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Resurrecting Inactive Antimicrobial Peptides from Lipopolysaccharide (LPS) Trap

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Running Title: β-boomerang motif conquers LPS barrier
Host defense antimicrobial peptides (AMPs) are a promising source of antibiotics for the treatment of multiple drug-resistant pathogens. Lipopolysaccharide (LPS), the major component of the outer leaflet of the outer membrane of Gram-negative bacteria, functions as a permeability barrier against a variety of molecules including antimicrobial peptides (AMPs). Further, LPS or endotoxin is the causative agent of sepsis killing 100,000 people per year in the USA alone. LPS can restrict activity of AMPs inducing aggregations at the outer membrane as observed for frog AMPs temporins and also in model AMPs. Aggregated AMPs, as ‘trapped’ by the outer membrane, are unable to traverse through the cell wall causing their inactivation. In this work, we show that these inactive AMPs can overcome LPS induced aggregations while conjugated with a short LPS binding β-boomerang peptide motif and become highly bactericidal. The generated hybrid peptides exhibit activity against Gram-negative and Gram-positive bacteria in high-salt and detoxify endotoxin. Structural and biophysical studies establish mechanism of action of these peptides in LPS outer membranes. Most importantly, this study provides a new concept for the development of potent broad spectrum antibiotic with efficient outer membrane disruption as the mode of action.
Introduction

Survival of bacteria requires integrity of the outer layer or cell wall surrounding the plasma membrane. The cell wall of Gram-positive bacteria contains a thick layer of a polymer, termed peptidoglycan. The coarse meshwork of peptidoglycan provides a structural support as well as balance osmotic pressure of cytosol and environment. By contrast, the cell wall of Gram-negative bacteria is typified by an asymmetric outer membrane and a thin peptidoglycan layer adjacent to the plasma or inner membrane. The inner leaflet of the outer membrane contains phospholipids similar to cytoplasmic membrane, whereas, the outer leaflet of the outer membrane is predominantly composed of lipopolysaccharide (LPS) [1, 2]. The cell wall or peptidoglycan of Gram-positive bacteria does not maintain a permeability barrier due to little resistance to the diffusion of antibiotics and antibacterial agents [3-5]. While LPS faces the external environment, it acts as a permeability barrier for the outer membrane of Gram-negative bacteria [3-5]. Molecules of \( \leq 600 \text{ Da} \) can freely diffuse through outer membrane, however, higher molecular weight compounds are found to make a limited access through the outer membrane [3-5]. LPS has an amphiphilic structure that can be divided into three regions: a conserved lipid A part, a highly variable polysaccharide or O-antigen and a core oligosaccharide [1,2]. Lipid A domain, a hexa-acylated glucosamine based phospholipid, anchors with the acyl chains of phospholipids of the inner leaflet of outer membrane. The core oligosaccharide domain is centrally located that is covalently bonded with lipid A and O-antigen. Apart from permeability barrier, LPS is also known as endotoxin, a potent inducer of innate immune system in humans [6-8]. While in blood stream, LPS recognizes a Toll-like receptor or TLR-4 in macrophages inducing production of variety of inflammatory agents like TNF-\( \alpha \), IL1-\( \beta \) [9,10]. These inflammatory agents are necessary to clear bacterial infection. However, a dysregulated immune system, often caused by a severe
Gram-negative infection, may result in over production of inflammatory molecules on-setting lethal septic shock syndromes [9,10]. Septic shock accounts for nearly 100,000 deaths annually in developed nations including USA [11,12]. At present treatment for septic shock is rather limited. Consequently, there are intense interests to develop drugs against sepsis [13-16].

Host defense antimicrobial peptides (AMPs), vital components of innate immune system, act as a first line of defense against invading pathogens in most of the organisms [17-19]. AMPs are considered to be highly important leads or potential therapeutics to control drug-resistant bacterial pathogens [20-24]. Bacterial cell lysis caused by AMPs is an outcome of disruption of the cytosolic membrane integrity. Cationic and hydrophobic characteristics of a vast majority of AMPs are suitable for insertion and perturbation of anionic bacterial membranes [25-27]. However, intra-cellular targets for AMPs are also identified [28-30]. Studies with model membranes, resembling cytosolic membrane of bacteria, suggest that AMPs mechanism of cell membrane perturbation may include barrel stave, torodial pore, carpet mode or general interfacial mode of action [31-33]. It has also been realized that the cell wall components of bacteria play important roles in insertion and translocation of AMPs towards cytosolic membrane [34-36]. Several studies have demonstrated higher MIC values of AMPs for Gram-negative bacteria in comparison to Gram-positive organisms [37-41]. In particular, LPS of the outer membrane of Gram-negative bacteria has been found to be actively involved in mode of action of AMPs [42-48]. Due to the LPS barrier there are less numbers of antibiotic precursors against multiple drug resistant strains of Gram-negative in the drug discovery pipelines [49, 50]. One of the mechanisms LPS-outer membrane employs toward reduction of efficacy of AMPs through inducing self-association or aggregations of peptides [51-54]. LPS induced aggregation of AMPs may result in their complete inactivation as
observed in temporins, a group of AMPs obtained from skin of European red frog and in
designed peptides [52-54]. Temporins are among the shortest AMPs demonstrating potential
in anti-bacterial therapeutics [54, 55]. However, a number of temporins including temporin-
1Ta (TA) and temporin-1Tb (TB) are only active against Gram-positive bacteria [52-54].
Similar observation has been made in designed Lys/Leu based AMP or KL12 whereby
peptide showed limited activity towards Gram-negative bacteria [51]. AMPs trapped in LPS
in oligomeric states are unable to traverse or translocate through the outer membrane possibly
cause their inactivation [54, 56, 57]. In this work, we demonstrate that a short LPS binding
peptide motif or β-boomerang motif, GWKRKRFG, designed in our laboratory [58,59]
rescues these inactive AMPs including TA, TB and KL12 peptide from LPS outer membrane.
Towards this, synthetic peptides are made containing β-boomerang motif at the C-termini and
investigated for bactericidal activities, cell lysis and mode of action. In the on-set of drug
resistance and LPS permeability, current study suggests that β-boomerang LPS binding motif
could be useful for the development of potent, salt resistant, non-toxic and broad spectrum
antimicrobial agents.

