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Research Overview

Antimicrobial Peptides: Resistant-Proof Antibiotics of the New Millennium

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Strategy, Management and Health Policy			
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ABSTRACT   This review encompasses an update on the worldwide research activities made towards developing antimicrobials against the persistent threats from multidrug-resistant gram-negative infection. The lethality of endotoxin or lipopolysaccharide and its conserved bioactive moiety, lipid A, is discussed. We include documentation of our own research efforts towards the development of novel endotoxin antagonists and antimicrobials such as Sushi peptides, derived from the horseshoe crab Factor C, which show great promise for future prophylactic/therapeutic applications. The mechanism of microbicidal action of the Sushi peptides is postulated. In addition to Sushi peptides, we also present other approaches we have undertaken, such as screening for anti-endotoxic peptides from a phage-display library; a computational approach towards rational design of anti-endotoxic and antimicrobial peptides; and finally, a novel alternative that is best analogized by the ancient Chinese medical practice of using ''poison to kill poison,'' which, in modern parlance, entails the innovative application of nonendotoxic *Helicobacter pylori* lipopolysaccharide/lipid A as a competitor to antagonize the endotoxicity of LPS, and hence, the display of antimicrobial properties. Drug Dev. Res. 62:317–335, 2004.   © 2004 Wiley-Liss, Inc.

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INTRODUCTION

All multicellular host organisms harbor innate immune system which constitutes germline-encoded proteins referred to as pathogen-recognition receptors, PRRs [Medzhitov and Janeway, 1997] and effector molecules, to defend themselves against invading pathogens. During infection, innate immune responses are initiated by PRRs that are able to recognize conserved molecular patterns found only in pathogens. These invariant molecules are referred to as pathogen-associated molecular patterns, PAMPs [Medzhitov and Janeway, 2002]. The defense strategies employed by higher living organisms include both innate immunity and adaptive immunity. Innate immunity, which offers immediate defense against acute microbial invasion, rapidly limits infection by pathogens, with a decisive impact on shaping subsequent adaptive immunity. Innate immunity has recently gained extensive research interest because of (a) high morbidity and mortality of septic shock resulting from bacterial infection and (b) multiple antibiotic resistance by microbial pathogens. These are serious threats to humans and pose recalcitrant problems in healthcare management, especially with rampant nosocomial infections [Horan et al., 1984].

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The understanding of the biochemical properties and molecular mechanisms underlying the action of PAMPs such as lipopolysaccharide (LPS) has provided us with a rich resource of information upon which much research has been directed for the design and development of antimicrobial agents. LPS is a conserved molecular pattern found on the outer membrane of gram-negative bacteria, which induces innate immune response and subsequent production of antimicrobial effectors. The LPS molecule and its pathophysiologically bioactive moiety, lipid A, have served as useful targets to design antimicrobial peptides. Because antimicrobial peptides with anti-LPS activity directly target the critically vital PAMP molecule to rapidly incapacitate gram-negative pathogens, various strategies have been employed to design, test, and develop such antimicrobial peptides as the future generation of novel antimicrobials that will be the "resistant-proof" antibiotic of the new millennium.

ENDOTOXIN, ENDOTOXEMIA, AND SEPTIC SHOCK

Endotoxin (or LPS), a constitutive component of the outer membrane of gram-negative bacteria, is shed during antimicrobial therapy and/or when the bacteria lyses [Tracey et al., 1987]. LPS from most species is composed of three distinct regions: the O-antigen region, a core oligosaccharide, and lipid A (LA). The latter is a highly conserved hydrophobic structure and is considered to be the toxic moiety of the LPS molecule [Galanos et al., 1985; Kotani et al., 1985; Frecer et al., 2000b; Yau et al., 2001]. LPS has been suggested to play a pivotal role in the pathophysiology of inflammation, sepsis, and shock [Houdijk et al., 1997; Breithaupt, 1999]. The acute phase plasma protein, LPS binding protein (LBP), binds circulating LPS [Yazawa, 2003; Kaksonen, 2003], to extract it from micelles and transfer it to CD14 receptor on the macrophages. The complex is thought to collaborate with Toll-like receptors to initiate intracellular signaling reactions, via transcription factor NF-κB [Ulevitch and Tobias, 1999]. Activation of protein kinases mediates the production of inflammatory cytokines, contributing to septic shock, which is among the leading cause of death in the developed world [Parillo, 1993].

Clinically, septic shock is characterized by a drastic decrease in blood pressure, cardiovascular collapse, and multiple organ failure [Bone, 1991; Kirikae et al., 1998; Parrillo et al., 1990] and is responsible for more than 100,000 deaths a year in the United States alone [Downey and Han, 1998]. Septic shock often creates more complications than the actual infection itself when massive amounts of LPS are released from bacteria disintegrated by antibiotics [Prins, 1996; Kirikae et al., 1998]. This condition is especially pronounced in children, the elderly, and immunocompromised patients. Thus, neutralizing the activity of LPS or its toxicophore, LA, and/or removing LPS from the body fluids of patients by synthetic analogues [de Haas et al., 1998; Scott et al., 2000] or by a novel class of cationic antimicrobial peptides [Tan et al., 2000b; Yau et al., 2001] may help to eliminate the risk of developing endotoxic shock during or after treatment of bacterial infections [Gough et al., 1996]. Consequently, LPS is a logical and useful target to develop antibacterial drugs. This review will focus on anti-LPS strategies.

ANTI-ENDOTOXIC ANTIMICROBIAL PEPTIDES

The LPS layer of gram-negative bacteria is essential to their growth, propagation, and survival. The massive release of LPS can be more deadly than the bacterial infection itself. The amounts of LPS released by antibiotics vary among different gram-negative bacterial strains. It is found that the amount of LPS released caused higher rates of lethality in mice than purified LPS alone [Kirikae et al., 1998]. Moreover, the release of LPS is also shown to be associated with an increase in bacteria count [Porat et al., 1991]. The mechanism of this phenomenon is still unknown. Antibiotic-induced LPS release perilously occurs as early as 6 h after treatment [Langevelde et al., 1998]. To fight against bacterial infection and PAMP molecules released by the pathogens, nature has developed in almost all forms of multicellular hosts an effective innate immune system, of which short antimicrobial cationic peptides play the key role [Hancock, 1999; Gura, 2001]. Because of high morbidity and mortality of LPS-induced septic shock, agents that can bind LPS and neutralize its toxic effects with low toxicity towards host cells are of clinical importance. While increased resistance of various bacteria towards available traditional antibiotics becomes a very serious challenge, antimicrobial peptides are thought to be a promising new generation of antibiotics. This is attributable to their unique structure and nature of interaction with bacteria, which makes them almost impossible for the bacteria to develop resistance by genetic recombination and mutation [Hancock, 1999; Gura, 2001; Yau et al., 2001].

Antimicrobial peptides fold into a variety of secondary structures: α-helical, β-sheet, cyclic and hairpin loop peptides with one or more disulphide bridges (e.g., magainins, cecropins, defensins, lactoferricins, tachyplesins, protegrins, thanatin), and others [Boman, 1995; Dimarcq et al., 1998]. Despite their structure and sequence diversity, most antimicrobial peptides share common features that include net positive charge and amphipathic character, which

segregates hydrophilic and hydrophobic residues to opposite faces of the molecule [Hancock, 1999; Oren and Shai, 1998]. Thus, antimicrobial peptides most probably also share common mechanism(s) of bactericidal action. Although the precise mode of their action is not fully understood, it has been proposed that they target bacterial membrane specifically [Boman, 1995; Hwang and Vogel, 1998; Oren and Shai, 1998]. The cationic peptides initially bind to negatively charged LPS or LA of gram-negative bacteria [Boman, 1995; Dimarcq et al., 1998; Hwang and Vogel, 1998; Oren and Shai, 1998; Hancock, 1999]. This binding leads to membrane permeation either through (a) minor disruption of phospholipid chain order and packing in the outer membrane, the "self-promoted uptaker" [Hancock, 1999]; (b) transmembrane channel formation via a "barrel-stave" or torroidal pore mechanism [Huang, 2000]; or (c) membrane destruction via a "carpet-like" mechanism [Oren and Shai, 1998], which ultimately kills the bacteria. Evidence has accumulated to suggest that aggregation of amphipathic peptides on the bacterial membrane surface may be important for their antimicrobial activity [Hwang and Vogel, 1998].

