Structural insights into the combinatorial effects of antimicrobial peptides reveal a role of aromatic–aromatic interactions in antibacterial synergism

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The recent development of plants that overexpress antimicrobial peptides (AMPs) provides opportunities for controlling plant diseases. Because plants employ a broad-spectrum antimicrobial defense, including those based on AMPs, transgenic modification for AMP overexpression represents a potential way to utilize a defense system already present in plants. Herein, using an array of techniques and approaches, we report on VG16KRKP and KYE28, two antimicrobial peptides, which in combination exhibit synergistic antimicrobial effects against plant pathogens and are resistant against plant proteases. Investigating the structural origin of these synergistic antimicrobial effects with NMR spectroscopy of the complex formed between these two peptides and their mutated analogs, we demonstrated the formation of an unusual peptide complex, characterized by the formation of a bulky hydrophobic hub, stabilized by aromatic zippers. Using three-dimensional structure analyses of the complex in bacterial outer and inner membrane components and when bound to lipopolysaccharide (LPS) or bacterial membrane mimics, we found that this structure is key for elevating antimicrobial potency of the peptide combination. We conclude that the synergistic antimicrobial effects of VG16KRKP and KYE28 arise from the formation of a well-defined amphiphilic dimer in the presence of LPS and also in the cytoplasmic bacterial membrane environment. Together, these findings highlight a new application of solution NMR spectroscopy to solve complex structures to study peptide–peptide interactions, and they underscore the importance of structural insights for elucidating the antimicrobial effects of AMP mixtures.

Despite progress in the field of antimicrobial therapeutics (1, 2), plant production remains profoundly affected by microbial disease outbreaks caused by pathogens of Xanthomonas and Pseudomonas species, leading to considerable losses in crop production and challenges for global food security (3). Finding new approaches for the management of microbe-borne diseases in plants remains a challenge, further complicated by the increasing occurrence of multidrug-resistant pathogens (“superbugs”) that are immune to available treatments (4, 5). Additionally, commonly used bactericides and fungicides pose considerable environmental constraints (6), necessitating the development of sustainable management techniques that can effectively combat such pathogens.

Antimicrobial peptides (AMPs) represent the oldest domain of the evolutionary tree, being structurally and functionally conserved across all forms of life (7–10). While having attracted research interest for several decades, peptide-based antimicrobial therapy has seen accelerated attention during the last few years, motivated by its potential applicability against drug-resistant strains (11–15). The direct interaction of AMP with bacterial membranes and membrane components provides broad-spectrum antimicrobial effects (16), and for many AMPs also anti-inflammatory and other such effects (17, 18). Despite such potent host defense functionality, the therapeutic

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The atomic coordinates and structure factors (codes 6KBO and 6KKB) have been deposited in the Protein Data Bank (http://wwwpdb.org/).

The three-dimensional LPS and 3:1 POPE/POPG vesicle-bound structures of the complex VG16KRKP–KYE28 have been submitted to Biological Magnetic Resonance Data Bank under codes 36265 for LPS-bound and 36266 for 3:1 POPE/POPG-bound.1

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The abbreviations used are: AMP, antimicrobial peptide; SEM, scanning electron microscopy; LPS, lipopolysaccharide; LUV, large unilamellar vesicle; FICI, fractional inhibitory concentration index; DLS, dynamic light scattering; PDI, polydispersity index; TOCSY, total correlation spectroscopy; trNOESY, transferred nuclear Overhauser effect spectroscopy; PRE, paramagnetic relaxation enhancement; SASA, solvent accessible surface area; MIC99%, minimal inhibitory concentration for 99% killing; TSP, 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt; POPE, 1-palmitoyl-2-oleoylsn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho(1′-rac-glycerol); CHAPS, 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonic acid; MTS, 1-(10-phenyl-10H-triporphyrin-2,5,10,15-tetramethyl-2,5-diisopropylpyridinium) methanesulfonothioate; DISC, 3,3′-dihydroxyphosphatidylcarboxylic acid; MBTH, 8-anilino-1-naphthalenesulfonic acid; PI, propidium iodide; H/D, hydrogen–deuterium; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; CFU, colony-forming unit; P/L, VG16KRKP–KYE28/LUVs.

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application of AMPs suffers from challenges related to dose-dependent toxicity, as well as scavenging and resulting activity loss by binding to anionic proteins, polysaccharides, and other compounds (19–21), which has motivated recent research on the combination of AMPs with drug-delivery systems (21–23).

Another prospective approach to address challenges with the development of AMP-based therapeutics could be to use a synergistic combination of AMPs for improved potency. The methodology of combination or poly-therapy, involving the rationale of using more than one drug, has attracted recent interest because naturally occurring AMPs are ubiquitously expressed together, and synergism between AMPs can very well explain their success in combating infections (24–27). In combinations, peptide interactions may lead to three primary outcomes, i.e. (i) additivity, (ii) synergism, and (iii) antagonism, in which the interacting peptides exhibit similar, stronger, and weaker activity, respectively, compared with their combined individual profiles at an equivalent dosage. The approach of peptide-based combinatorial therapy has been reported previously in the literature (28). So far, peptide synergism has not been conclusively correlated to the generation of specific structural motifs, which has resulted in other types of descriptions of synergisms, in which the peptides act cooperatively in destabilizing bacterial cell walls.

In previous work, we reported on the synthetically designed peptides VG16KRKP (VARGW/K/R/K/CPLF/GKGG) and KYE28 (KYEITTHN/FLR/KLTHLR/FR/NFGYTLR), exhibiting potent activity against devastating plant pathogens of the genus Xanthomonas, including Xanthomonas vesicatoria, Xanthomonas oryzae, and Xanthomonas campestris (29, 30). In doing so, we have also highlighted the biological effects of these peptides ex vivo and provided a structural correlation into the origin of these effects. In this study, we expand on these previously reported findings by combining the two peptides, VG16KRKP and KYE28, to demonstrate synergistic antimicrobial potency of the peptide mixture. In addition, we set out to obtain structural insights into the interaction of these peptides in the presence of bacterial membrane components and mimics and thus to establish the structural basis of the synergism observed in this particular system. As discussed below, the combination of the two peptides promotes killing of several important plant pathogens, as also established by scanning EM (SEM) imaging. Elucidating the molecular origin of these effects, structure-function correlation of the VG16KRKP–KYE28 system was obtained by solving the three-dimensional solution structure of the peptide complex in Escherichia coli total lipid extract bincelles, the major components of the outer membrane of Gram-negative bacteria, lipopolysaccharide (LPS) micelles, and a bacterial cytoplasmic membrane mimic, 3:1 POPE/POPG large unilamellar vesicles (LUVs), using nuclear magnetic resonance (NMR) spectroscopy. Through this, aromatic–aromatic interactions between Trp of VG16KRKP and Phe residues of KYE28, in conjunction with charge clustering, were demonstrated to be instrumental for structural stabilization of the complex formed between these two peptides and for the enhanced biological activity of the peptide combination. These findings were supported by results from site-directed mutagenesis.

Results

Combined antimicrobial effects of VG16KRKP and KYE28

Using binary combinations of the parent peptides, VG16KRKP and KYE28 (Fig. 1A), checkerboard analysis (31) was conducted to calculate the fractional inhibitory concentration index (FICI) (Fig. 1B and see Equation 3 under “Experimental procedures”). As shown in Fig. 1B, for plant pathogens of Xanthomonas and Pseudomonas genus, FICI results demonstrated “partial synergy” to “synergy” between the two parent peptides, with FICI values ranging between 0.32 and 0.60 (Table S1). The combination also showed a synergistic effect against the human fungal pathogen Cryptococcus grubii and a partial synergy against Candida albicans, as well as good plant protease stability (Fig. 1C). In this context, it should be noted that for highly potent peptides, such as VG16KRKP and KYE28, the synergistic factor usually found is considerably smaller as observed in this investigation.

Interaction of VG16KRKP–KYE28 with bacterial membrane components

VG16KRKP–KYE28 interacts and disrupts bacterial membranes—Scanning EM (SEM) analysis of X. vesicatoria cells before and after exposure to the combination of the parent peptides, VG16KRKP and KYE28, at their 0.5× and 1× MIC<sub>pp50</sub>, corresponding to 0.25 and 0.5 μM of each, respectively, demonstrated bacterial cell death to be associated with membrane disruption and leakage of intracellular content after only 1 h of treatment (Fig. 2, A–C). Analogous effects of membrane destabilization were observed for the live X. vesicatoria cells, probed using fluorescence-based experiments. As shown in Fig. 2D, the addition of the peptide combination at 1× and 2× MIC<sub>pp50</sub> concentrations (corresponding to 0.5 and 1 μM of each, respectively) to live X. vesicatoria cells led to immediate depolarization of the bacterial cells as indicated by an increase in the fluorescence intensity of DISC<sub>37</sub>, a membrane-potential sensitive dye (32). In yet other set of fluorescence experiments, the destabilizing effect of the peptide combination on X. vesicatoria bacterial membranes was confirmed by monitoring the kinetics of bacterial lipopolysaccharide and cytoplasmic membrane permeabilization by measuring the uptake of the fluorescent probes 8-anilino-1-naphthalenesulfonic acid (ANS), N-phenyl-1-naphthylamine (NPN), and propidium iodide (PI), respectively (30, 32, 33). Because dyes are membrane and/or nucleic acid–specific in nature, peptide-induced membrane disruption allows them to bind to their respective counterparts, thus exhibiting enhanced fluorescence. Fig. 2, E–G, displays the increase in fluorescence intensity of these dyes upon treatment of the bacterial cell with 1× and 2× MIC<sub>pp50</sub> Concentration, again demonstrating bacterial wall disruption induced by the combination of VG16KRKP and KYE28.