**Materials and Methods**

Peptides and rhodamine labeled peptides were synthesized commercially by GL Biochem
(Shanghai, China) and further purified by a reverse phase HPLC, Waters, using a C_{18} column
(300 Å pore size, 5 μm particle size). A linear gradient of acetonitrile/water mixture was used
for the purification and the peak eluting with highest purity was collected and freeze-dried.
LPS of *Escherichia. coli* 0111:B4, FITC-LPS of *E. coli* 055:B5, acrylamide, NPN (1-N-
phenylnaphthylamine) were purchased from Sigma. The bacterial strains were obtained from
American Type Culture Collection, VA, USA. SYTOX green was obtained from Invitrogen.
Antibacterial assay: The minimum inhibitory concentration (MIC) of hybrid peptides was determined using broth dilution method against four Gram-negative bacteria (*Escherichia. coli*, *Pseudomonas. aeruginosa* ATCC 27853, *Klebsiella. pneumoniae* ATCC 13883, *Salmonella. enterica* ATCC 14028) and four Gram-positive strains (*Bacillus. subtilis*, *Staphylococcus. aureus* ATCC 25923, *Streptococcus. pyogenes* ATCC 19615 and *Enterococcus. faecalis* ATCC 29212). Mid log phase cultures of these bacterial strains were obtained either in Mueller-Hinton (MH) or LB broth and diluted to an OD$_{600}$ of 0.01 (~10$^6$cfu/ml). Two-fold serial dilutions of the peptides were carried out in 96-well polypropylene microtiter plates in a total volume of 50 µL at concentrations from 200 µM to 0.1 µM. To this 50 µL of the diluted cells in MH or LB broth were added and incubated for 18 hrs at 37°C. Water in the place of peptides acted as negative control. The MIC values were computed spectrophotometrically and the concentration at which there is complete inhibition of growth of bacteria was considered as MIC of the peptide. In order to dissect bacterial killing and bacterial growth inhibiting activity, the wells before and after MIC values were streaked onto MH agar plates and incubated overnight at 37°C. The well that does not show visible bacterial growth was compared to the wells that were considered as MIC values.

Hemolytic assay: Blood was collected from healthy mice in a tube containing EDTA. RBCs with EDTA were centrifuged at 800 × g for 10 min to remove the buffy plasma coat layer. Then the resulting RBCs were resuspended in PBS (35 mM phosphate buffer, 150 mM NaCl, pH 7.0) and washed three times. Around 50 µL of suspended RBCs were added to equal volume of two-fold dilution of the peptides in 96-well micro titer plates and incubated for one hour. The final erythrocyte concentration is 4% (V/V). After one hour, the mixture was centrifuged and the release of hemoglobin in the supernatant was determined spectrophotometrically at OD$_{540}$. Buffer and 1% triton-X in the place of peptides served as
negative control and positive control respectively. The percentage of hemolysis was calculated using the following formula:

\[
\text{Percentage of Hemolysis} = \frac{\text{OD Peptide-OD buffer}}{\text{OD triton-X-OD buffer}} \times 100. 
\]

**LPS neutralization assay:** The strength of the peptides to neutralize LPS was assayed using commercially available LAL chromogenic kit (QCL 100 Cambrex). The protocol explained in the manufacturer’s instructions was strictly adhered. The endotoxic principle, LPS in Gram-negative bacteria activates a proenzyme in *Limulus* amoebocyte lysate (LAL). This activated enzyme in turn catalytically splits colored product para-nitroanilide (pNA) from the colorless substrate Ac-Ile-Glu-Ala-Arg-para-nitroanilide and is detected spectrophotometrically at OD\(_{410}\). The peptides were dissolved in the pyrogen free water supplied with the kit and pH was adjusted to 7.0 with 1N HCl or 1N NaOH (which is prepared in pyrogen free water). The increasing concentrations of the peptides were incubated with 1.0 EU (Endotoxin units) in a total volume of 50 µL for 30 minutes at 37 °C.