Earlier studies on peptides derived from putative LPS binding sequences of endotoxin-binding proteins indicated that an LPS/LA binding motif may be formed by amphipathic sequences rich in cationic residues [Hoess et al., 1993; Ried et al., 1996]. Recently, it has been proposed that even relatively short symmetric amphipathic peptide sequences containing cationic residues with β-sheet conformation will bind bisphosphorylated glucosamine disaccharide head group of LA, primarily via ion-pair formation between anionic phosphates of LA and the cationic side chains [Frecer et al., 2000b]. Therefore, design of novel antimicrobial peptides can be based on the similarities between the endotoxin-binding and antimicrobial cationic peptides, because both of these effects require similar structural features of the peptides, namely, cationic and amphipathic character. It can be expected that strong interaction of the peptides with LPS/LA will promote their destructive action on the bacterial membrane and reduce the risk of developing endotoxemia. Certain antimicrobial peptides show affinity not only to bacteria but also to higher eukaryotic cells in spite of the fact that the outer leaflet of normal mammalian cells is composed predominantly of neutral (zwitterionic) phospholipid [Oren and Shai, 1998; Huang, 2000]. Structure–activity studies on these antibacterial peptides indicate that changes in the amphipathicity could be used to dissociate antimicrobial activity from the hemolytic activity [Kondejewski, 1996]. Recently, it was shown that peptide cyclization increases the selectivity

for bacteria by substantially reducing the hemolytic activity [Oren and Shai, 2000; Frecer et al., 2004].

ANTIMICROBIAL SUSHI PEPTIDES

Development of Novel Sushi Peptides from Horseshoe Crab Factor C

LPS from gram-negative bacteria induces the hemocytes of horseshoe crab to aggregate and degranulate. This response underlies the important innate immune defense mechanism of the horseshoe crab against the invasion of GNB [Ding et al., 1995]. As a molecular biosensor, Factor C, a serine protease in the horseshoe crab hemocyte, can be autocatalytically activated by femtograms of LPS [Ho, 1983] to trigger the coagulation cascade [Ding et al., 1993; Fig. 1], suggesting that it contains structural domains that display profound affinity for LPS. Owing to its remarkable sensitivity to LPS, Factor C plays a crucial role in the detection of bacterial endotoxin in pharmaceutical products [Ding and Ho, 2001]. In our laboratory, we have characterized the LPS binding regions of Factor C [Ding et al., 1997; Pui et al., 1997; Roopashree et al., 1997a, 1997b; Tan et al., 2000a]. The multiple LPS-binding sites in the Sushi domains (spanning ≈ 300 amino acids) located at the N-terminus of the Factor C molecule has been subcloned and expressed [Tan et al., 2000a]. The recombinant fragment (SSCrFCES) of 38 kDa, which constitutes a signal peptide, a cysteine-rich region followed by epidermal growth factor-like domain and finally 3 Sushi domains, exhibits high affinity to LPS. On a molar basis, SSCrFCES displays greater potency than polymyxin B [Lehmann et al., 1987] and lactoferrin, LF 33 [Zhang et al., 1999], at suppressing LPS-induced Limulus amoebocyte lysate (LAL) coagulation and secretion of cytokines by human promonomyelocytic cell line THP-1 and normal human peripheral blood mononuclear cells [Tan et al., 2000a]. The Sushi domains bind LPS with high positive cooperativity, yielding K_d of 10^{-12} M. Furthermore, SSCrFCES is less cytotoxic than polymyxin B. On testing the anti-LPS potency of SSCrFCES in vivo, an intraperitoneal dose of 2.5 ng of *Escherichia coli* 055:B5 LPS plus 2–4 μM of SSCrFCES into D-galactosamine-sensitized C57BL/6J mice, was sufficient to confer > 90% survival of the mice, which would otherwise have succumbed to endotoxemia within 7 h.

In view of the antagonistic potency of SSCrFCES against LPS, a close examination of the amino acid sequences within Sushi 1 and Sushi 3 domains revealed two possible LPS-binding motifs. Consequently, two corresponding Sushi peptides, referred to as S1 (171-204: N′-GFKLKGMARISCLPNGQWSNFPPKCIRE-
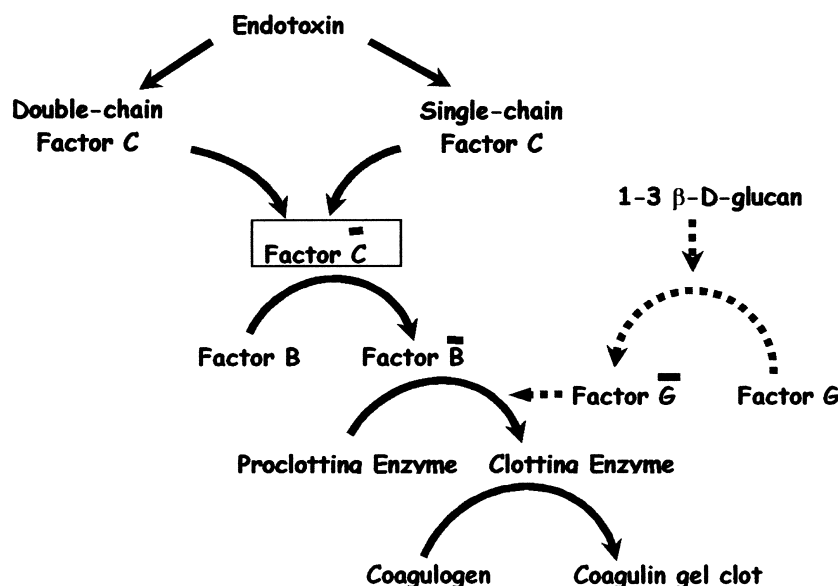
Fig. 1. The coagulation cascade reaction in the horseshoe crab amoebocyte lysate. Endotoxin activates both forms of Factor C. Single-chain Factor C exhibits a reversible activation reaction, which signifies a form of feedback regulation in the coagulation cascade [Ding et al., 1993]. Double-chain Factor C follows a path previously described for *Tachypleus tridentatus* amoebocyte lysate [Iwanaga et al., 1985]. The pathway with dotted arrows is an endotoxin-independent alternate mechanism, in which Factor G is activated by (1-3) β-D-glucan [Morita et al., 1981]. Adapted from Ding et al. [1993].
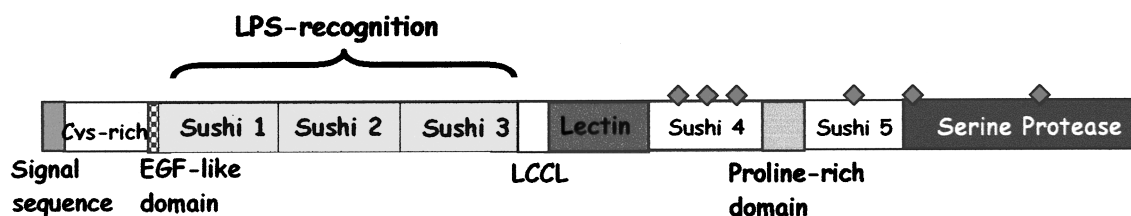

Fig. 2. The domain structure of the multifunctional Factor C molecule, an LPS-sensitive serine protease zymogen from the hemocyte of *C. rotundicauda*. The diamonds represent glycosylation sites. Adapted from Tan et al. [2000b].

CAMVSS-C′) and S3 (268-301: N′-HAEHKVKIG-VEQKYGQFPQGTEVTYTCSGNYFLM-C′), which correspond to the amino acid sequence of CrFC21 (GenBank Accession No: S77063), were chemically synthesized. Additionally, S1d and S3d (d=mutagenic variants of S1 and S3 where two lysine mutations were introduced) were synthesized with a view to improving binding affinity between peptide and LPS [Tan et al., 2000b]. All four Sushi peptides of 34 residues each contain the core LPS binding motif of Sushi 1 and Sushi 3 domains within SSCrFCES. Figure 2 shows the Sushi domains in the multidomain Factor C molecule. The Sushi peptides bind LPS with K_d of $10^{-6 \text{ to } -7}$ M. Circular dichroism spectrometry revealed that the Sushi peptides underwent conformational change in the presence of LA, transitioning from random coil to either α-helical or β-sheet structures. The Sushi peptides were able to inhibit LPS-induced LAL activity and suppress the LPS-induced hTNF-α secretion from THP-1 cells. It was envisaged that in the design of an improved LPS-binding and -neutralizing peptide, the maintenance of peptide negative–positive charge balance is a critical parameter, in addition to its structure. The modified S1d and S3d peptides also exhibit increased LPS neutralization potential, although their LPS binding affinities, derived from surface plasmon resonance (SPR) studies, at best indicated only a 10-fold improvement. Although it is tempting to attribute this improvement in LPS-neutralizing potency of S1d and S3d to the replacement of specific amino acids by lysine residues, as suggested by Hong et al. [1998], one cannot ignore the possibility that mutations to other residue of the peptide might elicit the same effect observed here. Thus, it is envisaged that the anti-endotoxin property of a peptide is also affected by the peptide–LA stoichiometric ratio and does not solely/

necessarily correlate with increased affinity for LPS. Nevertheless, the characterization of the minimal endotoxin binding motif of the horseshoe crab Factor C has provided a basis for designing small peptide molecules that could have prophylactic and/or therapeutic properties in humans for the management of gram-negative infection and septic shock.