VG16KRKP–KYE28 induces association of bacterial membrane mimics—Fig. 3A shows results from dynamic light-scattering (DLS) measurement performed with anionic LUVs (3:1 POPE/POPG) upon titration with increasing concentrations of the peptide combination. For better representation, the autocorrelation data (Fig. 3A, left) and the particle size distributions (Fig. 3A, right) pertaining to P/L (VG16KRKP–KYE28/LUVs)
The ratio of 1:1:20 (red), 1:1:10 (blue), 1:1:2 (green), and 1:1:1 (yellow) are shown, all at a constant VG16KRKP–KYE28 ratio of 1:1. Additionally, the same sample caused immediate vesicle flocculation even at the lowest P/L (VG16KRKP–KYE28/LUVs) ratio, leading to the generation of aggregates as indicated by the slowing down of the relaxation decay and increase in the delay time, τ. As can be seen from the figure, the LUVs used for the study displayed a quasi-perfect mono-exponentially decaying correlation graph on the linear-log scale and a low polydispersity index (PDI) of 0.13, indicating a narrow size distribution of spherical LUVs, with the mean hydrodynamic diameter centered around 149 nm. Increasing the total concentration of the 1:1 VG16KRKP–KYE28 peptide mixture resulted in particles ≤1 μm in diameter, and PDI ranging between 0.39 and 0.52 (data not shown). In contrast, the VG16KRKP or KYE28 peptides alone showed much smaller aggregation (Fig. S1).

To further extend our understanding of LUV aggregation induced by the VG16KRKP–KYE28 combination, we next employed 31P solid-state NMR and spectral-shape analysis for model bacterial membranes (Fig. 3 and Fig. S2) (34). The 31P spectrum of 3:1 POPE/POPG LUV or E. coli total lipid extracts showed a typical powder pattern spectrum (Fig. 3B, left) (35). Nonetheless, upon exposure to the peptide combination at 2 and 4 mol %, obvious and immediate changes were observed in the overall spectral shapes, indicating relative population changes of the orientations of phospholipid molecules. Careful comparisons of the spectra of 3:1 POPE/POPG vesicles showed the following: (i) a change in the intensity of the parallel (≈26 ppm) and perpendicular (≈−15 ppm) edges in the 31P spectra, and (ii) an increase in the spectral spans of the powder pattern (40 ± 0.5 ppm for 0 mol % and 44 ± 0.5 ppm for 4 mol %) upon peptide exposure. The relative intensity changes in the spectra implied that upon exposure to the VG16KRKP–KYE28 mixture, the vesicles underwent morphological changes that converted them from spherical LUVs to deformed membranes.

In addition, the increase in the spectral span observed reports on peptide-mediated vesicle aggregation, which retarded the motional averages of the vesicles due to the larger size. Such
Structural basis of peptide synergism

Figure 2. A, SEM images of *X. vescicaria* in the absence of both the peptides VG16KRKP and K YE28 (control) showed no visible morphological changes after 1 h of incubation. In contrast, cells exposed to 0.5 × MIC99% resulted in pronounced membrane perturbation with extensive release of intracellular constituents and loss of cellular morphology (C). The scale corresponds to 10 μm. Additionally, dye-based fluorescence assay with live *X. vescicaria* cells displayed that the peptide combination at 1 × and 2 × MIC99% concentration could effectively interact with bacterial cell membrane bringing about a change in membrane potential as indicated by an increase in DiscC fluorescence (D), thus disrupting the outer membrane comprising of lipopolysaccharide (E and F), and the inner membrane made of negatively charged lipid components (G). The control cell showed baseline fluorescence under all conditions. It should be noted that ANS and NPN are lipophilic dyes, which fluoresces strongly in nonpolar environments of the cell membrane, which gets exposed upon membrane disintegration. PI, however, binds to nucleic acid components of the dead cell conforming inner membrane disruption. Each experiment was recorded for 30 min at 298 K.

changes were expected as immediate aggregates were observed after peptide addition to the vesicles for all the samples during the sample preparation step for the NMR experiments (Fig. 3B, inset). This observation is consistent with the DLS data and SEM images. 

Comparison of the 31P spectra of 3:1 POPE/POPG LUVs with that of *E. coli* total lipid extract bicine showed that the latter underwent similar spectral shapes, but with a slightly increased spectral span (38 ± 0.5 ppm for 0 mol % and 44 ± 0.5 for 4 mol %), implying that the bicles undergo aggregation without any apparent change in the spherical shape when treated with the peptide combination, as also visible from the NMR samples remaining comparatively clear (Fig. 3B, inset). Such an observation highlighted an important aspect of peptide action through synergism, indicating that, in case of bicles, the two peptides can bind to the surface of the bicles and network with other such complexes, hence facilitating aggregate growth.

Probing peptide interaction with bacterial membrane components using NMR spectroscopy to identify the binding “hotspot”

Interaction of VG16KRKP–KYE28 with bacterial lipopolysaccharide—As a major component of the outer membrane in Gram-negative bacteria, LPS acts as an inevitable barrier for AMPs reaching the inner membrane (36, 37). Considering this, we next set out to obtain insights into the structural properties of the VG16KRKP–KYE28 combination, including any complex formed between these, by determining its three-dimensional LPS-bound structure.

LPS form high-molecular-weight aggregates at concentrations above its critical micelle concentration (1.3 μM) (38). As a result of peptide binding to LPS, as well as the large size and slow diffusion of LPS aggregates, successive addition of small aliquots of LPS to an equimolar solution of VG16KRKP and KYE28 led to line-broadening of the peptide proton resonances, indicating a fast-to–intermediate conformational exchange on the NMR time scale (Fig. S3A). Additionally, the binding of the individual VG16KRKP and KYE28 peptides to LPS was in the micromolar range, as determined from isothermal titration calorimetry experiment of the individual peptides with LPS. This suggests that transferred NOESY (tr-NOESY) experiments can be used to determine the three-dimensional structure of the peptide combination after LPS binding (39–41). In aqueous solution, the VG16KRKP–KYE28 displayed only intra-residual (NOEs between residues of individual peptide) αN(i,i + 1) backbone/side-chain NOEs, suggesting these to be unfolded and highly dynamic (Fig. S4A). As shown in Fig. S5A, spectral overlap was not observed (contrary to larger peptides), and the spectra could be assigned completely and unambiguously. A large number of intra-residual medium αN(i,i + 2/i + 3) NOEs, such as Ala-2/Gly-4, Arg-3/Trp-5, Arg-3/Lys-6, and Cys-9/Leu-11 in VG16KRKP and Tyr-2/Ile-4, Tyr-2/Thr-5, Ile-4/Ile-7, Ile-7/Asn-9, His-8/Phe-11, Leu-10/Arg-12, Arg-17/Phe-19, Arg-17/Arg-20, Phe-19/Asn-22, Arg-20/Phe-23, Asn-22/Gly-24, Phe-23/Thr-26, Gly-24/Thr-26, Gly-24/Leu-27, and Thr-26/Arg-28 in KYE28 were observed in the presence of LPS Fig. 4A, left. These provided a clear indication that the two peptides adopt combined and well-defined secondary structures when simultaneously present as a complex in LPS. In addition, several intra-molecular NN (i,i + 2) trNOEs between the residues Gly-4/Lys-6, Thr-5/Arg-7, Cys-9/Leu-11, Leu-11/Gly-13, and Gly-13/Gly-15 of VG16KRKP and Thr-6/His-8, Ile-7/Asn-9, His-8/Leu-10, Leu-10/Arg-12, Asn-22/Gly-24, and Phe-23/Tyr-25 of KYE28 (Fig. 4A, right, and Table S4) were also observed, indicating the occurrence of partial helical regions in both the peptides after LPS binding. Nevertheless, neither VG16KRKP nor its shorter analogs alone adopt α-helical conformation in the presence of LPS (29, 42), a change that could be therefore attributed to the interaction with KYE28.

The most striking observation was the inter-molecular (inter-peptide NOEs) long-range (i,i + 4/i + 5) side-chain/side-chain NOEs between residues Trp-5/Thr-5/VG16KRKP/Phe-23/KYE28, Tyr-6/KYE28/Thr-5/VG16KRKP/Phe-23/KYE28, Thr-5/VG16KRKP/Phe-23/KYE28, and Thr-6/KYE28/Thr-5/VG16KRKP that gave a clear indication that the two peptides interact with each other after simultaneous LPS binding. This interaction between VG16KRKP and KYE28 after LPS binding may therefore be responsible for stabilizing them and generating a unique orientation upon interaction with LPS (Figs. 4B and Fig. S6A).

Interaction of VG16KRKP–KYE28 with bacterial membrane mimics—The majority of antimicrobial peptides act via membraneolytic mechanisms, interfering with the integrity of both
bacterial cell wall and cell membrane. The inability to do so generally results in the loss of activity, a fact well-established by the example of the cyclic lipopeptide, daptomycin (43). Thus, to obtain in-depth insights into the mechanism of peptide synergism, to conclusively demonstrate the generation of a functional complex between the two peptides, as well as its importance for bacterial cell wall disruption, we set out to probe similar interactions with the cytoplasmic membrane. As in the

**Figure 3.** A, dynamic light-scattering experiment was performed with monodisperse population of spherical 3:1 POPE/POPG LUV. The concentrations stated indicate equimolar absolute concentrations of the two peptides individually, in μM. Upon titration with VG16KRKP and KYE28 mixtures, the vesicles showed significant changes in shape and size, as indicated by the increased PDI value and the increase in delay time (black broken line), respectively (left side). Each experimental set was done in triplicate. B, 31P solid-state NMR studies further supported VG16KRKP–KYE28–induced vesicle deformation and aggregation (right) as depicted by the change in intensity of the parallel (−30 ppm) and perpendicular (−15 ppm) edges and an increased spectral span. The inset shows immediate vesicle flocculation upon addition of the peptides, whereas the control vesicles remain unchanged. In contrast, as seen on the left side, the peptide combination could only cause flocculation but no morphological changes in the bicelles prepared from *E. coli* total lipid extract bicelle.

