *in vitro* studies using human cell cultures [108] to *in vivo* studies using mice or rabbits which are challenged with LPS/GNBs. In addition to this study, single amino acid substitutions and modifications may be performed to examine the function of critical amino acids in the Sushi peptides with a view to increasing their activity. Furthermore, the tertiary structure of the peptides should be resolved by NMR or X-ray crystallography in the presence and absence of LPS or lipid A to fully elucidate the structure-

activity relationship of the peptide-LPS complex. Maintenance of peptide solubility is another critical parameter in designing an improved antimicrobial peptide. It is important that the peptide mimic be non-toxic to humans and yet resistant to degradation by proteases *in vivo*, especially prior to reaching the site(s) of antimicrobial action.

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