Sushi Peptides Display Exceptionally Strong Bactericidal Activity Against Multidrug-Resistant Pseudomonas

Owing to the ease of acquisition and lack of lasting effective clinical management, nosocomial infection has drawn intense attention from the medical community. *Pseudomonas aeruginosa*, which is a fast-replicating bacterium that displays a short lag phase and doubling time, is the epitome of an opportunistic human pathogen [Horan et al., 1984] causing infections of the urinary tract, respiratory system, and soft tissue. It also causes dermatitis, bacteremia, and a variety of systemic infections, particularly in victims of severe burns [Trafny, 1998], patients with diabetes, and cancer and AIDS patients who are immunosuppressed. Hospitals provide an opportunistic reservoir for pseudomonads to develop a multitude of resistance mechanisms over the years to a variety of naturally occurring antibiotics used to combat them [Horan et al., 1984; Lorian, 1996; Arruda et al., 1999].

The urgency of developing potent anti-pseudomonas peptides cannot be overstated. The pathogenesis of Pseudomonas infections is multifactorial, as suggested by the wide array of its virulence determinants [Danel et al., 1998]. Owing to its antibiotic-resistant nature, a bactericidal agent with rapid action will be the most effective and appropriate countermeasure in controlling its spread from infected wounds. This is especially pronounced with secondary infections, such as those in cystic fibrosis patients [Arruda et al., 1999] and acute bacteremia in AIDS patients or those that occur near or in vital organs such as the cornea, as well as in exposed skin on burn patients. To date, only a few antibiotics remain effective against them. These include fluoroquinones, aminoglycosides, and imipenem [Horan et al., 1984; Bustamante et al., 1990; Lorian, 1996; Arruda et al., 1999]. However, resistance against these antibiotics is developing rapidly [Horan et al., 1984; Goldman et al., 1997; Trafny, 1998; Arruda et al., 1999]. A multifaceted approach to their eradication is essential to significantly reduce the possibility of the emergence of new resistant strains. Most antibiotics exert their bactericidal action by inhibiting a crucial biochemical enzyme [Udo and Dashti, 2000]. However, resistance can be attained throughout the acquisition of an antibiotic-resistance plasmid, for example β-lactamase, which expresses a new isoform of the targeted enzyme. Another mode of intervention is thus necessary to complement the current biochemical route.

Pseudomonads are naturally resistant to many antibiotics, because of the permeability barrier afforded by their outer membrane. Furthermore, their tendency and ability to colonize the surfaces of biofilms [Costerton et al., 1995] make them impervious to therapeutic concentrations of antibiotics. The futility of treating Pseudomonas infections with antibiotics is most dramatically illustrated in cystic fibrosis [Smith et al., 1996; Goldman et al., 1997] and bronchiecstasis [Hla et al., 1996] patients, virtually all of whom eventually succumb to infection with multidrug-resistant strains.

Sushi peptides exhibit excellent attributes of anti-Pseudomonas antimicrobials-cum-LPS-antagonistic properties. Recently, the concept of eradication via targeted disruption of bacterial LPS by cationic peptides and proteins was introduced [Chopra, 1998; Skerlavaj et al., 1999]. These peptides and proteins, which are mainly α-helical or β-sheet in structure, assert their effects by disrupting the bacterial membrane, causing pore formation that eventually leads to osmotic imbalance and cell death [Oren and Shai, 1998]. For effective antimicrobial therapy, such peptides and proteins need to satisfy several important criteria: (1) potent antimicrobial activity over a wide range of pH, (2) rapid killing rate, (3) low toxicity, (4) low hemolytic activity, and (5) delivery to the target site of infection without degradation of the peptide. Although numerous antimicrobial peptides such as lepidopteran cecropin [Teshima et al., 1986], magainin [Zasloff, 1987], FALL-39 [Agerberth et al., 1995], and SMAP-29 [Skerlavaj et al., 1999] have been reported, few display all of the abovementioned attributes. Thus, the search for new and more powerful, and yet safe antimicrobial peptides continues to be a worldwide priority in drug discovery programs.

Four peptides derived from the Sushi 1 and 3 domains of the Factor C protein sequence [Ding et al., 1995], namely, S1, S3, S1d, and S3d, exhibited high affinity for LPS. Further analyses of these Sushi peptides showed them to have low cytotoxicity; capability to neutralize the endotoxicity of LPS; to suppress LPS-induced cytokine production; and to confer protection against LPS-induced lethality in mice [Tan et al., 2000b]. Therefore, endotoxicity, as seen during the course of antibiotic treatment, will be dramatically reduced. This property would provide an advantage over existing antibiotics and most other non-LPS-sequestering cationic antimicrobial peptides, in

suppressing the adverse effects of LPS-induced septic shock during or after treatment. Thus, Yau et al. [2001] tested the antimicrobial activity of Factor C-derived Sushi peptides against clinical isolates of multidrug-resistant *P. aeruginosa*.

The antimicrobial potency of the Sushi peptides was challenged against 30 clinical isolates and a control strain of *P. aeruginosa* ATCC 27853. The resistance pattern of these strains gave a close representation of the resistant strains of *P. aeruginosa* found in Singapore [Yau et al., 2001]. The 30 clinical isolates showed very high resistance against most antibiotics used for the treatment of *P. aeruginosa*, yet the Sushi peptides exhibited low MBC_{90}s (0.06 to 0.25 µg/mL; 16–63 nM) for these multidrug-resistant strains of *P. aeruginosa*. It kills 90% of 10^9 CFU/mL of a clinical strain of *P. aeruginosa* within 7 min of incubation, and total eradication was achieved within 40 to 50 min. These MBC_{90}s obtained for the peptides are unsurpassed by any known antibiotics of metabolite or peptide origin. Comparatively, Sushi peptides are up to one to two orders of magnitude more effective than any other reported cationic antimicrobial peptides against *P. aeruginosa* [Catchpole et al., 1997; Sawa et al., 1998; Giacometti et al., 1999; Shin et al., 1999; Mosca et al., 2000]. Complete eradication of the Pseudomonas probably occurred within the first two generations of bacterial growth, which will reduce the possibility of mutation to resistance. Thus, this rapid killing rate should prevent the development of resistance, because it will require several precise mutations to occur at multiple enzymes along the LPS synthesis pathway to ultimately yield a modified LPS structure that is sufficiently different to evade Sushi peptide recognition.

The strong binding affinities of the Sushi peptides for LA [Tan et al., 2000b] suggest an explanation for the susceptibility of the clinical strains tested. The Sushi peptides probably act by disrupting the LPS-lamellar organization in the bacterial cell membrane by physical means, leading to osmotic imbalance and cell lysis.

The effectiveness of the Sushi peptides was well maintained over a broad range of pH and salt concentrations. All the peptides were bactericidal from pH 6.0 to 7.0 at their respective MBC_{90}s. S1, with a calculated pI of 9.85, is of particular interest, as it maintained its bactericidal potency across the pH range tested. As the pH approached the pI of the peptides, the loss of most cationic charges on the peptides led to a loss of ionic interaction with LPS, which thus affected their bactericidal action. Surprisingly, both S1d (pI=10.08) and S3d (pI=9.62), with pI relatively close to that of S1, did not perform as expected. They exhibited a bactericidal response at pH 6.0 to 7.0 and a bacteriostatic effect at pH 7.5 to 8.0.

The peptides were also resistant to high salt concentrations. At up to 300 mM NaCl, Sushi peptides (≤ 0.03 µg/mL) inhibited the growth of *P. aeruginosa* of an initial cell population of 10^5 CFU/mL. Again, the transition from bactericidal to bacteriostatic activity was probably due to the disruption of electrostatic interactions between the peptides and the bacterial LPS. Nevertheless, the peptides retained their bacteriostatic efficiency 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 [Smith et al., 1996; Goldman et al., 1997]. Hence, Sushi peptides can be developed for topical and aerosol applications.

The low MBC_{90} (0.06 to 0.25 µg/mL), rapid killing rate (40 to 50 min), versatility at high osmolarity (300 mM NaCl), tolerance of a broad pH range (6.0 to 8.0), and low or insignificant hemolytic activity as well as a lack of cytotoxic activity are excellent properties upon which Sushi peptides could be developed as highly effective and bactericidal candidate antibiotic against *P. aeruginosa*. The lack of cytotoxicity was confirmed both by in vitro and in vivo assays that showed minimal lysis of human monocytes, THP-1 cells, and also by the absence of aberrant behavior or death in C57BL/6J mice [Tan et al., 2000b].