**Figure 4.** A, two-dimensional 1H–1H trNOESY spectra of VG16KRKP–KYE28 complex in the LPS micelle, displaying the NOE contacts in the fingerprint region for Cα–NH and NH–NH resonances important for structure stabilization. B, parent peptide complex also displayed intra- and inter-molecular long-range NOE contacts, illustrating the importance of aromatic residues in the formation of a stabilized structure and justifying the strong synergistic interaction observed. The experiments were performed by titrating 0.6 mM of the total peptides with 10 μM LPS (LPS: each peptide 1:30) at pH 4.5, using a Bruker Avance III 700 MHz NMR spectrometer (NOESY mixing time 150 ms) and at 298 K (residues marked in blue are from VG16KRKP and in red are from KYE28).
case of LPS, tr-NOE experiments were used to determine the three-dimensional structure of the complex when bound to bacterial cytoplasmic membrane mimic, 3:1 POPE/POPG LUVs, and E. coli total lipid extract bicelles. Analogous to LPS, the peptide combination in 3:1 POPE/POPG LUVs displayed unambiguous and explicit intra-residual αN (i,i + 1) and an almost similar intra-residual medium αN (i,i + 2/3 + 1) NOEs (Fig. 5A, left, and Fig. S5B). Additionally, intra-molecular NN (i,i + 2) and inter-molecular long-range (i,i + 4/5 + 1) side-chain/side-chain trNOEs between the residues remained largely conserved (Fig. 5B and Table S4). However, the long-range interaction between the H,αβ of Thr-5 and Thr-6 and the aromatic ring proton of Trp-5 was lost, implying a structural re-organization of the VG16KRKP–KYE28 complex upon crossing the outer membrane.

These results obtained for VG16KRKP–KYE28 simultaneously present in bound complex with either LPS or 3:1 POPE/POPG suggest that the aromatic residues, because of their innate propensity to exclude water, play a central role in structural stabilization via generation of an aromatic zipper and a hydrophobic hub, effectively defining a structural hotspot for synergism in the VG16KRKP–KYE28 system. Also, this results in energetically-favorable salt-bridge interactions that have a substantial overall contribution in structure stabilization and hence activity. Of note, the indole ring proton (Nε), resonating at ~10.2 ppm, did not show any NOE contacts with other peptide residues in the complex. This is consistent with the observation for the individual peptide VG16KRKP interacting with LPS. Apart from that, the Cys–Pro bond maintained a trans-conformation in LPS as indicated by the presence of C9C≡H/P10C≡H NOE in all the three complexes.

In the case of E. coli total lipid extract bicelles, interference from CHAPS, the detergent used for bicelles preparation, structural destabilization, and the lack of unambiguous peaks were observed, thus impeding identification of a well-defined structure (Figs. S6C and S7, A–E). The latter finding suggests that the VG16KRKP–KYE28 complex is relatively subtle, sensitive to surfactant exposure, in line with well-established conformational changes in peptides and proteins observed by a wide range of surfactants (44–46).

**Aromatic cluster stabilizes the bound three-dimensional structure of the peptide complex**

Interaction of VG16KRKP–KYE28 with bacterial lipopolysaccharide—The 15-ensemble structures of VG16KRKP–KYE28 in LPS, 3:1 POPE/POPG LUVs, and E. coli total lipid extract bicelle were generated using CYANA (47), at an equimolar peptide ratio and an LPS/peptide mixture of 1:30, based on the NOE distance constraints obtained from the tr-NOE spectrum. In LPS, good convergence upon superposition of the backbone (Cα, N, and C') atoms was obtained, as expected due to the higher number of NOEs obtained (Fig. 6, A and B), as also demonstrated by the low value of root mean square deviation for backbone and heavy side-chain atoms (Table S6).

Closer inspection of the three-dimensional LPS-bound structure of VG16KRKP–KYE28 complex highlighted a marked change from their native LPS-bound structure (Fig. 6A and Fig. S8). In the binary peptide complex, VG16KRKP exhibited a more prominent structural change than KYE28 after LPS binding (Fig. S8, top). Notably, a helix Pro-10–Gly-16 was obtained at the C-terminal end of VG16KRKP to adopt a triad...
“Trp-5–Leu-11–Phe-12,” as opposed to a turn structure when present individually (Fig. 6C). In contrast, KYE28 in the peptide complex conserved its native “helix–loop–helix” motif with a minor (Glu-3–Ile-7) and a major (Leu-18–Leu-27) helix, also adopted when this peptide was present alone in LPS (Fig. S8, bottom). Importantly, the structural contribution of each residue in KYE28 in the VG16KRKP–KYE28 complex was not the same as in the case of the KYE28 peptide alone in LPS (Fig. 6D). A key structural change was observed in the central region of both VG16KRKP and KYE28, which together formed a dimer, characterized by a tightly packed aromatic cluster stabilized by residues Trp-5 and Phe-12 of VG16KRKP and Tyr-2, Phe-19, and Phe-23 of KYE28, together forming a hotspot (Fig. 6D). Additionally, a smaller and loosely arranged hydrophobic hub formed by Leu-10–Leu-14–Leu-18–Phe-19 of KYE28 was also observed.

It is worth mentioning that the orientation of aromatic residues within a distance of 4.5–7.0 Å plays an essential role in structural stabilization (48, 49). Among the three probable aromatic–aromatic orientations, i.e. T-shaped or edge-to-face, face-to-face, and parallel-displaced, the obtained distance constraints allowed the VG16KRKP–KYE28 complex to adopt an energetically favorable aromatic cluster geometry by forming a T-shaped interaction (50) between Phe-12<sup>VG16KRKP</sup>–Trp-5<sup>VG16KRKP</sup>–Phe-23<sup>KYE28</sup> (Fig. 6D). An additional cluster between Tyr-2<sup>KYE28</sup>–Phe-19<sup>KYE28</sup> was also observed between the helix-loop-helix structure of KYE28, allowing correct folding and providing scaffold stability (51). Although these interactions provide weak contributions individually, together they helped to maintain the VG16KRKP–KYE28 complex in a globally favorable conformation in LPS to provide stability and rigidity.

Opposing the hydrophobic hub, the positively charged Lys and Arg residues of both peptides in the LPS-bound VG16KRKP–KYE28 complex remained disseminated toward the solvent front, maintaining a distance of 10–15 Å between the headgroup, being almost identical to the distance between two-phosphate groups of the LPS (Fig. 6E) (39). An interesting
observation emerging here was the presence of two distinct clusters of concentrated positive charges at the two opposite faces of the adopted complex structure.

Collectively, the LPS-bound complex displayed an amphipathic structure, with a hydrophobic hub with well-separated charge distribution at the two termini, similar to some previously reported designed antimicrobial peptides, upon interaction with the bacterial membrane (39, 52). The electrostatic potential map confirmed the adoption of an amphipathic rigid structure in LPS that helped the two peptides to interact cooperatively with each other (Fig. 6).

Interaction of VG16KRKP–KYE28 with bacterial membrane mimics—The three-dimensional structure of VG16KRKP–KYE28 bound to a negatively charged bacterial cytoplasmic membrane mimic, 3:1 POPE/POPG LUV, exhibited marked changes from their native as well as their complex LPS-bound structure (Fig. 7). Although the distorted helix at the C terminus remains conserved, the “Trp-5–Leu-11–Phe-12” triad was lost (Fig. 7, A–C). Additionally, KYE28 adopted a helix–loop–helix orientation, with two short helices, between residues Ile-4–Leu-10 and Leu-18–Thr-26, distinct from the major and minor helices adopted in LPS, both when alone and in the complex (Fig. 7C). Of note, the hydrophobic cluster formed by Trp-5 and Phe-12 of VG16KRKP and Tyr-2, Phe-19, and Phe-23 of KYE28 in LPS remained conserved upon interaction with the bacterial cytoplasmic membrane mimic, thus forming a hotspot helping the peptide complex to penetrate deeper into the membrane bilayer (Fig. 7C). Despite this conservation of the hydrophobic hub, local reorientation of aromatic residues was observed, with the generation of three T-shaped or edge–to–face interactions, between residues Phe-12VG16KRKP–Trp-5VG16KRKP–Phe-23KYE28, Trp-5VG16KRKP–Tyr-2KYE28–Phe-12VG16KRKP, and Phe-19KYE28–Trp-5VG16KRKP–Phe-23KYE28, respectively (Fig. 7D).

The positively charged residues Lys and Arg remained flexible and oriented toward the solvent, causing the complex to adopt an overall amphipathic structure. Here again, the anticipated re-distribution in residue assembly was observed when compared with the LPS-bound complex. Thus, the two positively charged clusters found in the presence of LPS was dis-
ruptured, and instead a single face of high-charge density was observed, located opposite to the hydrophobic hub (Fig. 7E). Such local changes could be attributed to the difference in mechanism adopted by the peptide to establish an initial interaction with the microbial cell and thus leading onto the killing of the same.

On the contrary, the three-dimensional structure of the peptide complex bound to E. coli total lipid extract bicelles adopted a very loose and open structure, consisting of two very short helices, Thr-6–Asn-9 and Leu-18–Gly-24, generating a nearly helix–loop–helix motif for KYE28, whereas the helix at the C terminus of VG16KRKP remained conserved, with the “Trp-5–Leu-11–Phe-12” triad lost (Fig. S7F). The overall structure as a whole resonated more with the complex structure bound to 3:1 POPE/POPG membrane mimic, having a distinct dual-face residue alignment, a bulky hydrophobic hub, and a highly-flexible hydrophilic face consisting of positively charged amino acids.

As mentioned above, interference of the surfactant CHAPSO used in the bicelles destabilized the VG16KRKP–KYE28 dimer and precluded structure determination. Considering this, further studies with this system were not undertaken, but the findings are nevertheless interesting in that they indicate that the inclusion of the potent VG16KRKP. This observation was also evident in their trNOESY profile, lacking inter-molecular medium- and long-range contacts (Fig. S10A).

Next, the three-dimensional LPS-bound structure of the mutants in complex with either of the parent peptides was calculated (Table S7). The VG16F12A–KYE28 complex adopted a comparatively relaxed structure, being held together by the aromatic tetrad formed by residue Trp-5 of VG16F12A with Phe-11, Phe-19, and Phe-23 of KYE28, which may explain its biological activity (Fig. S9, C and D, and Fig. S11, A and B, left). Apart from this, the overall structure of the complex-forming peptides remained conserved, with VG16F12A remaining in an extended conformation and KYE28 adopting a helix–loop–helix structure via formation of a major (Glu-3–Leu-14) and a minor (Phe-F23–Leu-27) helix (Fig. S9, C and D).