About 50 µL of LAL reagent was added to peptide-EU complex and further incubated for 10 minutes followed by addition of 100 µL substrate. After incubation of six minutes for the reaction, the release of colored product was recorded at OD\(_{410}\). Water in the place of peptides served as negative control (blank) that is considered as 0% inhibition and percentage of LPS neutralization was calculated by

\[
\% \text{ of LPS neutralization} = \frac{\text{OD blank- OD peptide}}{\text{OD blank}} \times 100. 
\]

**Fluorescence studies:** All of the fluorescence studies were carried out in Cary Eclipse fluorescence spectrophotometer (Varian, Inc) in 10 mM phosphate buffer, pH=7.0 unless otherwise specified.
Aggregation state of the peptides using rhodamine fluorescence: 2 μM of the purified rhodamine labeled peptides were added to increasing concentrations of LPS and the fluorescence was monitored at excitation of 485 nm and emission at 550-620 nm in 10 mM phosphate buffer, pH=7.0.

Membrane permeabilization assays: E. coli BL21 (DE3) cells were grown to mid logarithmic phase in LB broth and diluted to an OD$_{600}$ of 0.5. For outer membrane permeabilization using NPN dye, about 500 μL of the cells were added to 10 μM of NPN (1-N-phenylnaphthylamine) dissolved in acetone) and basal fluorescence was recorded with excitation at 350 nm and emission maximum at 420 nm. An increasing concentration of hybrid peptides was added to cells with NPN and fluorescence intensity after addition of each concentration was recorded. For membrane permeability using SYTOX green dye, 0.5 OD$_{600}$ cells were added to 1 μM dye and incubated with shaking at 37 °C for 15 min. Basal fluorescence was recorded by excitation at 485 nm and emission at 520 nm followed by the addition of increasing concentration of peptides.

Membrane depolarization: The assay to measure membrane depolarization ability was performed in intact E. coli cells and its spheroplast. For intact cells, the bacteria were grown to mid log phase, centrifuged and suspended in 5 mM HEPES and 20 mM glucose at pH=7.4. After a brief wash, the cell pellets were suspended in the same buffer containing 100 mM KCl to an OD$_{600}$ nm of 0.05. Spheroplast was prepared by suspending the log phase grown bacterial pellets in 10 mM Tris, 25 % sucrose pH=7.4. The pellets were washed twice and resuspended in the same buffer containing 1 mM EDTA. The cells were incubated for 15 min with shaking and centrifuged. The pellets were immediately dissolved in ice-cold water and further incubated for 10 min at 4°C. The cells devoid of outer membrane i.e. spheroplasts
were collected by centrifugation and the pellets were dissolved to an $OD_{600}$ of 0.05 in same buffer (5 mM HEPES, 20 mM glucose, 100 mM KCl) as intact cells. The depolarization detecting dye DiS-C$_{3}$-5 (3,3′-diethylthiodicarbocyanine iodide) dye was added to either intact cells or spheroplasts and basal fluorescence was recorded by exciting the dye at 622 nm and emission collected at 630-700 nm. This was incubated for about 45-60 minutes until the decrease in fluorescence in stable i.e. the dye partitions into the membrane. This was then followed by the addition of increasing concentration of different peptides and the depolarizing ability was recorded as the measurement of increase in fluorescence intensity.

Measurement of dissociation of LPS micelles: The ability of the conjugated peptides to dissociate LPS micelles was studied fluorometrically using FITC conjugated LPS and also by dynamic light scattering (DLS) measurements. In FITC-LPS, the fluorescence of FITC is self-quenched in LPS micelles which upon dissociation of micelles would dequench. Basal fluorescence of 0.5 µM of FITC-LPS was recorded at an excitation of 480 nm and emission at 520 nm. The dissociation of LPS micelles was recorded as a function of increase in fluorescence intensity with addition of increasing concentration of peptides. In DLS measurements, the distribution of various sizes of LPS micelles was determined with 0.5 µM of LPS. The change in this distribution was observed after addition of peptides in different ratios e.g. 1:0.5, 1:1 and 1:2 (LPS: Peptide). The scattering was measured with Dynamic Light Scattering software provided with the instrument (Brookhaven Instruments Corp., Holtsville, NY) and the scattering data was analyzed with CONTIN method.

Intrinsic tryptophan fluorescence and acrylamide quenching: The binding of hybrid peptides to LPS was determined using tryptophan fluorescence. Tryptophan is an intrinsic fluorescent probe and is extremely sensitive to polarity of the environment. About 5 µM of the peptide
was taken with 500 µL buffer and the emission of tryptophan fluorescence was noted in free-
state with excitation at 280 nm and emission at 300-400 nm. Each peptide sample was titrated
with increasing concentrations of LPS and fluorescence emission was recorded from 300-400
nm. Fluorescence quenching of tryptophan residue was carried out by adding increasing
concentrations of acrylamide from stock of 5 M to solution containing only peptide and also
to peptide-LPS (1:4) complexes. The Stern-Volmer constant (Ksv) values were then
calculated using $\frac{F_0}{F} = 1 + Ksv [Q]$ where $F_0$ and $F$ are the fluorescence intensities before
and after addition of quencher and [Q] is the quencher concentration.