The high affinity of Sushi peptides for *E. coli* LA also implies that the bactericidal potency of the peptides can be applied to other gram-negative bacteria, without the risk of LPS release during the bactericidal action. Such peptides not only afford effective bactericidal action against *P. aeruginosa*, but also confer protection to the host against endotoxemia and septic shock. The therapeutic indices [Yau et al., 2001]) of the Sushi peptides further demonstrate their promising therapeutic potential in controlling the emergence of such multidrug-resistant pseudomonads. These peptides are minimally hemolytic against both rabbit and human erythrocytes even at concentrations up to 1,600-fold their MBC_{90}s. Both in vitro and in vivo studies indicate that cytotoxic effects are small even at 1,000-fold their MBC_{90}s. Furthermore, the Sushi peptides are tolerant of high-salt and adverse pH conditions. Such stringent attributes of the Sushi peptides would assure their versatility, potency, and worthiness of being developed into powerful and "resistant-proof" antimicrobial peptides against multidrug-resistant pseudomonas.

Mechanism of Anti-endotoxic and Antimicrobial Action of Sushi Peptides

Recent approaches to develop drugs to neutralize LPS have concentrated on characterizing the LA moiety of LPS using synthetic and recombinant

peptides derived from sequences of polymyxin B sulfate, *Tachypleus* anti-LPS factor, recombinant LALF, BPI, CAP-18, LBP [Epand, 1999], and Sushi peptides [Tan et al., 2000b; Yau et al., 2001]. These peptides are believed to induce complete lysis of the microorganism by rupture of the membrane or perturbation of the membrane lipid bilayer, which leads to leakage of bacteria cell components as well as dissipating the electrical potential of the membrane [Nizet, 2001]. The efficacy of these peptides results from their ability to disrupt prokaryotic membranes at concentrations that are not harmful to mammalian host membranes [Maloy and Kari, 1995]. The molecular basis for this selectivity and the underlying molecular mechanism of peptide-induced permeation of bacterial membranes remain ill-defined, although it is believed that the less negatively charged mammalian cell membranes spares the host from such undesired membrane perturbation [Rina, 2000; Gidalevita, 2003]. To date, two main hypotheses have been proposed for membrane perturbation: "barrel-stave" and "carpet" [Oren et al., 1997].

The LPS binding motif of Sushi3 domain of Factor C resides within 34 amino acid sequence of the Sushi3 peptide (S3). Like most antimicrobial peptides, the mechanism of action of S3 is thought to be influenced by its interaction with LPS as the target molecule [Tan et al., 2000b]. S3 has been demonstrated to bind and kill gram-negative bacteria [Yau et al., 2000]. S3 contains a single cysteine; thus, it is conceivable that in solution, S3 exists in two possible forms: monomeric peptide without disulfide bond or dimeric peptide linked by intermolecular disulphide bond. Consistent with other reports that some multimeric antimicrobial peptides are more active than their corresponding monomers [Lai 2003; Situ 2003; Wu 2003], the cysteine residue in S3 peptide can affect its LPS-binding and antimicrobial activity. Thus, in order to gain a global perspective on the tolerance of cysteine residues in S3 to amino acid substitutions, Li et al. [in press] designed a mutant peptide where cysteine at position 27 was changed to serine and the structure–activity relationship was analyzed. This mutant peptide is named $S3C_{27}S$. The effects of the S–S bridged S3, the conformational change of mutant $S3C_{27}S$, and their biological activities were investigated with a view to elucidating the mechanism of action of the S3 peptide.

S3 dimer and LPS interact via electrostatic and hydrophobic forces

LA is the biologically active moiety and most conserved part of LPS. The diphosphate head groups on the LA molecule confer a net negative charge while its acyl chains are hydrophobic. The N-terminus of S3 is positively charged while the C-terminus of S3 is more hydrophobic. In aqueous solutions, binding to LA appears to involve a two-step process [Frecer et al., 2000a, 2000b, 2004], through preliminary electrostatic interaction between the cationic amino acids at the N-terminus of S3 and the diphosphoryl head groups of LA, followed by stabilization of the resulting molecular complex through hydrophobic interactions between the hydrophobic C-terminus of S3 and fatty acyl chains of LA. This concurs with the observation that mutations of the N-terminus to introduce two extra lysine residues into S3 resulted in an increase in LPS neutralizing activity [Tan et al., 2000b].

Although an LPS monomer is ≈ 10 kDa, it has great propensity to form stable polymeric aggregates, which is controlled by nonpolar interactions between neighboring alkyl chains as well as bridges generated among phosphate groups by bivalent cations [Altenbach, 1994]. These aggregates are similar to LPS assembled on the outer membrane of GNB, and can serve as the mimetic to GNB membrane. By fluorescence correlation spectroscopy, FCS [Li et al., unpublished data; personal communication] showed that LPS attains a "detergent-like" activity after a threshold critical micellar concentration has been reached, which is consistent with the report by Bechinger [1997]. It is possible that at high concentrations, S3 binds the acyl chain of LA, thus disrupting the critical force, which maintains the stability of the LPS micelles. Because S3 dimer is symmetrical, it is conceivable that each of the two S3 binds one LPS molecule to form a trio of "LPS:S3 dimer:LPS" complex.

Based on secondary structure observations, more than 30% of the S3 dimer is in β-sheet form, which is stabilized by one disulfide bond. Many shorter peptides such as magainins and cecropins are unstructured in aqueous solution. In contrast, small β-sheet peptides, such as tachyplesin, protegrin, and lactoferricin B, are already in a more or less defined amphipathic sheet structure in aqueous solution [Epand, 1999]. The presence of one or two disulfide linkages may thus help to stabilize these structures [Tam, 2002]. Presumably, this structure contributes to the interaction with LPS, and the β-sheet in S3 dimer may form the β-barrel structure to shield the hydrophobic acyl chains of LPS [Li et al., unpublished data; personal communication].

Disruption of LPS micelles by disulfide bonded S3 dimer

Although there is general agreement over the importance of molecular charge [Hancock, 1997; Hong et al., 2001] of antimicrobial peptides and electrostatic

interactions in initiating membrane association of peptides as well as their selective toxicity towards microorganisms, opinions differ with regard to the subsequent events that eventually lead to the lysis of microbes [Gidalevita, 2003]. The central dispute remains on whether peptides disintegrate the membranes by forming multimeric solvent-permeable pores, leading to leakage of intracellular components ("barrel-stave" hypothesis), or simply by densely covering the outer leaflets of the membranes ("carpet" hypothesis) without forming discrete membrane channels. The pore-forming postulate proposes that structural amphiphilicity, viz., spatial segregation of clusters of cationic residues from patches of hydrophobic groups, is the most important prerequisite for stable pore formation in the membrane. Many defensins indeed form amphiphilic dimers and sometimes higher-order amphiphilic oligomers in solution. However, growing evidence appears to support the existence of multiple mechanisms of action for different peptides. Even for a particular peptide, the mechanism may vary for different bacterial species it targets. In fact, Dempsey [2003] suggested that linear analogs and their disulfide bonded parent molecules can follow different mechanisms of action in bacterial killing.

Using FCS, Li et al. (personal communication) have investigated the kinetics of S3 peptide binding to LPS molecules and disruption of LPS micelles, which mimics the perturbation of the outer membrane of gram-negative bacteria, ultimately leading to its lysis. Two realtime systems were employed to monitor the interaction between peptide and LPS by using either one of the two fluorescent-labeled reacting components: rhodamine-S3 peptide or fluorescein isothiocyanate (FITC)-LPS. In the rhodamine-S3 system, LPS micelles were detected in solution, which appear as large fluorescent peaks among the intensity signal, which is attributable to the binding of rhodamine-S3 to LPS aggregates. In the FITC-LPS system, the function of S3 peptide on FITC-LPS micelle was examined. When compared to $S3C_{27}S$ mutant monomer, the S3 dimer specifically caused a drastic reduction of the large fluorescent peak intensity, and the number of fluorescent particles in the observation volume increased during the S3 dimer-induced LPS micelle disruption. This indicates that the S3 mutant monomer is incapable of interacting with LPS and confirms the importance of disulfide bonding in the S3 dimer, which enables it to perturb the gram-negative bacterial membrane.

At low ratio of peptide:LPS, S3 binds LPS micelles, but, at high ratio, S3 not only binds LPS, but also disaggregates the LPS micelles. This suggests that the S3 dimer interacts preferentially with LPS micelle aggregates on GNB membrane, thereby perturbing the fluidic integrity of the bacterial membrane via "carpet-like" mechanism. This is facilitated by the disulfide linkage, which stabilizes the S3 dimer to maintain the pore in the membrane, which is consistent with the findings of Dempsey [2003] and Qiu [2003]. In conclusion, Li et al. suggest that the ability of the native S3 dimer peptide to bind and perturb the micelle of endotoxin equips it to preferentially damage the membranes of bacteria during the antimicrobial assault, and that this capability is related to its secondary structure.

Scaled-up Production of Sushi Peptides by Recombinant Expression

Since chemically synthesized Sushi peptides derived from Factor C were shown to preferentially bind LPS micelles and neutralize its activity [Tan et al., 2000b; Li et al., unpublished data; personal communication], an initiative was taken to scale up the production of Sushi peptide, S3. Chemical synthesis is generally an uneconomical approach to obtain large quantities of peptides, whereas recombinant expression in *E. coli* may be more cost effective [Latham, 1999]. However, the yield from *E. coli* may be low and unstable [Le and Trotta, 1991]. Thus, expression of the multimers of peptides would circumvent the above-mentioned problems [Kajino et al., 2000]. A more important attribute for recombinant multimer of S3 is that enhanced LPS-binding affinity and activity are expected through synergistic effect of multiple LPS-binding units [Mauro and Pazirandeh, 2000].