Although the helical regions of KYE28 remain conserved in both the VG16KRKP–KYE28 and VG16F12A–KYE28 complexes in LPS, a slight change in the focus and overall orientation allows conserving the variants in their bioactive conformation, resulting in partially maintained antimicrobial potency. This structural re-arrangement could also justify the relatively relaxed and dynamic orientation of the VG16F12A–KYE28 complex structure, as peptides and proteins have evolved to prioritize functional aspects by compensating in terms of structural stability (53). Exemplifying this, the mutation of the Phe-12 residue in this complex generated a change in the overall geometry and orientation of aromatic residues, so that the inter-helical interaction between Tyr-2xKYE28 and Phe-19KYE28 was lost to maintain the T-shaped or face–to–edge interaction between Phe-23KYE28 and Phe-19KYE28 (Fig. S9C) in order to retain the synergism and the activity of VG16F12A. In line with this, the lack of a well-folded three-dimensional structure is consistent with the inactivity of the VG16WFA–KYE28 complex against the plant pathogens and the apparent lack of synergism.

Additionally, as predicted from the lack of inter-molecular NOE contacts in the VG16KRKP–KYE28A complex and the tendency of protein/peptide folding toward functional conservation, these peptides adopt their native secondary struc-
ture mutually exclusive of the other, conforming with the low antimicrobial activity displayed (Fig. S11, right). Circular dichroism (CD) studies supported the adoption of an extensive $\alpha$-helical conformation for the VG16KRKP–KYE28, VG16F12A–KYE28, and VG16KRKP–KYE28A complexes in LPS (Fig. S12). Intriguingly, VG16F12A–KYE28, and to some extent VG16KRKP–KYE28, displayed a diffuse CD spectral pattern, which could be corroborated to their interaction with the other peptide partner in the complex, the same being absent in VG16KRKP–KYE28A.

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**Figure 8.** A, PRE NMR was performed using MTSL-tagged VG16KRKP with KYE28 in LPS. The trNOESY experiment was conducted under similar conditions with the unlabeled peptide to compare residue-specific $T_2$ relaxation, depicted as $I/I_0$. The residues in the vicinity of the labeled Cys-9 residue exhibited almost complete relaxation as shown by broadened Ho/NH trNOESY peaks. B, similarly, H/D experiments performed throughout 5 h corroborated with the solved structure with residues involved in the formation of the hydrophobic hub and those engaged in $\pi$-cation interaction remaining shielded from the exchange kinetics. C, FRET experiment showed a linear change in the emission intensity of the Trp residue of the donor peptide, VG16KRKP upon titration with increasing concentrations of dansylated acceptor KYE28, up to 0.5 mol fraction. The FRET experiment was performed at 298 K in 10 mM potassium phosphate buffer (pH 7.4).
teracting with or were in close juxtaposition to Cys-9 of VG16KRKP and its nearby amino acid groups, also experienced faster $T_2$ relaxation of the Ca-H. This mainly includes residues in the extreme N- and C-terminal of KYE28, which underwent complete relaxation.

In comparison, the residues at further distances experienced a suppressed effect. The same was found true for the Cβ/γδ-H, which because of the tapered relaxation effect was not affected to a similar extent as Ca-H (Fig. S14A). Nonetheless, the observation that the end-to-end distance from MTSL-C9 is ~25 Å (Fig. S14B) helps to justify the phased out quenching pattern observed for all the residues and hence corroborates well with the complex three-dimensional structure discussed above.

Hydrogen/deuterium (H/D) exchange studies—NMR-based H/D exchange experiment helped to assess the degree of protection of the LPS-bound VG16KRKP–KYE28 complex. Careful examination of the $^1$H-$^1$H TOCSY spectra, recorded through 5 h of exchange, demonstrated that the backbone amide protons of several residues, notably Ala-2/Trp-5/Phe-12 (from VG16KRKP) and Phe-11/Phe-19/Phe-23 (from KYE28), undergo slow solvent exchange, as evident from their estimated protection factors (Fig. 8B and Fig. S15A). These residues play a central role in forming the $\alpha$-helix and are involved in structure stabilization and folding through aromatic–aromatic and aromatic–aliphatic interaction, as well as through $\pi$–cation interactions, as also indicated by the protection factor (Fig. S15A). Moreover, the solvent-accessible surface area (SASA), calculated using the POPS* software (55), indicated that the residues involved in the peptide–peptide and the peptide–LPS and peptide–peptide–LPS interactions are characterized by an overall decrease in SASA on complexation. In contrast, the cationic residues in both the peptides forming the complex demonstrated an enhanced value of the same (Figs. S15B and S16). Collectively, these results show that such interactions play a vital role in maintaining the conformational stability of the peptide complex.

FRET experiments indicate complex formation—The interaction between the parent peptides and their mutant analogs to come together and generate synergistic antimicrobial activity was further investigated by fluorescence-based FRET analysis (56). Interaction between the Trp of donor peptide (VG16KRKP) and dansyl of N-terminal labeled acceptor peptide (KYE28) was probed by monitoring the quenching of Trp emission intensity upon titration with the acceptor dansyl in the presence of E. coli LPS, cytoplasmic membrane mimic 3:1 POPE/POPG, and E. coli total lipid-extract bicelles. The VG16KRKP–KYE28 complex showed highly quenched Trp emission upon addition of increasing concentrations of dansylated KYE28 (dans-KYE28), giving a linear relationship between the quenching of donor and concentration of the acceptor molecule (Fig. 8C). The interaction between VG16KRKP and the parent KYE28 exhibited a strong FRET effect amounting to a maximum quenching of 0.56, corroborating to a distance of 16.5 Å, in satisfactory agreement with the 3D structure (Fig. S17, A and F). The slight deviation from the NMR-derived structure could be attributed to the motional freedom and difference in orientation of the fluorophore groups attached. In contrast to these findings for LPS-bound peptide complexes, FRET analysis in 3:1 POPE/POPG and E. coli total lipid-extract bicelles did not reveal a strong interaction between VG16KRKP and KYE28.

The same could be true for VG16F12A–KYE28, which exhibited a modest FRET effect surmounting to a distance of 15.6 Å, in satisfactory agreement with the 3D structure (Fig. S17, E and F). Strikingly, the VG16KRKP–KYE28A complex did not show any FRET effect, in line with the absence of any significant interaction observed on the NMR timescale (Fig. S17B).

Discussion

Matsuzaki et al. (57) did the first pioneering work to study peptide synergism and attempted to reach an understanding of the mechanism of the same by trying to elucidate the molecular and structural basis of peptide synergism, using the two amphibian antimicrobial peptides, magainin 2 and PGLa. Initial studies with the two peptides displayed maximum synergy at a 1:1 molecular ratio, hinting toward a probable heterodimer complex formation (57, 58). The three-dimensional structure of the hybrid peptides generated in the presence of SDS and DPC micelles, however, failed to demonstrate any stable heterosupramolecular structure, underlying the synergism observed (59). Following this early work, studies of the structural aspect of synergism remained largely unexplored because of the limitations associated with the available biophysical techniques. Therefore, Matsuzaki et al. (57) also highlighted the importance of well-defined NMR experiments required for obtaining unambiguous NOE peaks, which can make structure determination more precise. Later, Bhunia et al. (56) studied the synergistic interaction between two amphibian peptides, temporin TL (TL) and temporin Tb (TB), using NMR in conjunction with other biophysical experiments, showing that the peptide TL affects the LPS-mediated oligomerization state of TB, while maintaining its native antiparallel dimer helix. However, the group did not report on the complex structure or its implications.

Also addressing the structural origin of AMP synergy, Ulrich and co-workers (60), working on PGLa–MAG2 peptide combination, highlighted that the GXXG motif plays an important role in synergy. These authors proposed the formation of a functionally active PGLa–MAG2 complex, in which an antiparallel PGLa dimer stabilized by the GXXGX motif was generated and interacted with MAG2 monomers via its C terminus (60). In addition to studying the structural features underlying synergy, various groups have highlighted the importance of intrinsic membrane curvature for AMP synergy (61–63). Here, Bechinger and co-workers (64) have performed breakthrough work, associating peptide synergism with the “Soft Membranes Adapt and Respond, also Transiently” ("SMART") model to gain mechanistic insights for peptide synergism. This highlights the importance of both the peptide and the interacting lipid partners in deciding the mode of action. Additionally, work from these authors has identified the importance of individual peptides, which do not form complexes but do interact at the membrane interface to generate peptide synergism. For example, through their work on the PGLa–MAG2 system, Bechinger and co-workers (64) highlighted that magainin displays stable membrane surface alignment, whereas PGLa undergoes
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re-alignment when in the presence of magainin, but only in the unsaturated lipid membrane, adopting a mesophase arrangement.

In this study, probing the structural basis of synergism via three-dimensional structure calculation in bacterial outer and inner membrane components highlighted the underlying key aspect of observed synergism to be lying in its aromatic core, which formed the hotspot region for peptide activity. Studies have associated overall high hydrophobicity of small peptides with an enhanced ability to self-associate and precipitate, thus negating availability and decreasing the antimicrobial efficacy (65). Nevertheless, the fact that VG16KKRP–KYE28 complex generated an aromatic packing cluster that remained globally conserved while incorporating local changes in all three structures calculated, as well as in the mutant complex studied, highlighted the importance of these hotspot residues. Experimental and structural analyses have previously established the role of such interaction and the resulting higher-order clusters in generating a super-secondary structure in α-helices of the type “helix–loop–helix” in proteins (66) as observed for our peptide combination.

A comparison between the LPS-bound and the bacterial membrane mimic-bound structure of the VG16KKRP–KYE28 complex further indicated defined differences between the two, which might help to deduce the mode--of--action of the peptide complex. A key change observed was in the segmentation of positive charges in the three-dimensional LPS-bound structure. Although the complex maintained its amphipathicity, the hydrophilic, cationic residues generated two clusters of mild positive charges when bound to LPS as opposed to a highly-concentrated charged region adopted in a bacterial membrane mimic. The end--to--end distance between the two clusters was ~29 Å, closer to the LPS bilayer thickness of 32 Å, implying that the complex as a whole could mount itself into the bilayer and generate transient pores and traverse further to reach the inner membrane. Such charge segmentation could be looked upon as an approach to help prevent excess peptide oligomerization (60), so that higher numbers of peptide complexes could be generated, attacking the Gram-negative bacteria’s first line of defense, the LPS. Upon reaching the inner membrane, composed of a 3:1 mixture of bulky POPE and smaller POPG, the peptide undergoes structural reorganization as explained above. Such clustering of positive charges helps the peptide complex to interact with a negatively charged phosphate head-group of the lipid, by launching themselves into the spatial voids. Such voids are generated due to the difference in size of the lipid headgroup constituting the inner membrane, and peptide interaction at these voids would cause further membrane incompatibility leading to increased mobility and stress through generation of positive curvature, thus affecting lipid alignment in the membrane, membrane thinning, and manifesting in a detergent-like mechanism of action (67). This structural attribute correlated very well with the 31P NMR data, which indicated similar deformation of the membrane mimics.