Circular dichroism spectroscopy: Conformational change of the peptides upon binding to
membranes was detected using CD spectroscopy. Data were collected using a Chirascan CD
spectrometer (Applied Photophysics Ltd., UK). The peptide and peptide/micelle complex
were scanned from 190 to 240 nm wavelengths in a 0.01 cm path length cuvette for an
average of 3 scans. Baseline scans were acquired using 10 mM phosphate buffer, pH=7.0.
Samples containing only LPS were also acquired with same settings. About 25 µM of
peptides were used with 30 µM of LPS to obtain secondary structure of the peptides. The
appropriate baselines were used to subtract the data and the corrected data were converted to
molar ellipticity (deg.cm².dmol⁻¹).

Isothermal titration calorimetry (ITC): Binding of peptides with LPS micelles were
Peptides and LPS were dissolved in 10 mM phosphate buffer, pH=7.0 and filtered. LPS at a
concentration of 10 µM was loaded into the sample cell and the reference cell was filled with
the buffer. The syringe was filled with 1mM peptide stock. Typically 25 injections of 3.5 µL
of the peptides were made into the sample cell at 25°C. The sample cell was stirring
continuously at 300 rpm. Raw data was collected and fitted using single site binding model in Microcal Origin 5.0 software (Origin Lab Corporation, Northampton, MA). Association constant (Kₐ) and enthalpy change (ΔH) were directly obtained from the software used. ΔG and ΔS were calculated using the fundamental equations of thermodynamics, ΔG = -RTlnKₐ and ΔS=(ΔH-ΔG) respectively.

Electron microscopy: Mid log phase grown *E. coli* cells were incubated with various concentrations of LG21 peptide (3 μM, 8 μM and 15 μM) and 50 μM of LG21R19A for 2 hours. The cells were centrifuged and the pellets were dissolved in 10 μL 10 mM Phosphate buffer, pH 7.0 and a drop containing the bacteria were loaded onto carbon-coated EM grids. The grids were then negatively stained with 2% phosphotungstic acid and examined using Jeol JEM-1230 electron microscope.

**Results and Discussion**

Peptide Design: In previous studies, we have designed *de novo* a series of 12-amino acid long cationic/hydrophobic peptides with antimicrobial and anti-endotoxic activity [58, 59]. A sequence motif WKRKRF located at the center of the primary structures of the designed peptide has been demonstrated to be critical for the structure in LPS and activity. An octa-peptide or GG8, G-WKRKRF-G, interacted with LPS and adopted boomerang-like conformation in LPS. Amphipathic structure of the boomerang motif is demarcated by close packing of the aromatic side chains of residues W and F whereas the side chains of the four basic amino acids are distally located. GG8 peptide or boomerang motif is devoid of antimicrobial and antiendotoxic activities, but its atomic resolution structure in LPS indicated
specific interactions. We have surmised that inclusion of boomerang motif sequence in TA, TB and KL12 based peptide may abolish LPS induced aggregation yielding broad spectrum AMPs (Fig. 1). We have obtained synthetic hybrid peptides of TA, TB, KL12 containing the boomerang motif at the C-terminus (Table 1). Two analog peptides containing Ala replacements are also synthesized to understand role of the β-boomerang motif sequence in activity (Table 1).

Antimicrobial Activity and Red Blood Cell Lysis by Hybrid Peptides: Table 2 summarizes toxicity of the hybrid peptides, LG21, FG21, KG20 and LG21R19A and LG21W15AF20A against bacterial strains, in MH broth, and red blood cells. Remarkably, hybrid peptides, LG21, FG21 and KG20, demonstrate potent antibacterial activity, with low MIC values, including Gram-negative strains in comparison to the parent peptides (Table 2). It may be noted that the parent peptides, TA, TB and KL12, were found to be largely ineffective against Gram-negative bacteria [51-53, 60]. Interestingly, the mutated analogs of LG21, LG21R19A and LG21W15AF20A exhibited largely limited bacterial cell killing activity (Table 2). The role of outer membrane towards bactericidal activity of the parent peptides and hybrid peptides correlate well with the membrane depolarization, studied with a fluorescent dye DiS-C3-5, of *E. coli* cells and spheroplast i.e. *E. coli* cells lacking outer membrane [51]. The hybrid peptides efficiently depolarizes *E. coli* cells and spheroplast, whereas, parent peptides show lower depolarization of *E. coli* cells and more depolarization of the spheroplast (Fig. S1). The LG21R19A peptide was least active to depolarize the cells and spheroplast (Fig. S1). These data indicate that the outer membrane, as a permeability barrier, plays essential roles in insertion of the peptides.
In hemolysis assays, the hybrid peptides, LG21, FG21 and KG20, demonstrate very low activity (Table 2). At 100 µM concentration of peptides, lysis of red blood cells has been estimated to be only <10%. However, a somewhat higher hemolysis has been detected for the mutated analogs LG21R19A and LG21W15AF20A peptides (Table 2).