Besides Factor C, the tachylectin family members identified in circulating hemocytes and hemolymph of the horseshoe crab also contribute to the recognition of invading pathogens. Five types of lectins, named tachylectin-1 to -5, have different specificities for carbohydrates exposed on pathogens. Interestingly, all of these lectins contain different numbers of tandem repeats in their structure [Iwanaga, 2002]. Thus, Li et al. [2003] investigated the tandem repeats of S3 with a view to providing explanations as to why these proteins adopt repetitive structure, and how they contribute strategically towards pathogen recognition and antimicrobial potencies.

Many methods can be applied to construct the tandem repeats of a peptide [Dolby et al., 1999; Lee et al., 2000; Mauro and Pazirandeh, 2000]. Li et al. [2003] chose the amplification vector that readily allows the construction of various tandem multimers of the S3 gene in *E. coli*. Furthermore, a DP (asp-pro) linker was incorporated between the repetitive units of S3, to facilitate subsequent convenient cleavage of the multimeric peptide under mildly acidic buffer to release the

monomeric S3 peptides. As the copy number of S3 increases, the expression level improved dramatically, especially with the S3 tetramer, where the expression level reached 25% of the total cell proteins. However, further doubling to octamer reduced the expression level, suggesting that the copy number is not always proportional to the expression level for this peptide. Thus, induced expression of the most robust tetramer clone was scaled-up. Recombinant S3 tetramer (rS3-4mer) was purified and digested into monomers (rS3-1mer or rS3) by acid treatment. Endogenous LPS from the *E. coli* host was exhaustively removed [Liu et al., 1997; Ding et al., 2001; Goh et al., 2002]. Both the parent tetramer and resultant monomer peptides were tested for their LPS-binding and -neutralizing activities.

By enzyme-linked immunosorbent assay–based LPS binding test and SPR analysis of realtime biointeraction between rS3 and LPS, differential binding efficiencies for LPS was observed among the rS3-4mer, rS3-1mer and the chemically synthesized S3 (cS3). Comparatively, rS3-4mer demonstrated eight-fold higher efficacy in LPS binding. This suggests positive cooperativity between the repetitive units in rS3-4mer. The 50% saturation binding concentrations of the tetramer and monomer are 27 nM and 260 nM, respectively. The 50% endotoxin-neutralization concentrations are 0.31 μM and 2.3 μM for the tetramer and monomer, respectively. At 2.5 μM and 18.3 μM, rS3-4mer and rS3-1mer achieved 50% suppression of LPS-induced hTNF-α secretion, respectively. These biological activities provide compelling evidence for the superiority of rS3-4mer in LPS-binding and –neutralization. Both the tetramer and monomer peptides exhibit low cytotoxicity and no hemolytic activity on human cells.

Apart from designing, synthesizing, and testing peptides based on LPS-binding proteins, other approaches are employed to judiciously obtain potential antimicrobial peptides. The remaining part of this review describes rational computational approach, phage-display peptide library screening, and finally, testing of isolated nonendotoxic LPS as a means of anti-LPS *prototype drugs* for further development.

COMPUTER-ASSISTED DESIGN OF ANTIMICROBIAL V-PEPTIDES

From the understanding of amino acid sequences in the Sushi domains of Factor C molecule, a rational design strategy based on the presumed mechanism of antibacterial effect was adopted to design cationic antimicrobial peptides capable of binding LPS through tandemly repeated sequences of alternating cationic and nonpolar residues [Frecer et al., 2004]. The

peptides were designed to achieve enhanced antimicrobial potency through initial binding of the bacterial membrane, with a reduced risk of endotoxic shock. Recently, it has been proposed that even relatively short symmetric amphipathic peptide sequences containing cationic residues such as *HBHPHBH* or *HBHBHBH* (**B**–cationic, *H*–hydrophobic, *P*–polar residue) with β-sheet conformation will bind bisphosphorylated glucosamine disaccharide head group of LA, primarily via ion-pair formation between anionic phosphates of LA and the cationic side chains [Frecer et al., 2000b]. In fact, potent cyclic antimicrobial peptides selective for gram-negative bacteria based onÿthe β-stranded framework mimicking the putative LPS-binding sites of the family of LPS-binding proteins have been successfully developed [Muhle and Tam, 2001].

Frecer et al. (2004) reported de novo design of synthetic cationic peptides with two LPS/LA binding sites, which show structural similarity to cyclic β-sheet defense peptides such as protegrin-1, thanatin, or androctonin [Dimarcq et al., 1998]. Systematic modifications of molecular properties were performed by varying amino acid residues of the amphipathic LPS/LA binding motifs while preserving the size, symmetry, and amphipathic character of the peptides. The purpose of these substitutions was to demonstrate whether improving the binding affinity to endotoxin could enhance the antimicrobial activity of cyclic cationic peptides. Furthermore, whether directed substitutions can selectively increase the antimicrobial potency independent from the hemolytic activity of the peptides was investigated. Thus, Frecer et al. [2004] rationally designed and synthesized seven cationic peptides cyclicized via a disulphide bridge, collectively referred to as V peptides (Fig. 3). The biological properties of the V peptides were characterized. Quantitative structure–activity relationships (QSAR) were obtained by linking the experimental potencies to simple physicochemical molecular properties that were easily derived from the peptide sequences. Implications for the molecular mechanism of antibacterial effect were suggested, and practical guidelines for design of nonhemolytic highly active antimicrobial peptides were proposed.

Binding affinities of V-peptides to LA

The binding affinities of the V-peptides computed for the LA monomer and the dissociation constants derived from SPR measurements on LA monolayer represent two diverse models for the prediction of peptide interaction with the outer bacterial membrane. The first theoretical model simulates binding of single free LA molecule to the V-peptide forming a 1:1
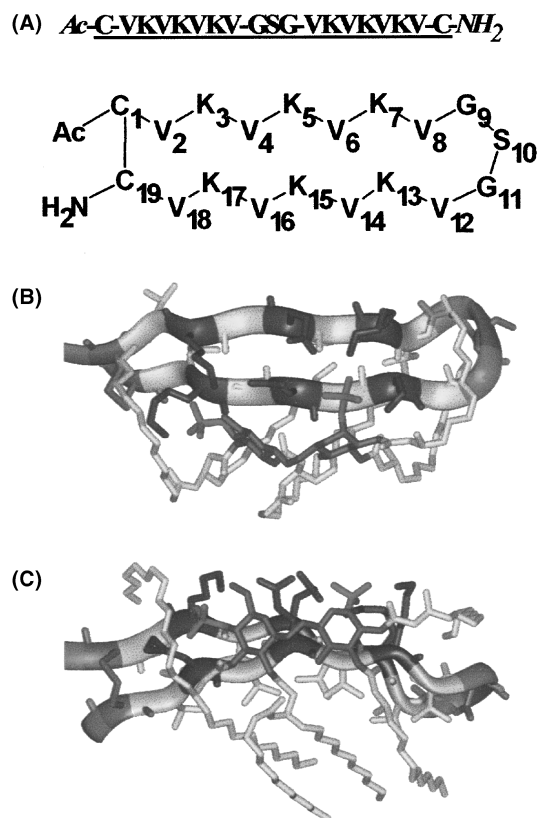
(A) *Ac*-C-VKVKVKV-GSG-VKVKVKV-C-*NH*₂

(B)

(C)

Fig. 3. V-peptide: **(A)** Sequence and chemical structure of designed cyclic cationic peptide **V**1. The V-peptides are composed of two symmetric amphipathic LPS/LA binding motifs *HBHB(P)HBH* with **B**–basic, *P*–polar, *H*–hydrophobic residues, which form two strands of a β-hairpin joined by $-G_9S_{10}G_{11}-$ turn on one side and disulfide bond C_1-C_{19} bridging the N- and C-terminal residues on the other side. **(B)** Computer model of peptide:LA complex derived by molecular dynamics assisted docking in water. In the model, LA is attached to the dual LPS/LA binding sequence of peptide V-peptide (V4). The ribbon shows the backbone conformation of the V4 peptide Ac-C-VKVQVKV-GSG-VKVQVKV-C-NH₂, which acquired β-hairpin-like structure in the 1:1 complex with LA monomer in water. The β-hairpin supersecondary structure of V4 was induced by the amphipathic LA counterpart. Residue side chains and LA are shown in stick representation. Hydrophobic Val residues interact primarily with fatty acid chains of the LA. Cationic Lys residues (blue) form ion-pairs with the anionic phosphate groups of LA headgroup. Hydrogen atoms were omitted for clarity. **(C)** Side view of the V4:LA complex. Adapted from Frecer et al. [2004].

complex in a dilute aqueous solution. In the second experimental model, the K_d constants from SPR measurement reflect the adsorption of V-peptide upon the monolayer of sterically hindered LA immobilized on the biosensor chip. Thus, for example, the K_ds from SPR do not take into account the contribution from hydrophobic interactions between the acyl chains of LA and side chains of nonpolar residue of the peptides, in contrast to the relative binding affinity. The measured K_ds and computed binding affinities repre-

sent complementary data and confirmed that the designed cationic peptides exhibit strong affinity for *E. coli* LA that is comparable to the affinity of the reference antiendotoxin agent, polymyxin B [Rustici et al., 1993; Srimal, 1996].