An important point to note here is the fact that the electrostatic interaction observed emerging from the positive charge cluster and their orientation in different membrane mimics, although important for launching an initial assault on the membrane, remained secondary in importance, contributing indirectly to synergy. This could be attributed to the observation that the mutant peptides, lacking critical aromatic residues, displayed diminished or no synergy.

VG16KKRP and KYE28 individually are capable of forming membrane defects and killing cells by a detergent-like or toroidal-pore mechanism (29, 30, 68). As clearly demonstrated in this investigation, the VG16KKRP–KYE28 complex similarly destabilizes both bacterial cell walls and model membrane mimics. Strikingly, this is paralleled by the generation of defined complexes, involving VG16KKRP–KYE28 dimer formation. Although some features of these binary peptide complexes are the same in the LPS and plasma membrane environment, notably in relation to the overall symmetry and the aromatic hub, there are also distinct differences between these environments, as is evident by the changed orientation of particular residues, especially the polar cationic ones which differ locally, upon interaction with a different region of the bacterial membrane. When added together to a bacterial suspension, the two peptides first interact with the bacterial outer membrane-forming heteropeptide complex that disrupts LPS assembly, followed by diffusion deeper into the bacterial cell wall to reach the inner membrane. Here, the complex disturbs membrane fluidity as found by 31P NMR experiments, causing membrane deformation and ultimately lysis, in turn contributing to the observed synergism. Such a scenario is also compatible with findings of both structure, membrane disruption, and antimicrobial effects of combinations of native and mutated VG16KKRP and KYE28 analogs.

Although synergy between VG16KKRP and KYE28 thus seems to involve formation of a well-defined structure in the LPS region of the bacterial wall, which is partly retained and partly transformed when reaching the cytoplasmic membrane environment, a key question is that of the generality of this mechanism of AMP synergy. Here, it should be noted that aromatic–aromatic interactions have been observed to be widely important for structural stabilization and hydrophobic domain formation in AMPs, e.g. the peptide combination used in this study, VG16KKRP and KYE28 as well as their shorter analogs, when present individually in LPS, exhibit similar structural characteristics (29, 30, 52, 68). One should also note that previous work on AMP synergy has only partly succeeded in identifying structural features associated with synergy, which in turn has led to other approaches, such as that based on local curvature (62, 64). Also, as shown for the results obtained for the E. coli total lipid extract bicelles, the presence of the surfactant CHAPSO was sufficient to destabilize the dimer formed by VG16KKRP–KYE28 in either LPS or cytoplasmic membrane mimic environments. This, in turn, suggests a relatively low stability of the binary structure formed. Adding these considerations together, further work is therefore needed to assess whether the structure formed in this study is a rare exception or whether related aromatic–aromatic hotspot formation through dimerization can also be found in other AMP systems.

Furthermore, a useful understanding of the interplay in mixtures of AMP will require wider considerations than addressed here, e.g. relating to pharmacodynamic aspects of such interactions. Synergism between two to three peptides, including nat-
urally occurring AMP, as well as between AMP and either antibiotics or nanoparticles, has been reported (69, 70). Exemplifying this, interaction studies between peptides of the dermaseptin family, between β-defensins and the cationic protein BPI, and between the α-helical peptides magainin and PGLa have all been reported before (64, 71, 72). In none of these cases, however, has a well-defined structure of the type reported here been observed.

Within the context of plant protection, it should also be noted that transgenic plants with individual defense genes have been found to display only partial resistance against key plant pathogens (73–75). Therefore, combining heterologous defense genes from various sources offers a more promising alternative for enhanced resistance in transgenic plants. Simultaneous expression of chitinase and glucanase is one such example of synergism-induced enhanced resistance against fungus infection in the transgenic tomato (76). Similarly, using a synergistic combination of Fusarium-specific antibody linked to antifungal peptides was found to boost resistance against Fusarium oxysporum f. sp. matthiola in Arabidopsis (77).

In conclusion, the studies above demonstrate that the synergistic antimicrobial effects observed for VG16KRKP and KYE28 in combination are related to the formation of a well-defined amphiphilic dimer in the presence of LPS, which is partially retained upon interaction of this peptide pair in the cytoplasmic membrane environment. In addition, the adoption of such an aromatic zipper motif in the three-dimensional LPS-bound structure was demonstrated to induce antimicrobial potency in an inherently inactive peptide VG16F12A. Although only one peptide pair was demonstrated, both the structural requirements and the effects of peptide amino acid changes suggest these effects to be of potentially wider importance, applicable also to other peptide combinations. That said, additional work is required to elucidate the potential generalization of the effects observed in this investigation.

**Experimental procedures**

**Reagents**

*E. coli* O111:B4 LPS and polymyxin B were purchased from Sigma. 3-(Trimethylsilyl)propionic-2,2,3,3-d₄ acid (TSP) sodium salt and deuterium oxide were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury). MTS-L was purchased from Santa Cruz Biotechnology, Inc. (Dallas). DISC₃, ANS, NPN, and PI were purchased from Thermo Fisher Scientific (Waltham, MA). All buffer components, including salts, were purchased from Merck (Kenilworth, NJ). CHAPSO was purchased from Sigma. POPE and POPG were obtained from Avanti Polar Lipids (Alabaster, AL).

Bacterial and fungal media components were purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). VG16KRKP and its analogs were obtained from GL Biochem (Shanghai, China), and KYE28 and its variant were from Biopeptide Co. (San Diego), both with ≥95% purity. Peptide purity was confirmed by RP-HPLC, MS, and NMR spectroscopy (78). Peptide stock solutions were prepared either in autoclaved water or phosphate buffer (pH 4.5 and 7.4, respectively) and filter-sterilized unless stated otherwise.

**Bacterial and fungal strains and reagents**

Plant pathogenic strain *X. campestris pv. vesicatoria* was a kind gift from Dr. Christian Lindermayr, Institute of Biochemical Plant Pathology, Germany. *X. campestris pv. campestris* was isolated locally from fields of Kalyani, West Bengal, India, and *X. oryzae pv. oryzae* was kindly provided by Professor Sampa Das, Bose Institute, India. *Pseudomonas syringae* DCM 300 was a gift from Dr. Pallob Kundu, Bose Institute, India. Fungal strains *C. albicans* SC5314 and *Cryptococcus neoformans* var. *grubii* H99 were the kind gift from Prof. Kaustuv Sanyal, JNCASR, India.

For microbroth dilution and checkerboard activity assay, *X. vesicatoria, X. campestris,* and *X. oryzae* were grown in Nutrient Broth, PS broth (1% peptone and 1% sucrose), and PSP broth (0.5% peptone, 2% sucrose dissolved in starch obtained from boiling 200 g of potato/liter of media), respectively, and maintained at 301 K under shaking. *P. syringae DCM 300* was grown in King’s Broth supplemented with 50 µg/ml rifampicin and kanamycin, respectively, and maintained at 301 K under shaking. *C. albicans* and *C. grubii* were grown in YPDU broth (1% yeast extract, 1% peptone, 2% dextrose, and 20 µg/ml uridine) and maintained at 301 K under shaking.

**Microbroth dilution assay**

To assay for antimicrobial activity of the peptides, standard microdilution broth assay was used, as described previously (79). Overnight-grown cultures of the respective plant pathogens, *X. vesicatoria, X. campestris,* and *X. oryzae,* and *P. syringae* DCM 300, were used to obtain mid-log–phase cultures. The cell suspensions were centrifuged at 6000 rpm for 5 min, after which cell pellets were washed three times with 10 mM phosphate buffer (pH 7.4), followed by resuspension in the same buffer to 10⁵ CFU/ml. The reactions were set in a 96-well flat-bottom plate by incubating 50 µl of the cell suspension with 2-fold increasing concentrations of the respective peptides (ranging from 1 to 100 µM) prepared from 1 mM peptide stock in phosphate buffer (pH 7.4) and incubated at 301 K, 150 rpm shaking for 4 h. For negative and positive controls, samples containing only cell suspension and 10 µM polymyxin B–treated cell suspension, respectively, were included. Next, 150 µl of suitable media was added to each well and incubated for 48 h with shaking at 301 K. Bacterial growth was monitored by measuring the absorbance at 630 nm. All absorbance readings were normalized using the negative control polymyxin B. The peptide concentration at which 99 ± 0.5% growth inhibition was observed served as its MIC₉⁹. All experiments were performed in triplicate.

**Checkerboard dilution assay**

A modified version of a standard microdilution broth assay was used to analyze the possible synergistic interaction between the parent peptides (VG16KRKP and KYE28) and their mutant variants using two-dimensional checkerboard dilution assay (80). Mid-log phase cultures of each organism were obtained by inoculating the overnight grown cultures of respective pathogens in their respective growth medium. The cell suspensions were centrifuged at 6000 rpm for 5 min, followed by washing three times with 10 mM phosphate buffer (pH 7.4) and resus-
pension in the same buffer to 10⁵ CFU/ml for bacteria and 10⁴ CFU/ml for fungi. In a 96-well plate format, 50 μl of this suspension was incubated with varying concentrations of VG16KRKP against a 2-fold dilution range of KYE28 to obtain MIC₉₀₉₀ of the combination by incubating at their respective temperatures for 4 h. A negative control containing only cell suspension, as well as a positive control containing 10 μM polymyxin B with cell suspension, was included as well. 150 μl of suitable media was added to each well and incubated overnight with shaking at their respective temperatures. All experiments were performed in triplicates. Peptide interaction was assessed by calculating the fractional inhibitory concentrations of the two-peptide combinations, according to Equations 1–3,

\[
\text{FIC}_{\text{peptide1}} = \frac{\text{MIC of peptide1 in combination}}{\text{MIC of peptide1 alone}} \quad (\text{Eq. 1})
\]

\[
\text{FIC}_{\text{peptide2}} = \frac{\text{MIC of peptide2 in combination}}{\text{MIC of peptide2 alone}} \quad (\text{Eq. 2})
\]

\[
\text{FIC index (FICI)}_{\text{combination}} = \text{FIC}_{\text{peptide1}} + \text{FIC}_{\text{peptide2}}
\]

FICI, calculated as the sum of each individual fractional inhibitory concentration, was interpreted as follows: FICI < 0.5 synergy; 0.5 ≤ FICI < 1 partial synergy; 1 ≤ FICI < 4 additive effect or indifference; and 4 ≤ FICI antagonism.