We have also tested antibacterial activity of the active hybrid peptides LG21, FG21 and KG20 in LB medium containing 150 mM NaCl. As can be seen, LG21 and FG21 peptides are able to exert antimicrobial activity even in the presence of salt, with MIC value ranging from 8 to 10 µM, against test bacteria (Table 3). However, KG20 peptide has been observed to be salt sensitive showing a rather high MIC values (Table 3). A number of AMPs are known to be inhibited under physiological salt concentrations including β-defensins, magainins, indolicidin etc. [61-63]. Taken together, these results demonstrate that the hybrid peptides, LG21, FG21 and KG20 are bestowed with broad spectrum of bactericidal activity and notably activities are retained, for LG21 and FG21, even in the presence of salt. Further, it may be noted that TA and TB were found to be poorly hemolytic whereas KL12 peptide exhibited strong hemolysis, ~80% at 100 µM concentration [51, 55]. In other words, LG21 and FG21 retain low hemolytic activity akin to TB and TA peptides. Further, the inclusion of the boomerang motif in KL12 peptide has significantly lowered the hemolytic property of the hybrid KG20 peptide. The lowered hemolytic activity of the KG20 peptide, in comparison to KL12, may be arising due to the preferential interactions, as demonstrated in previous studies [58,59], of the boomerang motif of the hybrid peptide with negatively charged lipids of bacterial membranes over zwitterionic lipids in mammalian cells. The inability of the mutated peptides, LG21R19A and LG21W15AF20A, to exert bactericidal effect implicates the vital role of the cationic residues and aromatic residues W15 and F20 in the short boomerang motif sequence for the activity of the hybrid peptides. It may be noted that despite the same
cationicity of the LG21W15AF20A peptide akin to LG21, the mutated peptide lacks bactericidal activity. Most strikingly, a single mutation of R19A in LG21R19A peptide also severely reduces the bactericidal activity. The poor bactericidal activity and relatively higher hemolytic activity of the mutated peptides, LG21W15AF20A and LG21R19A, may be related the altered interactions and structures in the context of different cell types. To gain potential correlation in structure-activity, we have utilized LG21R19A peptide along with LG21, FG21 and KG20 peptides for further studies as described below.

*Endotoxin Neutralization by Hybrid Peptides:* LPS or endotoxin neutralization by the hybrid peptides are examined using LAL assays [64]. LAL assay is highly sensitive in detecting free LPS at concentration as low as 1pM [65]. LPS neutralizing proteins and peptides can sequester LPS reducing free endotoxin in solution [66,67]. All three hybrid peptides, LG21, FG21 and KG20, demonstrate inhibitory activity in neutralizing LPS (Fig. S2). The LG21 peptide shows a higher potency in endotoxin neutralization in comparison to FG21 and KG20 (Fig. S2). LG21 exhibits inhibition of LPS even at 3 µM concentration. At 10 µM concentration of LG21 >75% inhibition of LPS has been detected. Overall, there has been an increase of inhibition with increasing concentrations of LG21, FG21 and KG20 peptides (Fig. S2). The mutated peptide LG21R19A does not show LPS inhibitory activity in a significant way (Fig. S2). Note, previous study showed that LPS neutralization ability of TB and TA peptides is highly limited [53]. These hybrid peptides, LG21, FG21 and KG20, contain ability to neutralize toxicity of LPS. It may be worthwhile to consider that the incorporation of the boomerang motif in other AMPs may enhance their antimicrobial and anti-endotoxic activities with potentially lowering toxicity.
Self-association of Hybrid Peptides in LPS: Rhodamine labeled peptides are utilized to assess self-associations of hybrid peptides in presence of LPS. The fluorescence emission intensity of rhodamine is highly sensitive to molecular aggregations whereby aggregations of rhodamine labeled peptides would show a decrease in fluorescence intensity of the fluorophore due to self-quenching [52]. Fig. 2 shows a plot indicating differences in fluorescence intensity (ΔF) of rhodamine, measured at emission maxima of 590 nm, for rhodamine labeled peptides in the absence of LPS and in presence of LPS, at 1 µM, 2 µM, 4 µM, 8 µM and 10 µM concentrations. As can be seen, there has been an increase in fluorescence intensity of rhodamine for LG21, FG21 and KG20 hybrid peptides with increasing concentrations of LPS, demonstrating plausible absence of aggregations in LPS for these peptides. By contrast, fluorescence intensity of rhodamine has been found to be decreased for LG21R19A peptide, implying aggregation of the mutated peptide in complex with LPS (Fig. 2). Collectively, aforementioned results suggest that the incorporation of LPS binding boomerang motif abolish LPS induced aggregations in hybrid peptides. The ability to kill Gram-negative bacterial strains by the hybrid peptides correlate well with their lack of aggregations in LPS. The self-association of LG21R19A peptide in LPS appears to be responsible for its reduced bactericidal activity.

Permeation of E. coli Cell Membrane and Membrane Disruption by Hybrid Peptides: In order to determine membrane damage caused by the hybrid peptides, we have carried out fluorescence studies, with E. coli cells, employing membrane probes: NPN, SYTOX green. NPN and SYTOX green are unable to enter into an intact cell unless the membrane integrity is compromised by additions of membrane disrupting compounds. The fluorescence intensity of NPN delineate a drastic increase while bound to membrane lipid components whereas
fluorescence intensity of the cationic dye SYTOX green enhances in complex with the intracellular nucleic acids.