While the V-peptides acquired flexible ring-shaped conformation in water, they underwent conformational transition and attained a different secondary structure in the vicinity of heterogeneous membrane-mimicking interface upon association with the amphipathic LA or PC molecules. Similar observations have been made for other linear and cyclic peptides [Woody, 1995]. Molecular modeling suggested that in the 1:1 complexes with LA, the peptide backbone underwent conformational transition to an amphipathic β-hairpin with distinct polar and nonpolar faces induced by the amphipathic LA molecule. In these complexes, both LPS/LA-binding motifs, *HBHB(P)HBH*, were involved in the interaction with a single LA molecule. The presence of a central hinge of the type –GIG– similar to the –GSG– loop of the V-peptides was found to be responsible for the effective antibiotic activity of a composite 20-residue synthetic peptide cecropin-A(1-8)-magainin-2(1-12) with a helix–hinge–helix structure [Shin et al., 2000], indicating that structural flexibility is related to antimicrobial potency.

QSAR analysis of V-peptides

The validity of the QSAR model for antimicrobial potencies of the V-peptides against gram-negative bacteria was verified on the set of cyclic cationic amphipathic peptides designed by Muhle and Tam [2001]. These peptides displayed potent activities against gram-negative bacteria, *E. coli* and *P. aeruginosa*, with the lowest minimum inhibitory concentration (MIC) starting at 160 nM concentration. The correlation equation used by Frecer et al. [2004] for the MIC was able to reproduce the qualitative rank order of antimicrobial potencies at low salt concentration for eight of nine peptides: R6F > R6A > R5-Y ≅ R5W > R4A ≅ R4Y > K4A > K5L, and only failed to rank correctly one peptide R5L [Muhle and Tam, 2001].

Antimicrobial Activity of the V-Peptides

For comparison of the MIC of the V4 peptide with some clinically used antibiotics, the MIC values were estimated against three gram-negative bacteria. The MICs of five antibiotics against *E. coli, K. pneumonia,* and *P. aeruginosa* were reported by Lancini et al. [1995] to be: erythromycin (85 μg/mL), tetracycline (6 μg/mL), chloramphenicol (3 μg/mL), rifampin (1 μg/mL), and gentamicin (0.17 μg/mL). In comparison, the most potent peptide, V4, with MIC of

10 ng/mL, displayed 17 to 8,500-fold greater anti-bacterial potency than the five established antibiotics against these three bacterial species. Muhle and Tam [2001] designed amphipathic cyclic cationic antimicrobial peptides similar to the V-peptides with sequences such as c(PAC**R**C**R**AG-PA**R**C**R**CAG) constrained by two cross-linking cysteine bonds that mimicked the LPS-binding β-strands of LPS binding proteins, which showed MIC values of 20 nM against *E. coli* comparable to the MIC of the **V**4. Bactericidal/permeability increasing protein (BPI), a 60-kDa LPS-binding protein, displays MIC of <1 nM against gram-negative bacteria [Tobias et al., 1997].

The V-peptides with the highest affinity to LA of *E. coli* did not necessarily show the highest antimicrobial effect. This indicates that initial binding to the outer membrane of gram-negative bacteria, which differ in their LPS composition, does not solely determine the overall antimicrobial effect. This observation opposes earlier assumptions that strong initial binding of cationic amphipathic peptides to outer membrane components may interfere with the antimicrobial activity [Oren and Shai, 2000; Wu and Hancock, 1999]. In fact, higher affinity to the outer bacterial membrane seems to be a favorable prerequisite for the antimicrobial effect because the V-peptides displayed low micromolar K_ds to the LA of *E. coli* and MICs against *E. coli* are in the nanomolar range.

Strategy for dissociation of antimicrobial and hemolytic effects

The QSAR correlation equations obtained for the peptides V1–V7 permit quantitative prediction of biological activities of analogs of synthesized and tested V-peptides and can help to elucidate whether site-directed replacements of residues in the polar and nonpolar faces of the peptides (preserving the molecular charge and overall symmetry of the LPS/LA binding motifs) may lead to independent variations in antimicrobial and hemolytic potencies of cationic amphipathic peptides.

The QSAR correlation equations applied to analogs of V1–V7 predicted rapid increase of the MIC* (predicted MICs* were lower than that of V4) upon increasing molecular charge (Q_M) over 4 è when amphipathicity and hydrophobicity were kept constant at the levels of the most promising peptide V4. Model analogs of **V**4 that shared the polar *HKHQHKH* motif and differed only in the hydrophobic residues $H=$\{Val, Tyr or Ala\}, which retain amphipathicity index of V4 and display decreasing lipophilicity index due to variable *H* residues, were predicted to possess decreasing hemolytic activities while their predicted antimicrobial and cytotoxic activities remained basically

unchanged. In other analogs of **V**4, the replacement of the two central Gln residues by more polar Asn (*HKHNHKH* motif with $H=$\{Val, Tyr, or Ala\}) with increased peptide amphipathicity were predicted to attain significantly increased antimicrobial potencies independent of the *H* residue type.

Thus, the variation of *H* residues forming the hydrophobic face of the analogs V4 mainly affected the hemolytic activity, which was shown to depend strongly on the $\Pi_{o/w}$ (lipophilicity index), but did not affect the predicted MIC* of the analogs. Because the hemolytic activity correlates strongly with the lipophilicity index of V-peptides and is less dependent on Q_M (molecular charge) and AI (amphipathicity index), it was concluded that the lysis of human erythrocytes is probably caused by enhanced penetration of more hydrophobic peptides into the phospholipid membrane of the eukaryotic cells. Therefore, Frecer et al. [2004] proposed that substitutions of the *H* residues with less hydrophobic residues in the nonpolar face of the amphipathic analogs (while keeping the polar face unchanged) seems to be a suitable design strategy for reduction of the hemolytic activity of the V-peptides while preserving their antimicrobial potencies.

On the other hand, directed substitutions of the *B*/*P* residues in the polar face of the V-peptides by more polar residues that increase the peptide amphipathic character (more negative AI values) while preserving the net charge, symmetry of the binding motifs, and composition of the hydrophobic face are predicted to bring about a significant increase in the antimicrobial potency. Thus, the rational strategy of residue substitution directed either to polar or nonpolar faces of the cyclic V-peptides might result in dissociation of antimicrobial and hemolytic effects of cationic amphipathic V-peptides.

Implications for hypothetical mechanism of antimicrobial effect of V peptides

Amphipathicity of molecules has been related to their ability to form aggregates and supramolecular complexes [Israelachvili et al., 1980]. Increased aggregation or formation of assemblies containing amphipathic particles on the surface of bacterial membrane may therefore be responsible for their antimicrobial effect [Hwang and Vogel, 1998; Oren and Shai, 1998]. Aggregation of cationic peptides alone is less probable at neutral pH. However, introduction of anionic amphipathic LA molecules into such assemblies may facilitate formation of aggregates by cationic peptides. Recently, it was shown that polymyxin B is able to sequester LA from the surface of liposomes containing dimyristoylphosphatidylethanolamine [Thomas et al., 1999]. Because antimicrobial activity of the V-peptides

strongly increases with increasing amphipathicity of the molecules at constant Q_M and $\Pi_{o/w}$, Frecer et al. [2004] hypothesized that aggregates of V-peptides rather than individual molecules exert their strong antimicrobial effect. The low nanomolar range of the MIC of the V-peptides suggests that a more intricate interaction with the bacterial membrane may be involved in the mechanism of antimicrobial action of these peptides rather then the nonspecific membrane disruption. In fact, it was reported [Matsuzaki, 1998] that antimicrobial peptides such as magainin-2 and PGLa form peptide-lipid heterosupramolecular pores in the phospholipid bilayers, which explains the observed synergism in their antimicrobial effects. Membrane pores formed by α-helical magainin as well as β-hairpin-shaped protegrin-1 were previously detected by neutron diffraction [He et al., 1995; Huang, 2000]. These peptides produced a diffraction pattern similar to the one generated by the well-established transmembrane gramicidin channel [Yang et al., 1998]. Similar conclusions, which relate the ability of cationic peptides to form aggregates to their antimicrobial potencies, has been recently presented for dermaseptin S4 [Feder et al., 2000], protegrin-1 [Roumestand et al., 1998], and human defensins [Skalicky et al., 1994].