**Plant extract stability assay**

Stability of VG16KRKP and KYE28 against degradation by plant proteases obtained from plant leaf extract was investigated using a modified version of a previously reported experiment (81). For this, 1 g of a 1-month-old tomato (Pusa Ruby) leaf was ground in liquid nitrogen, followed by extraction with 2 ml of 10 mM phosphate buffer (pH 7.4). The mixture was centrifuged at 13,500 rpm (5 min, 277 K), and the supernatant was collected and saved in aliquots at −80 °C until further use. To analyze peptide stability, 1 mM of the respective peptide was incubated with 20 μg/ml of the leaf extract (concentration determined using BSA assay) at 310 K, and aliquots of 100 μl were withdrawn at the respective time points. The reaction was stopped by adding trifluoroacetic acid (TFA) to a final concentration of 1%. Water and plant extract (20 μg/ml) were used as blank and negative control, respectively. The reaction mixture was next loaded onto a Phenomenix C₁₈ column (Dimension 250 × 10.0-mm, pore size 100 Å, particle size 5 μm), at room temperature, to be analyzed by an LC-20AT reverse phase-HPLC system (SHIMADZU, Japan) using dual solvent (acetonitrile and water in 0.1% TFA) linear gradient elution at a flow rate of 1 ml/min. To determine the remaining peptide in percentage (%), the peak obtained at 220 nm was integrated using the SPINCHROME CFR software, and the area obtained was compared with that of a free peptide in the absence of plant extract.

**Scanning Electron Microscopy**

*X. vesicatoria* cells were cultured in nutrient broth (NB) to mid-logarithmic phase and were harvested by centrifugation at 6000 rpm for 5 min, washed twice with phosphate buffer (10 mM (pH 7.4)), and resuspended in the same to a final concentration of 10⁶ cells per ml. 50 μl of this suspension was incubated with 0.5× and 1× MIC₉₀₉₀ concentration of the peptide combination, VG16KRKP and KYE28, for 1 h at 310 K. Untreated cells were set as control. After treatment, the cells were fixed with 4% (v/v) glutaraldehyde, and 15 μl of the same was spotted on clean poly-l-lysine–coated glass slides and dried overnight. Next, the slides were washed twice with phosphate buffer, followed by dehydration with a graded series of ethanol (30, 50, 70, 90, and 100%), each for 15 min. The sample slides were then air-dried, followed by gold coating, and observed by SEM (FEI, QUANTA 200).

**Fluorescence spectroscopy**

All studies were conducted with *X. vesicatoria* live cell. The cells were grown and maintained as mentioned above, and a final concentration of 10⁶ or 10⁷ cells per ml was used for experimental purposes until unless specified otherwise. Measurements were performed at 298 K by monitoring the emitted fluorescence, at the excitation wavelength of the dye used, upon peptide treatment of the cells using a Hitachi F-7000 FL spectrometer.

**Cytoplasmic membrane depolarization assay**—Mid-logarithmic phase *X. vesicatoria* cells were washed in 5 mM HEPES buffer (pH 7.4) supplemented with 5 mM glucose and resuspended in the same along with 100 mM potassium chloride in 1:1:1 (v/v) ratio to a final concentration of 10⁶ cells per ml. Next, 1 μM of the membrane potential sensitive dye, DISC₃, was added to the cell suspension and incubated in the dark for an hour. Fluorescence from DISC₃ was recorded for 30 min at an excitation wavelength of 622 nm, an emission wavelength of 670 nm, and a slit width of 10 and 5 nm, respectively. The uptake of the dye by the cell resulting in self-quenching properties are dependent on the potential of the membrane. Upon maximum uptake, indicated by a decrease in the inherent fluorescence of the dye, the peptides were added to the cells at 1× and 2× MIC₉₀₉₀ concentration, and the decrease in membrane potential as indicated by an increase in dye fluorescence was recorded.

**Outer-membrane disruption assay**—The experiment for outer membrane interaction and disruption by the peptide combination was performed using ANS and NPN dyes. 10 μM of the respective dye was added to the cell suspension and incubated in the dark for 60 and 15 min, respectively, to allow equilibration. Next, the cells were titrated with 1× and 2× MIC₉₀₉₀ concentration of the parent peptides, and the increase in fluorescence intensity was recorded for 30 min.

Fluorescence from ANS was recorded at an excitation wavelength of 380 nm, an emission wavelength of 470 nm, and a slit width of 1 nm. Fluorescence from NPN was recorded at an excitation wavelength of 350 nm, an emission wavelength of 400 nm, and a slit width of 5 nm.

**Inner-membrane permeabilization assay**—PI dye, which binds DNA/RNA of dead cells, was used to probe disruption of the inner membrane. 10 μM of the dye was added to the cell suspension (10⁷ cells/ml) and incubated in the dark for 15 min for equilibration. Next, the cells were titrated with 1× and 2×
MIC$_{99\%}$ concentration of the parent peptides, and the increase in fluorescence intensity of PI was recorded for 30 min. Fluorescence from PI was recorded at an excitation wavelength of 535 nm, an emission wavelength of 617 nm, and a slit width of 10 nm.

**Liposome preparation**

Approximately, 1 mg of POPE and POPG was weighed and dissolved in chloroform to obtain stock solutions and further mixed to obtain the model liposomes, a 3:1 molar ratio of anionic POPE/POPG, which is reported to be used extensively as a model system for negatively charged bacterial membranes in the literature (82). Next, uniform lipid films were obtained by drying the lipid suspension of model liposomes in a nitrogen stream, followed by overnight lyophilization. The film was resuspended in 10 mM sodium phosphate buffer at pH 7.4 by vigorous vortexing for 30 min, followed by five freeze-thaw cycles in liquid nitrogen to obtain vesicles. To isolate unilamellar vesicles, the suspension was passed through a mini-extruder (Avanti Polar Lipids, AL) fitted with two 1.0-ml Hamilton gastight syringes (Hamilton, NV). Samples were typically subjected to 23 passes through the filter. Such an odd number of passages helps to avoid contamination of the sample by vesicles that have not passed through the first filter. The VG16KKRP or KYE28 or both peptide solutions, dissolved in similar buffer, were added to the LUVs to prepare the appropriate final peptide concentration/lipid molar ratio, making a final sample volume of 120 μl.

**Dynamic light scattering**

The experiment was performed on a Malvern ZetasizerNano S (Malvern Instruments) provided with a 4-milliwatt He-Ne laser (λ = 633 nm) and a back-scattering angle of 173°. DLS experiments were performed using Malvern ZetasizerNano S (Malvern Instruments) provided with a 4-milliwatt He-Ne laser (λ = 633 nm) and a back-scattering angle of 173°. LUVs (pH 7.4) were titrated with increasing concentrations of the peptide solution, prepared in the same buffer (phosphate buffer (pH 7.4)), and DLS measurement was performed in triplicate and three independent experimental sets were recorded. All samples were filtered using Millipore 0.45-μm polycarbonate membrane filters, degassed before use, and measured at 298 K in low-volume disposable sizing cuvettes. For data analysis, the viscosity and refractive index of 10 mM Tris buffer containing 100 mM NaCl buffer (pH 7.4) were taken to be 0.89 and 1.33, respectively. The particle size distributions were generated using the autocorrelation data from the non-negative least-squares fit algorithm provided by the instrument manufacturer (83).

**Circular dichroism spectroscopy**

CD spectra of the peptide combination were obtained using a JASCO 814 spectrometer (Japan). For this, 25 μM of both peptides, prepared in 10 mM phosphate buffer (pH 7.4), was titrated with increasing concentrations of LPS, prepared in the same buffer. The spectra were recorded in a 0.2-cm cuvette, at 298 K with three scans accumulating at a speed of 100 nm/min, scanning from 190 to 260 nm, at a data interval of 1 nm. The spectra generated were corrected using a baseline spectrum for each combination of peptide, and the ellipticity in milli-degrees was plotted against wavelength in nanometers.

**Solid-state NMR spectroscopy**

**NMR sample preparation**—Sample preparation for $^{31}$P solid-state NMR was done by dissolving 3:1 POPE/POPG in chloroform. The mixed samples were dried under a stream of nitrogen followed by vacuum-dry oven (30 °C) overnight to completely remove any residual solvent. 10 mM Tris buffer (pH 6.0) was added to the samples to hydrate the lipid film. The hydrated samples were next vortexed for 2 min above the lipid-phase transition temperature and freeze-thawed for a minimum of four times using liquid nitrogen, ensuring a generation of a single lamellar vesicle. To obtain LUVs with a uniform size of 1 μm in diameter, the sample was extruded through polycarbonate filters (pore size of 1 μm, Nuclepore®, Whatman, NJ) mounted on a mini-extruder (Avanti Polar Lipids, AL) fitted with two 1.0-ml Hamilton gastight syringes (Hamilton, NV). Samples were typically subjected to 23 passes through the filter. Such an odd number of passages helps to avoid contamination of the sample by vesicles that have not passed through the first filter. The VG16KKRP or KYE28 or both peptide solutions, dissolved in similar buffer, were added to the LUVs to prepare the appropriate final peptide concentration/lipid molar ratio, making a final sample volume of 120 μl.