Fig. 3 (panels A,B) shows difference in fluorescence intensity (ΔF) of NPN (Fig. 3A), SYTOX green (Fig. 3B) vs concentrations of hybrid peptides. As can be seen, fluorescence intensities of NPN and SYTOX green are significantly increased upon additions of increasing concentrations of LG21, FG21 and KG20 peptides. These results strongly demonstrate that the hybrid peptides, LG21, FG21 and KG20, efficiently disrupt integrity of bacterial outer and inner membranes causing an influx of fluorescent molecules NPN and SYTOX green into the cells. Notably, in these assays, there are no significant changes of fluorescence intensity of these probes upon treatment with inactive LG21R19A peptide (Fig. 3, panels A, B). Further, EM images are obtained for E. coli cells either in absence or upon treated with LG21 peptide at three different concentrations, 3 µM, 8 µM and 15 µM and LG21R19A at 50 µM concentration for 2 hours (Fig. 4). Morphology of the cells of the LG21 peptide treated bacteria is distinctly modified in comparison to control (Fig. 4). Plausible membrane or cell wall damage can be seen at 3 µM (close to MIC) concentration of LG21 (Fig. 4B), in comparison to untreated cells (Fig. 4A). At higher concentrations, 8 µM and 15 µM, of LG21 cell shape and integrity changes and ghost like images are clearly observed (Fig. 4C and Fig. 4D). By contrast, the inactive LG21R19A peptide does not impart any discernable damage to the E. coli cells (Fig. 4E).

**Binding Affinity of Hybrid Peptides with LPS:** In order to better understand superior activity of hybrid peptides against Gram-negative bacteria, LPS binding parameters are obtained using ITC experiments (Fig. S3). LPS-peptide interactions are endothermic in nature for the active peptides, LG21, FG21 and KG20, as disclosed by upward trend to the ITC heat peaks
(Fig. S3 A-C, top panels). Usually, entropy driven or endothermic binding has been observed for LPS-AMPs interactions with gel phase of LPS at 25°C [68,69]. Interestingly, the inactive peptide LG21R19A shows an opposite trend whereby LPS interactions appear to be exothermic in nature (Fig. S3 panel D, top). The apparent dissociation constant (K_d) values and thermodynamic parameters of LPS-peptide interactions are estimated from ITC data (Table 4). The hybrid peptides, LG21, KG21 and FG20, interacted with LPS with sub micromolar affinity with K_d of 0.6 µM, 0.6 µM and 0.3 µM, respectively, whereas, LG21R19A delineates a comparatively much weaker binding to LPS with K_d of 10 µM (Table 4).

Localization of Hybrid Peptides in LPS: We have further assessed insertion of hybrid peptides in LPS micelles using intrinsic tryptophan fluorescence experiments. Fluorescence emission spectra of Trp for LG21, FG21, KG20 and LG21R19A were obtained at various concentrations of LPS (ranging from 1 µM to 20 µM) (Fig. S4). As seen, tryptophan residue has experienced a marked blue shift, i.e. emission maximum shifted toward a shorter wavelength in comparison to free peptide, for LG21, FG21 and KG20 peptides in LPS. In marked contrast, tryptophan residue of the mutated peptide LG21R19A exhibited a highly limited spectral shift in LPS (Fig. S4, Table 5). The solvent accessibility of the tryptophan residue was judged by fluorescence quenching studies in presence of acrylamide either in free solutions or in LPS containing solutions. The quenching constant (Ksv) values of LG21, FG21 and KG20 peptides in LPS are estimated to be significantly lower in comparison to Ksv values determined in free solution (Table 5). By contrast, LG21R19A peptide showed comparable Ksv values for tryptophan residue in LPS and in buffer solution (Table 5). These observations demonstrate that in active peptides, LG21, FG21 and KG20, tryptophan residue is inserted into non-polar environment of LPS with restricted exposure to solvent whereas tryptophan residue of the inactive peptide, LG21R19A, lacks non-polar environment of acyl
chains of LPS. In other words, the LG21R19A peptide is predominantly localized at the outer
surface or hydrophilic sugar region of LPS with higher degree of solvent exposure.

Disaggregation of LPS Aggregates by Hybrid Peptides: High-binding affinity and insertion
into hydrophobic lipid A domain of LPS of the active hybrid peptides may cause structural
changes of LPS. We have examined perturbation of LPS using dynamic light scattering (DLS)
and fluorescence of FITC labelled LPS. DLS experiments examine size distribution of LPS
aggregates in presence of peptides (Fig. 5). In free solution, LPS is highly polydisperse with
an average diameter of 806 nm (Fig. 5A). There has been a drastic reduction in size of LPS
aggregates with concomitant reduction in polydispersity in the presence of LG21 (Fig. 5B),
FG21 (Fig. 5C) and KG20 (Fig. 5D) peptides. By contrast, changes of LPS aggregates appear
to be largely limited; with an average diameter of 521 nm, in the presence of mutated
LG21R19A peptide (Fig. 5E). The average diameter of LPS micelles has been estimated, for
each of the active peptide, to be 85 nm, 87 nm and 134 nm for LG21, FG21 and KG20,
respectively. Hybrid peptide induced disaggregation of LPS micelles has also been observed
from fluorescence intensity of FITC in FITC conjugated LPS. Fluorescence intensity of FITC
in FITC-LPS is largely quenched in solution due to aggregation of LPS. Binding of proteins
or peptides causing perturbation of LPS aggregated structures may enhance fluorescence
intensity [59,70,71]. Proteins or peptides mediated dissociation of LPS have been correlated
with their anti-endotoxin activity [59,70,71]. Figure 5F shows change in fluorescence
intensity (ΔF) of FITC-LPS with increasing concentrations of LG21, FG21, KG20 and
LG21R19A peptides. As can be seen, there have been drastic increases in ΔF for active
hybrid peptides, LG21, FG21 and KG20, in a dose dependent manner, indicating potential
dissociation of LPS aggregates (Fig. 5F). On the other hand, the inactive peptide, LG21R19A,
does not cause dissociation of LPS as suggested by the lack of increase of fluorescence intensity of FITC (Fig. 5F).