In conclusion, the computationally designed antimicrobial V-peptides display binding affinities to LPS and LA in the low micromolar range, and by means of molecular modeling were predicted to form amphipathic β-hairpin-like structures when binding to LPS or LA. The V-peptides also exhibit strong antimicrobial effect against gram-negative bacteria with MIC in the nanomolar range, and low cytotoxic and hemolytic activities at concentrations significantly exceeding their MIC. QSAR analysis of peptide sequences and their antimicrobial, cytotoxic, and hemolytic activities revealed that site-directed residue substitutions in the hydrophobic face of the amphipathic peptides with less lipophilic residues selectively decreases the hemolytic effect without significantly affecting the antimicrobial and cytotoxic activities. On the other hand, the antimicrobial effect can be enhanced by substitutions in the polar face with more polar residues, which increase peptide amphipathicity. Thus, the findings by Frecer et al. [2004] highlight the importance of the peptide amphipathicity and propose a rational method to dissociate the antimicrobial and hemolytic effects of cationic peptides. The de novo designed V-peptides harbor potencies unsurpassed by any known antibiotics of metabolite or peptide origin. The general sequence pattern (Ac-C-HBHB(P)HBH-GSG-HBHB(P)HBH-C-NH₂) may be adopted for further rational design of a repertoire of anti-endotoxin peptides from which candidates with potent antimicrobial activities of high specificity index may be selected through empirical tests.

SCREENING FOR ANTIMICROBIAL PEPTIDES FROM PHAGE-DISPLAY PEPTIDE LIBRARY

For a better understanding of the selective interaction of antibacterial peptides with the gram-negative bacterial outer membrane, many structure–activity relationship studies have been carried out to relate the structural parameters such as peptide charge, helicity, hydrophobicity, and hydrophobic moment to the activity and selectivity [Dathe and Wieprecht, 1999; Tossi et al., 2000; Frecer et al., 2000b, 2004]. However, most studies have not considered or solved the problem that sequence modifications usually result in complex changes of more than one structural parameter, making it very difficult to trace the activity differences to a specific structural motif [Dathe and Wieprecht, 1999].

By molecular modeling of peptide-LA interaction, our earlier study [Frecer et al., 2000b, 2004] predicted that the minimum LA binding motif, ***bhphb***, has the best affinity for LA among various derived or predicted LPS/LA-binding motifs, such as ***bhhhbhhhb, bhb(p)hb, bbbbb***, and ***hhhhh*** (***b***-basic, ***p***-polar, ***h***-hydrophobic residue). By biopanning of LPS/LA binding peptides from a random phage display peptide library, Zhu et al. [2003] further tested the above computational prediction. The analysis of dodecapeptide sequences screened from this phage-displayed random library was expected to help to elucidate the physicochemical nature of binding of LPS/LA, via possible specific binding motifs. Furthermore, in combination with SPR technology, this approach allows a rapid initial search for potential new anti-endotoxin peptides that have high affinity for LPS/LA, and provide guidance for the rational design and development of new peptides.

Although all identified natural linear short peptides adopt α-helical structure upon interaction with negatively charged membrane, different synthetic peptides containing 6–15 residues [Ono et al., 1990; Castano et al., 2000] that adopt a β-sheet structure in the presence of lipid were reported to exhibit variable antimicrobial potentials. Because the length of 12 residues is sufficient to assume a distinct secondary structure, Zhu et al. [2003] have used an empirical approach involving biopanning of a phage-displayed dodecapeptide library to select LPS/LA-binding peptides to study the minimum LA/LPS-binding motif. This study was carried out with the long-term aim of searching for potential pharmaceutical anti-endotoxin peptides with antimicrobial therapeutic value. In amplification of the phage of interest, it was found

that indeed under the same conditions, different phage clones were amplified to varying titer with more than a 1,000-fold difference (2.4×10^8 pfu/mL to 3.6×10^{11} pfu/mL), suggesting variable antibacterial potentials among the phage-displayed dodecapeptides. By SPR analysis using BIAcore technology, the selected dodecapeptides showed binding in realtime to LPS/LA.

Indeed, the pattern of alternate distribution of basic and hydrophobic residues, *b(p)hb(p)hb(p)* that were computationally predicted by Frecer et al. [2000b], was also found to be present in LPS/LA binding dodecapeptide sequences selected by biopanning. In addition, other patterns similar to *hhhhh*, *bbbbb* were also observed. Sequence alignment revealed various potential secondary structures with these selected dodecapeptides, which contain specific signature motifs such as *b(p)hb(p)hb(p)*, *bbbb*, *hhhh* capable of binding LPS/LA. However, none of these peptides exhibit a significant calculated structural amphiphilicity while assuming a secondary structure. Thus, Zhu et al. [2003] suggest that for these short dodecapeptides to bind LPS/LA, the potential for their structural adaptation is more important than an amphipathic structure. Thus, using a strategic combination of empirical and theoretical approaches, Zhu et al. [2003] revealed a more remarkable diversity of sequence and structure propensities than expected, not being limited to an amphipathic structure (α-helix or β-sheet).

It is noteworthy that many of the dodecapeptide sequences contain clusters of basic and hydrophobic amino acids, especially histidine, proline, and tryptophan, highlighting apparent amphiphilicity and bacterial membrane activity, such as the natural histidine-rich peptide, histatin, which shows no homology to other known peptides and does not have an amphipathic character [Brewer et al., 1998]. Furthermore, contrary to expectation, the structural amphipathicity, which is usually quantitatively measured by hydrophobic moment, is also generally far from being significantly high in the selected dodecapeptides. Many efforts have been undertaken to improve the activity of antimicrobial peptides with emphasis on the modulation of hydrophobic moment [Dathe and Wieprecht, 1999]. However, based on the findings of Zhu et al. [2003], an amphipathic structure may be too simple to account for LPS/LA binding of peptides, and the importance of the hydrophobic moment might have been overemphasized.

Thus, although the growing opinion of antimicrobial peptides is that their biological activity is a function of amphipathic structure and high cationic charge, these LPS/LA binding dodecapeptide sequences have no predictable secondary structure in solution, indicating the importance of a random structure prior to their interaction with LPS/LA. The widely accepted dogma of structure–activity relationship was recently challenged by Rao [1999], who suggested that the maintenance of balance between peptide hydrophobicity and hydrophilicity is a critical parameter in addition to structure. This property is also observed in the dodecapeptides selected in this study, and is deemed to be an important consideration in future design of peptides with pharmaceutical relevance. This idea is further corroborated by Bessalle et al. [1990], in their study of short peptides of 9 or 10 residues with little or no α-helicity, where they argued that it might be an overinterpretation to correlate bioactivity strictly with structural amphiphilicity. Further supporting evidence comes from a study of synthetic diastereomeric peptides, which also showed that derivatives that are totally devoid of α-helical structure but have a high ratio of hydrophilic to hydrophobic residues still retain the full antimicrobial activity of the parent peptide [Oren et al., 1997].

Although the dodecapeptides were selected based on their affinity for LPS/LA, their antibacterial potential may not be directly related to their affinity for LPS/LA. However, identical membrane affinity and permeabilization efficiency revealed in other studies [Dathe et al., 1996] suggest that high affinity is sufficient to destabilize highly negatively charged lipid membranes despite distinct structural differences. In consideration of the difficulty and limitations in structure–activity-based modulation of antibacterial activity and selectivity [Dathe and Wieprecht, 1999] due to the fact that replacement of residues often does not only affect a single structural parameter, Zhu et al. [2003] proposed that screening a random peptide library in combination with BIAcore technology may be a very efficient initial approach towards the development of anti-endotoxin peptides. This approach aids further investigations using chemically synthesized peptides based on the identified phage-displayed sequences to determine the structure and absolute binding affinity to LPS/LA and antibacterial activity.

The foregoing part of this review has given an assessment of some conventional and recent approaches in antimicrobial drug discovery and development. The final part of this review will focus on a novel alternative that is best analogized by the ancient Chinese medicinal strategy of using "poison to kill poison." In modern parlance, this innovative technique entails the enslavement of a "*non*-endotoxic" LPS molecule derived from a bacterial pathogen (*H. pylori*) as a competitor to antagonise the endotoxicity of LPS targets.

H. pylori LPS AS AN ENDOTOXIN-ANTAGONIST WITH POTENTIAL ANTIMICROBIAL PROPERTIES

It has been estimated that half the world population is infected by *H. pylori* [Lee, 1996]. This bacterium colonizes the human gastric mucosa and is the etiological agent of active chronic gastritis [Warren and Marshall, 1983]—the common precursor for peptic ulcer disease and possibly, gastric cancer [Forman and Goodman, 2000]. *H. pylori* (LPS) may be an important pathogenic factor and as such, attempts have been made to relate its structure to its biological functions [Moran, 1996; Moran and Aspinall, 1998]. Although LPS is an essential component of virtually all GNB cell membrane, structural variations of the LPSs have been well documented [Westphal et al., 1983; Raetz, 1990; Moran et al., 1997; Suda et al., 1997]. Based on both in vitro and in vivo observations, much of the pathogenicity and virulence of *H. pylori* LPS has been speculated to be linked to its O-specific chain and core oligosaccharide [Moran and Aspinall, 1998]. The *H. pylori* LPS has significantly lower endotoxic and immunological activities compared to those of the enterobacterial LPS [Moran 1995, 1996; Moran and Aspinall, 1998].