**Solution-state NMR spectroscopy**

All NMR experiments were carried out at 298 K using a Bruker Avance III 700 MHz NMR spectrometer, equipped with 5-mm cryogenic cooled triple resonance probe. NMR samples were prepared in 10% deuterated water (pH 4.5), and TSP was used as an internal standard (0.0 ppm). Two-dimensional $^1$H-$^1$H nuclear Overhauser effect spectroscopy (2D NOESY) with a sweep width of 12 ppm in both directions was recorded for the peptides VG16KKRP and KYE28 at a 1:1 molar ratio in aqueous solution. The number of scans was fixed at 16 per $t_1$ increment with 16 dummy scans. Next, to probe the synergistic interaction of the two peptides with LPS, 1D proton NMR spectra of 0.6 mM VG16KKRP and KYE28 were recorded alone and upon titration with increasing concentrations of E. coli LPS. The 1D spectra were acquired using excitation-sculpting scheme for water suppression and the States-TPPI for quadrature detection in the $t_1$ dimension (84). The molar ratio of LPS/
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VG16KRKP/KYE28 of 1:30:30 was used for two-dimensional $^1$H–$^1$H total correlation spectroscopy (2D TOCSY) (mixing time = 80 ms) and two-dimensional $^1$H–$^1$H trNOESY (mixing times = 100, 150, and 200 ms) experiments, keeping all other parameters the same. The number of scans was fixed to 16 and 72, respectively, for TOCSY and trNOESY experiments. 512 increments in $t_1$ and 2048 data points in $t_2$ dimension along with States TPPI for quadrature detection in $t_1$ dimension and excitation-sculpting scheme for water suppression were used to record the experiments. The experiments were processed and analyzed using Bruker TopSpin™ 3.1 and Sparky (85). Similar TOCSY and trNOESY NMR experiments were also recorded for VG16KRKP-KYE28A, VG16F12A-KYE28, and VG16WFA-KYE28 to study the role of aromatic residues in the interaction of peptides with lipopolysaccharide using Bruker Avance III 700 MHz NMR spectrometer.

**Calculation of NMR-derived structures**

The LPS-bound structure of the individual peptides VG16KRKP, KYE28, and KYE28A have already been reported with Protein Data Bank accession codes of 2MWL, 2NAT, and 2NAU, respectively (29, 30). To get structural insights into their mode of interaction and the combination effects, we calculated the LPS-bound three-dimensional conformation of the complexes VG16KRKP-KYE28, VG16KRKP-KYE28A, VG16F12A-KYE28, and VG16WFA-KYE28, generated by linking the two peptides via 10 poly-glycine residues, using CYANA version 2.1 software (47). It could be noted here that the choice of glycine poly-linker merely aids in structure calculation by helping to comprehend the influence of the peptides on their interaction abilities with each other, as well as with LPS, and to ensure that the peptide complex remains flexible and unbiased. For this, the inter-proton upper bound distances were calculated initially by sorting trNOESY cross-peaks based on their intensities into strong (3.0 Å), medium (4.0 Å), and weak (5.0 Å). The lower-bound distance constraint was kept fixed at 2.0 Å. The backbone dihedral angles, $\phi$ (between $-30$ and $120^\circ$ and/or $-80$ and $-60^\circ$) and $\psi$ ($120^\circ$ and $-120^\circ$ and/or $-40^\circ$ and $-20^\circ$), were kept flexible for all nonglycine residues so as to limit the conformational space. The distance constraints were then used for iterative refinement at each round of structure calculation. Hydrogen bonding and stereo-specificity were excluded from the structure calculation. The ensemble structure was generated from the 15 lowest-energy structures, which excluded from the structure calculation. The ensemble structure was generated from the 15 lowest-energy structures, which were analyzed using PyMOL, MOLMOL, and Chimera software. PROCHECK server was used to authenticate the backbone stereochemistry (47, 86–88).

**PRE NMR**

To conduct PRE NMR, site-directed spin labeling was done using a paramagnetic quencher, MTSL, attached to the thiol group of the cysteine residue present in VG16KRKP. This was done by incubating a 10:1 molar ratio of the peptide VG16KRKP and MTSL in 50% acetone overnight in the dark at 298 K under a mild shaking condition. The resulting solution was diluted and subjected to RP-HPLC (LC-20AT Phenomenix C-18 column, SHIMADZU, Kyoto, Japan) to purify labeled VG16KRKP from unlabeled peptide and free MTSL and characterized using MS in a Bruker autoflex speed TOF/TOF in a positive reflector mode. MTSL-labeled VG16KRKP was then dissolved in deuterated water in an equimolar ratio with KYE28 (0.6 mM (pH 4.5)) and $^1$H–$^1$H TOCSY, and trNOESY experiments were recorded, keeping all the recording parameters similar as described previously. NMR data were analyzed using Bruker TOPSPIN™ 3.1 and SPARKY (85). The H$_\alpha$/NH cross-peak intensities of MTSL-labeled and unlabeled complex in LPS were calculated, and the remaining amplitudes ($I/I_0$) in percentage was calculated using Equation 4,

$$\text{% remaining amplitude } \left( \frac{I}{I_0} \right) = \left( \frac{I_{\text{H}_\alpha/\text{NH(MTSL-labeled)}}}{I_{\text{H}_\alpha/\text{NH(unlabeled)}}} \right) \times 100 \quad \text{(Eq. 4)}$$

where $I_{\text{H}_\alpha/\text{NH(MTSL-labeled)}}$ and $I_{\text{H}_\alpha/\text{NH(unlabeled)}}$ indicate the H$_\alpha$/NH peaks intensities of each amino acid in the unlabeled and MTSL-labeled peptide complexes, respectively. No attempts were made to back-calculate theoretical $I/I_0$ values due to uncertainties related to $R_2$ relaxation effects and INEPT transfer calculations from 2D trNOEY spectra.

**Hydrogen/deuterium exchange NMR**

To gain insight into the stability and solvent accessibility of the VG16KRKP–KYE28 peptide complex, amide proton exchange was monitored. For this, 0.5 mM VG16KRKP/KYE28 was suspended in deuterium oxide (pD 4.5), and a series of 2D TOCSY (80-ms mixing time) was recorded until 5 h with an interval of 1 h each. The number of scans and dummy scans were kept fixed at 8 and 16, respectively, with 256 and 2048 data point increments in $t_1$ and $t_2$ dimensions, respectively. NMR data were analyzed using Bruker TOPSPIN™ 3.1 and SPARKY (85). The hydrogen exchange rates ($k_{ex}$) were obtained by fitting to a single-exponential decay equation, and the protection factor was determined by taking a ratio of the calculated intrinsic exchange rate and the experimentally determined exchange rate.

**SASA calculations**

Online POPS* algorithm was used to calculate the SASA value of each peptide, individually as well as the peptide complex in LPS (55).

**FRET analysis**

FRET experiment between the Trp of donor peptide and dansyl of the N-terminally-labeled acceptor peptide was carried out by monitoring the quenching of Trp emission intensity upon titration with the acceptor dansyl in the presence of *E. coli* LPS. 5 µM of the donor peptide, VG16KRKP, in the presence of LPS, 3:1 POPE/POPG vesicle, or *E. coli* total lipid extract bicelle was titrated with increasing concentrations of dansyl–KYE28, and the emission was recorded at a 300–550-nm range after exciting at 280 nm wavelength in a Hitachi F7000 instrument (Tokyo, Japan) at room temperature. The normalized quenching value ($F/F_0$) was plotted as a function of mole fraction ($P_o$) of the acceptor molecule. $F$ and $F_0$ represent the intensity of fluorescence emission of Trp in the presence and absence of the dansyl group, and $P_o$ was calculated by Equation 5,
This quenching graph was extrapolated linearly to obtain the value of $E(1 - Q)$, with $Q$ being the maximum quenching value. Next, using Förster equation, the inter-chromophore distance between Trp and the dansyl group was calculated by Equation 6,

$$r = R_0 (E^{-1} - 1)^{1/6} \quad (\text{Eq. 6})$$

where $r$ = distance between the two chromophores (Trp and dansyl in this case), $R_0$ = Förster distance (23 Å for Trp-dansyl) and $E$ = efficiency of FRET obtained for the quenching plot. The same experiment was carried out for a combination of two peptides, and in all cases the inter-chromophore distance between Trp and the dansyl group was calculated.

Statistical analysis

All biological experiments were repeated three times. Data were analyzed by using SigmaPlot 12.0 (Copyright 2011 Systat Software, Inc., Germany) and Origin Pro 2018 (Northampton, MA).