Secondary Structures of the Hybrid Peptides: CD spectroscopy, at far UV region (240-190 nm), was carried out to assess global conformations of hybrid peptides in free solution and in complex with LPS micelles. CD spectra, in free solution, of the hybrid peptides, LG21, FG21, KG20 and LG21R19A, delineate a single band ~195 nm, indicating random conformations (Fig. S5). CD spectra obtained for the hybrid peptides and mutant analog either in LPS or in detergent micelles are characterized by two negative bands at ~220-225 nm and ~208-210 nm, indicating predominantly helical conformations. Intriguingly, active hybrid peptides, LG21, FG21 and KG20 as well as the inactive mutant LG21R19A assume helical conformations in LPS. It may be noted that TA, TB and KL peptides demonstrated helical conformations in LPS and random conformations in water [51,53]. In other words, irrespective of the activity, interactions of these peptides with LPS induce conformational transitions into helical states. At present, it is not clear from the CD studies whether the LPS binding motif assumes the boomerang-like conformations in LPS micelles. Atomic resolution structures of these hybrid peptides and the mutated peptides in complex with LPS micelles are required to be determined for specific structural features.

Conclusion

The lower activity of AMPs toward Gram-negative bacteria may arise from their self-association in LPS outer membrane. In this work, we demonstrate that an LPS binding peptide motif when conjugated with Gram-negative inactive AMPs temporins and KL12 abolished LPS-induced aggregations yielding broad spectrum salt resistant hybrid AMPs. Hybrid peptides demonstrate neutralization of endotoxin and poor in hemolysis. As a mode of
action, hybrid peptides bind LPS with high-affinity and disrupt the higher order structures of LPS. These traits of the hybrid peptides are correlated with efficient disruption of the outer membrane and cell permeabilization. The boomerang motif is critically involved in broad spectrum activity of the hybrid peptides as a single mutation LG21R19A in LG21 produced an inactive peptide that shows self-association in LPS. The lack of bactericidal and endotoxin neutralization activity of the LG21R19A peptide may stems from its low affinity binding to LPS micelles and limited perturbation of LPS structural states. We surmise that hybrid peptides obtained in this work and their mode of action through LPS outer membrane would be useful to design new class of cell wall permeabilizing AMPs. Such AMPs can be tested further for in vivo efficacy in animal models.

**Acknowledgment:** This work is supported by a grant from the Ministry of Education (MOE), Singapore (RG11/12).


Table 1: Amino acid sequences of the hybrid peptides LG21, FG21, KG20 and mutated analogs of LG21 with Ala replacement.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LG21</td>
<td>LLPIVGNLLKSLLGWKRKFAG</td>
</tr>
<tr>
<td>FG21</td>
<td>FLPLUGRVLGLLGWKRKFAG</td>
</tr>
<tr>
<td>KG20</td>
<td>KLLKLKLLKLLKGWKRKFAG</td>
</tr>
<tr>
<td>LG21R19A</td>
<td>LLPIVGNLLKSLLGWKRKFAG</td>
</tr>
</tbody>
</table>

*The amino acid sequences from parent peptides, TB (in LG21), TA (in FG21) and KL12 (in KG20) are highlighted in blue, whereas β-boomerang motif is in red. Mutations to Ala are italicized in LG21R19A and LG21W15AF20A. All peptides are amidated at their C-termini.
Table 2: Minimal inhibitory concentration (MIC, in µM) in MH broth and hemolysis of hybrid peptides. MIC values are also determined for the parent peptides TA, TB and KL12 peptides for comparison.

<table>
<thead>
<tr>
<th>Parent Peptides</th>
<th>Hybrid peptides and mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB</td>
<td>TA</td>
</tr>
<tr>
<td>R19A</td>
<td>W15AF20A</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gram-negative bacteria</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli (lab strain)</td>
<td>100</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (ATCC27853)</td>
<td>100</td>
</tr>
<tr>
<td>Klebsiella pneumonia (ATCC13883)</td>
<td>100</td>
</tr>
<tr>
<td>Salmonella enterica (ATCC14028)</td>
<td>200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gram-positive bacteria</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis (lab strain)</td>
<td>25</td>
</tr>
<tr>
<td>Staphylococcus aureus (ATCC25923)</td>
<td>25</td>
</tr>
<tr>
<td>Streptococcus pyogenes (ATCC19615)</td>
<td>25</td>
</tr>
<tr>
<td>Enterococcus faecalis (ATCC29212)</td>
<td>50</td>
</tr>
</tbody>
</table>

% of hemolysis at 100 µM: 8.5, 9.9, 2.4, 25, 60
Table 3: Minimal inhibitory concentration (MIC, in µM) in salt containing LB medium of hybrid peptides.