Compared to enterobacterial LA, *H. pylori* LA isolated from two *H. pylori* strains is shown to be underphosphorylated and underacylated [Moran et al., 1997; Suda et al., 1997]. Such structural deviations from conventional enterobacterial LA have been implicated as the molecular basis for the low endotoxicity of *H. pylori* LA and LPS [Moran et al., 1997; Pérez-Pérez et al., 1995; Ogawa et al., 1997]. Thus, the *H. pylori* LPS/LA may have anti-endotoxic potentials, suppressing the action of potent enterobacterial LPS. On the assumption that *H. pylori* LPS/LA competes with endotoxin for cellular receptors, it could be further developed as an analogue that antagonizes endotoxin-induced cell activation and endotoxemia-associated inflammatory responses. The following part of this review describes our recent work (Ho et al., unpublished data; personal communication) on the investigation of the antiendotoxin properties of *H. pylori* LPS/LA for development into a potent endotoxin-antagonist with potential antimicrobial activity.

The anti-endotoxin effects of H. pylori LPS/LA was displayed by two-dimensional electrophoretic analysis of cell lysates of the LPS-treated human macrophage cells, THP-1. Compelling evidence was provided for the suppression of signal transduction proteins, hence preempting endotoxin-induced inflammation. In addition to its antagonistic ability, *H. pylori* LPS/LA was found to be noncytotoxic, nonhemolytic,

and nonhemagglutinating even at 100-fold the maximum normal antagonist concentration used. These properties confer functional advantages to *H. pylori* LPS for potential therapeutic applications.

Structural variations of *H. pylori* LPS influence its endotoxicity

H. pylori LAs isolated by Ho et al. are invariably underphosphorylated at the nonreducing glucosamine residue and underacylated and lack acyloxyacyl groups. Moreover, the 16- or 18-carbon acyl chains attached to the backbones are longer than those of the endotoxic enterobacterial LAs. Intrastrain microheterogeneity of the LA moiety is observed for *H. pylori* strains NCTC 11637A and 233. The most obvious difference is the presence of an (R)-3-(octadecanoloxy)octadecanoic acid in place of an (R)-3-hydroxyoctadecanoic acid at position 2′ of the reported structure. Such microheterogeneity in LA structures of the same *H. pylori* strain cultured under the same conditions could be attributed to the different growth stages of the bacteria during which subtle differences may occur to the de novo synthesis of the LAs. Upon repeated subculture on solid growth medium, it had been reported that *H. pylori*-type strain produces low-molecular-weight R-LPS while NCTC 11637A and B express S-LPS. However, NCTC 11637A LPS harbors shorter Le^x O-specific chains than the LPS of NCTC 11637B. With the exception of strain 233, all of the *H. pylori* isolates cultured on chocolate blood agar expressed short O-specific chains, which are of interest, because the shorter O-specific chains are expected to pose less steric hindrances to the bioactive LA during endotoxin neutralization. This is verified with the increased anti-inflammatory activity (efficacy in inhibiting endotoxin-induced hTNF-α) of *H. pylori* NCTC 11637A LPS compared to *H. pylori* 233 LPS. Both LPS species harbor the same LA moiety as well as Le^x antigen in the O-specific chain. The only difference is that 233 LPS harbors more repeating subunits of the Le^x antigen than NCTC 11637A LPS. Hence, the difference in the endotoxin neutralization capabilities of the two LPS species is attributed to the difference in the O-specific chain length, which probably results in different degrees of steric hindrances to the LA moiety.

H. pylori LPS is an effective endotoxin antagonist

Potential therapeutic use of *H. pylori* LPS/LA for the treatment of human septicemia would require it to be effective against endotoxin-induced activation of the cytokine response without appreciable cytokine induction on its own. Our work [Ho et al., unpublished communication] showed that LPS/LA from various *H. pylori* isolates were 1,000-fold weaker than the

endotoxic *E. coli* O55:B5 LPS in LAL activation. In addition, *H. pylori* LPS/LA is a very weak inducer of phagocyte activation [Birkholz et al., 1993; Pece et al., 1995; Pérez-Pérez et al., 1995; Ogawa et al., 1997]. Ho et al. showed that 0.5 nM *E. coli* O55:B5 LPS induced 1123.46 pg/mL of hTNF-α. *H. pylori* LPS/LA, however, induced less than 30% of that amount of hTNF-αeven at a concentration of 800-fold excess of *E. coli* LPS. Being negligibly endotoxic and non-inflammatory, *H. pylori* LPS/LA may be developed as a potential antagonist or a prophylactic agent in the treatment of endotoxemia. To avoid potential steric hindrance due to the additional O-specific chain and the core oligosaccharides in *H. pylori* LPS, which may attenuate its anti-endotoxic potentials, it would be pertinent to base future studies on the bioactive LA moiety alone, taking into consideration the charge modifications so as to assess the contribution of charge to endotoxin neutralization efficacy.

Proteome profiling of macrophages treated with *H. pylori* LPS/LA indicates suppression of endotoxin-induced proinflammatory response: *E. coli* endotoxin induces the proinflammatory response by regulating the signal transduction pathways of the immune-competent cells. Thus, suppression of such proinflammatory response by *H. pylori* LPS/LA would be regarded as its ability to "rescue" changes towards the unchallenged norm. The proteome profiles of cells treated with *H. pylori* LPS alone and with simultaneous mixtures of *H. pylori* and *E. coli* LPS were compared with the established "normal" and "disease" state standards to determine the changes in the protein profile. Kinetic differences were observed between the profiles of macrophages treated with LPSs from *E. coli* and *H. pylori*. The protein expression peaked at the 6th h for *E. coli* and at the 3rd h for *H. pylori* LPS treatments. This implies that (1) *H. pylori* LPS interacts rapidly with its target molecule that led to its "avoidance"/"interception" of inflammation/sepsis. Thus, it effectively competes against *E. coli* endotoxin, which takes a longer time to produce a proteomic change. Simultaneous treatment for 6 h with 0.5 nM *E. coli* LPS and 400 nM *H. pylori* LPS resulted in an intermediate number of total proteins expressed, indicating the competition between *E. coli* LPS and *H. pylori* LPS for the cellular receptors; and (2) *H. pylori* LPS treatment could have hastened protein expression from 6 to 3 h. If 6 h was the critical time-point for inflammatory response, as was demonstrated by hTNF-α secretion, then *H. pylori* LPS treatment would have disrupted the chronological order of events, thereby avoiding sepsis. Nevertheless, it must be acknowledged that both the *E. coli* and *H. pylori* LPSs could have acted via different mechanisms/

pathways such as the CD14-dependent, TLR-2, TLR-4 or scavenger receptor pathways.

Since the proteome profile of macrophages treated with 400 nM *H. pylori* LPS alone exhibited closer resemblance to that of the control untreated sample, it would be obvious that the *H. pylori* LPS is nonendotoxic and/or exhibits anti-inflammatory effects. Furthermore, the proteome profiles of macrophages treated simultaneously with *E. coli* LPS and *H. pylori* LPS were remarkably similar to those treated with *H. pylori* LPS alone, viz., the *E. coli* LPS-upregulated proteins were "rescued" to basal levels by treatment with *H. pylori* LPS. In these simultaneous combinatorial treatments, it appears that the nonendotoxic *H. pylori* LPS has preempted the endotoxic effects of *E. coli* LPS via competitive binding of receptor targets that are necessary for the activation and subsequent transduction of signal cascade. Such finding is consistent with the suppression of *E. coli* endotoxin-induced LAL activation and hTNF-α secretion by *H. pylori* LPS/LA. These results provide the basis for beneficial antagonistic relationship between *H. pylori* LPS and the endotoxic LPSs. Such dynamic kinetics of protein expression levels demonstrates the complexities of the molecular mechanisms involved in the proinflammatory response.

In conclusion, the simultaneous treatment of macrophages with *E. coli* LPS agonist and *H. pylori* LPS antagonist resulted in the attenuation of the otherwise up-regulated signal transduction proteins. This implies that the *H. pylori* LPS antagonist is able to suppress the induction of the proinflammatory signal transduction intermediates such that progress to the status of inflammation or apoptosis is pre-empted. With the structural elucidation of the highly efficacious bioactive *H. pylori* LA, chemical analogues could be developed with high yields and purity for the scaled-up treatment of macrophages and in vivo animal studies.

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