<table>
<thead>
<tr>
<th>Gram-negative bacteria</th>
<th>LG21</th>
<th>FG21</th>
<th>KG20</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> (lab strain)</td>
<td>8</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (ATCC27853)</td>
<td>8</td>
<td>8</td>
<td>50</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em> (ATCC13883)</td>
<td>8</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> (ATCC14028)</td>
<td>8</td>
<td>10</td>
<td>200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gram-positive bacteria</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> (lab strain)</td>
<td>10</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (ATCC25923)</td>
<td>12.5</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> (ATCC19615)</td>
<td>8</td>
<td>12.5</td>
<td>100</td>
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<tr>
<td><em>Enterococcus faecalis</em> (ATCC29212)</td>
<td>12.5</td>
<td>10</td>
<td>50</td>
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</table>
Table 4: Thermodynamic parameters and binding affinity of the hybrid peptides with LPS as determined from ITC.

<table>
<thead>
<tr>
<th>Peptide-LPS interaction</th>
<th>$K_a$</th>
<th>$\Delta H$</th>
<th>$T\Delta S$</th>
<th>$\Delta G$</th>
<th>$K_d$</th>
</tr>
</thead>
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<tr>
<td></td>
<td>$\mu M^{-1}$</td>
<td>kcal.mol$^{-1}$</td>
<td>kcal.mol$^{-1}$deg$^{-1}$</td>
<td>kcal.mol$^{-1}$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>LG21</td>
<td>1.64</td>
<td>0.58</td>
<td>8.9</td>
<td>-8.4</td>
<td>0.6</td>
</tr>
<tr>
<td>FG21</td>
<td>1.6</td>
<td>4.4</td>
<td>12.8</td>
<td>-8.4</td>
<td>0.6</td>
</tr>
<tr>
<td>KG20</td>
<td>3.2</td>
<td>3.4</td>
<td>12.2</td>
<td>-8.8</td>
<td>0.3</td>
</tr>
<tr>
<td>LG21R19A</td>
<td>0.099</td>
<td>-1.7</td>
<td>5.1</td>
<td>-6.8</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 5: Emission maximum wavelength ($\lambda_{\text{max}}$) and $K_{sv}$ values of Trp residue of hybrid peptides in buffer (Free) and in LPS micelles.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>K$_{sv}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
<td>LPS</td>
</tr>
<tr>
<td>LG21</td>
<td>358</td>
<td>338</td>
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<tr>
<td>FG21</td>
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<td>338</td>
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<tr>
<td>KG20</td>
<td>354</td>
<td>336</td>
</tr>
<tr>
<td>LG21R19A</td>
<td>356</td>
<td>354</td>
</tr>
</tbody>
</table>
Figure 1: Proposed model of activation of inactive AMPs aggregated in LPS by β-boomerang motif. Cartoon showing aggregation of AMP in LPS (left panel) and disruption of LPS outer membrane by non-aggregated AMP upon conjugation with β-boomerang motif (right panel).
Figure 2: Association of hybrid peptides with LPS by rhodamine fluorescence. Plot shows changes in fluorescence intensity, at the emission maxima ($\Delta F_{590}$ nm), of rhodamine labeled LG21 (rhLG21), FG21 (rhFG21) and KG20 (rhKG20) and LG21R19A (rhLG21R19A) peptides as a function of concentration of LPS. Experiments are done in 10 mM sodium phosphate buffer, pH 7.0. 2 µM of rhodamine labeled peptides were individually titrated with increasing concentrations of LPS. Fluorescence was monitored at excitation of 485 nm and emission at 550-620 nm.
Figure 3: **Cell permeabilization by hybrid peptides.** (panel A) Plot shows changes in fluorescence intensity of NPN (1-N-phenylnaphthylamine) at emission 420 nm with increasing concentrations of LG21, FG21, KG20 and LG21R19A peptides in presence of *E. coli* cells. *E. coli* cells were incubated with 10 µM of NPN and increasing concentrations of peptides were titrated, in individual experiments, measuring NPN fluorescence. (panel B) Plot shows changes in fluorescence intensity of SYTOX green at emission 520 nm with increasing concentrations of LG21, FG21, KG20 and LG21R19A peptides in presence of *E. coli* cells. *E. coli* cells were incubated with 1 µM SYTOX green at 37 °C for 15 min. Fluorescence emission of SYTOX green was recorded in the absence of peptides and with increasing concentrations of peptides.
Figure 4: Cell damage by LG21 in EM studies. Electron micrograph of negatively stained *E. coli* cells in the absence (panel A) and at 3 µM (panel B), 8 µM (panel C) and 15 µM (panel D) concentrations of LG21 peptide and 50 µM (panel E) LG21R19A peptide.
Figure 5: **Disaggregation of LPS by hybrid peptides.** (panels A-E) Bar diagrams showing diameter versus intensity of scattered light for LPS alone (panel A) and in presence of LG21 (panel B), FG21 (panel C), KG20 (panel D) and LG21R19A (panel E). (panel F) Changes in fluorescence intensity of FITC-labeled LPS as a function of various concentrations of LG21, FG21, KG20 and LG21R19A peptides. All experiments were carried out in 10 mM sodium phosphate buffer, pH=7.0.