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References

1. Chiang, C. Y., Uzoma, I., Moore, R. T., Gilbert, M., Duplantier, A. J., and Panchal, R. G. (2018) Mitigating the impact of antibacterial drug resistance through host-directed therapies: current progress, outlook, and challenges. MBio 9, e01932-17 CrossRef Medline
2. Spaulding, C. N., Klein, R. D., Schreiber, H. L., 4th, Janetka, J. W., and Hultgren, S. J. (2018) Precision antimicrobial therapeutics: the path of least resistance? NPJ Biofilms Microbiomes 4, 4 CrossRef Medline
3. Gustavsson, J., Cederberg, C., Sonesson, U., Otterdijk, V. R., and Maybeck, A. (2011) Global Food Losses and Food Waste—Extent, Causes, and Prevention. Food and Agriculture Organization of the United Nations, New York ISBN 978-92-5-107205-9
4. Levy, S. B., and Marshall, B. (2004) Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. 10, S122–S129 CrossRef Medline
5. Strange, R. N., and Scott, P. R. (2005) Plant disease: a threat to global food security. Annu. Rev. Phytopathol. 43, 83–116 CrossRef Medline
6. McNamara, P. S., Stockwell, V. O., Sundin, G. W., and Jones, A. L. (2002) Antibiotic use in plant agriculture. Annu. Rev. Phytopathol. 40, 443–465 CrossRef Medline
7. Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. Nature 415, 389–395 CrossRef Medline
8. Strominger, J. L. (2009) Animal antimicrobial peptides: ancient players in innate immunity. J. Immunol. 182, 6633–6634 CrossRef Medline
9. Hancock, R. E., and Sahl, H. G. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. 24, 1551–1557 CrossRef Medline
10. Mahlapuu, M., Häkkanson, J., Ringstad, L., and Björn, C. (2016) Antimicrobial peptides: an emerging category of therapeutic agents. Front. Cell Infect. Microbiol. 6, 194 CrossRef Medline
11. Hancock, R. E., and Patrzykat, A. (2002) Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics. Curr. Drug Targets Infect. Disord. 2, 79–83 CrossRef Medline
12. Hancock, R. E. (2001) Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect. Dis. 1, 156–164 CrossRef Medline
13. Hancock, R. E., Brown, K. L., and Mookherjee, N. (2006) Host defence peptides from invertebrates—emerging antimicrobial strategies. Immunobiology 211, 315–322 CrossRef Medline
14. Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3, 238–250 CrossRef Medline
15. Sahi, H. G. (2006) Optimizing antimicrobial host defense peptides. Chem. Biol. 13, 1015–1017 CrossRef Medline
16. Shai, Y. (2002) Mode of action of membrane active antimicrobial peptides. Biopolymers 66, 236–248 CrossRef Medline
17. Kalle, M., Papareddy, P., Kasetty, G., van der Plas, M. J., Mörgelin, M., Malmsten, M., and Schmidtchen, A. (2014) A peptide of heparin cofactor II inhibits endotoxin-mediated shock and invasive Pseudomonas aerugi-nosa infection. PLoS ONE 9, e102577 CrossRef Medline
18. Bhattacharjya, S. (2010) De novo designed lipopolysaccharide binding peptides: structure–based development of antiendotoxic and antimicrobials drugs. Curr. Med. Chem. 17, 3080–3093 CrossRef Medline
19. Wimley, W. C., and Hristova, K. (2011) Antimicrobial peptides: successes, challenges and unanswered questions. J. Membr. Biol. 239, 27–34 CrossRef Medline
20. Aoki, W., and Ueda, M. (2013) Characterization of antimicrobial peptides toward the development of novel antibiotics. Pharmaceuticals 6, 1055–1081 CrossRef Medline
21. Nordström, R., Nyström, L., Andrén, O. C. J., Malkoch, M., Umbersk, A., Davoudi, M., Schmidtchen, A., and Malmsten, M. (2018) Membrane interactions of microgels as carriers of antimicrobial peptides. J. Colloid Interface Sci. 513, 141–150 CrossRef Medline
22. Biswaro, L. S., da Costa Sousa, M. G., Rezende, T. M. D. S., and Franco, O. L. (2018) Antimicrobial peptides and nanotechnology, recent advances and challenges. Front. Microbiol. 9, 855 CrossRef Medline
23. Kovalainen, M., Mönkäe, J., Riikonen, J., Pesonen, U., Vilson, V., Sal-lonen, J., Lehto, V.-P., Järvinen, K., and Herzig, K.-H. (2015) Novel delivery systems for improving the clinical use of peptides. Pharmacol. Rev. 67, 541–561 CrossRef Medline
24. Yu, G., Baeder, D. Y., Regoes, R. R., and Rolf, J. (2016) Combination effects of antimicrobial peptides. Antimicrob. Agents Chemother. 60, 1717–1724 CrossRef Medline
25. Yan, H., and Hancock, R. E. (2001) Synergistic interactions between mammalian antimicrobial defense peptides. Antimicrob. Agents Chemother. 45, 1558–1560 CrossRef Medline
26. Siavoshi, M. A., Prokopczuk, F. I., Del Rosario, N.-A., Abasi, L., and Franco, O. L. (2018) Antimicrobial peptides and nanotechnology, recent advances and challenges. Front. Microbiol. 9, 855 CrossRef Medline
27. Grassi, L., Maisetta, G., Esin, S., and Batoni, G. (2017) Combination strategies to enhance the efficacy of antimicrobial peptides against bacterial biofilms. Front. Microbiol. 8, 2409 CrossRef Medline
28. Choi, H., and Lee, D. G. (2012) Synergistic effect of antimicrobial peptide arenicin-1 in combination with antibiotics against pathogenic bacteria. Res. Microbiol. 163, 479–486 CrossRef Medline
29. Datta, A., Ghosh, A., Airoldi, C., Sperandeo, P., Mroue, K. H., Jiménez-Barbero, J., Kundi, P., Ramamoorthy, A., and Bhunia, A. (2015) Antimicrobial peptides: insights into membrane permeabilization, lipopolysaccharide fragmentation and application in plant disease control. Sci. Rep. 5, 11951 CrossRef Medline
30. Datta, A., Bhattacharyya, D., Singh, S., Ghosh, A., Schmidtchen, A., Malmsten, M., and Bhunia, A. (2016) Role of aromatic amino acids in lipopoly-saccharide and membrane interactions of antimicrobial peptides for use in plant disease control. J. Biol. Chem. 291, 13301–13317 CrossRef Medline
31. Bremder, D. N., Bauer, K. A., Pouch, S. M., Thomas, K., Smith, D., Goff, D. A., Pancholi, P., and Balada-Llasat, J. M. (2016) Correlation of checker-board synergy testing with time-kill analysis and clinical outcomes of extensively drug-resistant Acinetobacter baumannii respiratory infections. Antimicrob. Agents Chemother. 60, 6892–6895 CrossRef Medline
Structural basis of peptide synergy

32. Yralagadda, V., Akrapeddi, P., Manjunath, G. B., and Haldar, J. (2014) Membrane active vancomycin analogs: a strategy to combat bacterial resistance. J. Med. Chem. 57, 4558–4566 CrossRef Medline

33. Thennarasu, S., Lee, D. K., Tan, A., Prasad Kari, U., and Ramamoorthy, A. (2005) Antimicrobial activity and membrane selective interactions of a synthetic lipopeptide MSI-843. Biochim. Biophys. Acta 1711, 49–58 CrossRef Medline

34. Schiller, J., Muller, M., Fuchs, B., Arnold, K., and Huster, D. (2007) 31P NMR spectroscopy of phospholipids: from micelles to membranes. Curr. Anal. Chem. 3, 283–301 CrossRef

35. Bechinger, B., and Salnikov, E. S. (2012) The membrane interactions of antimicrobial peptides revealed by solid-state NMR spectroscopy. Chem. Phys. Lipids 165, 282–301 CrossRef Medline

36. Snyder, D. S., and McIntosh, T. J. (2000) The lipopolysaccharide barrier: correlation of antibiotic susceptibility with antibacterial permeability and fluorescent probe binding kinetics. Biochemistry 39, 11777–11787 CrossRef Medline

37. Rosenfeld, Y., Barra, D., Simmaco, M., Shai, Y., and Mangoni, M. L. (2006) A synergism between temporins toward Gram-negative bacteria overcomes resistance imposed by the lipopolysaccharide protective layer. J. Biol. Chem. 281, 28565–28574 CrossRef Medline

38. Yu, L., Tan, M., Ho, B., Ding, J. L., and Wohland, T. (2006) Determination of critical micelle concentrations and aggregation numbers by fluorescence correlation spectroscopy: aggregation of a lipopolysaccharide. Anal. Chim. Acta 556, 216–225 CrossRef Medline

39. Bhunia, A., Domadia, P. N., Torres, I., Hallock, K. J., Ramamoorthy, A., and Bhattacharya, S. (2010) NMR structure of pardaxin, a pore-forming antimicrobial peptide, in lipopolysaccharide micelles: mechanism of outer membrane permeabilization. J. Biol. Chem. 285, 3883–3895 CrossRef Medline

40. Ghosh, A., Datta, A., Jana, J., Kar, R. K., Chatterjee, C., Chatterjee, S., and Bhunia, A. (2014) Sequence context induced antimicrobial activity: insight into lipopolysaccharide permeabilization. Mol. Bioyst. 10, 1596–1612 CrossRef Medline

41. Chowdhury, R., Ilyas, H., Ghosh, A., Ali, H., Ghoni, A., Midya, A., Jana, N. R., Das, S., and Bhunia, A. (2017) Multivalent gold nanoparticle–peptide conjugates for targeting intracellular bacterial infections. Nanoscale 9, 14074–14093 CrossRef Medline

42. Datta, A., Jaiswal, N., Ilyas, H., Deb Nath, S., Biswas, K., Kumar, D., and Bhunia, A. (2017) Structural and dynamic insights into a glycine-mediated short analog of a designed peptide in lipopolysaccharide micelles: correlation between compact structure and anti-endotoxin activity. Biochemistry 56, 1348–1362 CrossRef Medline

43. Li, J., Koh, J. J., Liu, S., Lakshminarayanan, R., Verma, C. S., and Beuerman, R. W. (2017) Membrane active antimicrobial peptides: translating mechanistic insights to design. Front. Neurosci. 11, 73 CrossRef Medline

44. Luo, R. R., Dales, N., and Andrews, P. C. (1994) Surfactant effects on protein structure examined by electrospray ionization mass spectrometry. Protein Sci. 3, 1975–1983 CrossRef Medline

45. Sjögren, H., Ericsson, C. A., Evenås, J., and Ulvenlund, S. (2005) Interactions between charged polypeptides and nonionic surfactants. Biophys. J. 89, 4219–4233 CrossRef Medline

46. Mcdonnell, P. A., and Opella, S. (1993) Effect of detergent concentration on multidimensional solution NMR spectra of membrane proteins in micelles. J. Magn. Reson. Series B 102, 120–125 CrossRef

47. Güntert, P., Mumenthaler, C., and Wüthrich, K. (1997) Torsion angle dynamics for NMR structure calculation with the new program DYANA. J. Mol. Biol. 273, 283–298 CrossRef Medline

48. Burley, S. K., andPetsko, G. A. (1986) Amino-acidic interactions in proteins. FEBS Lett. 203, 139–143 CrossRef Medline

49. Anjana, R., Vaishnavi, M. K., Sherlin, D., Kumar, S. P., Naveen, K., Kanth, P. S., and Sekar, K. (2012) Aromatic–aromatic interactions in structures of proteins and protein-DNA complexes: a study based on orientation and distance. Bioinformation 8, 1220–1224 CrossRef Medline

50. Samanta, U., Pal, D., and Chakrabarti, P. (1999) Packing of aromatic rings against tryptophan residues in proteins. Acta Crystallogr. D Biol. Crystallogr. 55, 1421–1427 CrossRef Medline

51. Serrano, L., Bycroft, M., and Ferstl, A. R. (1991) Aromatic–aromatic interactions and protein stability. Investigation by double-mutant cycles. J. Mol. Biol. 218, 465–475 CrossRef Medline

52. Datta, A., Kundu, P., and Bhunia, A. (2016) Designing potent antimicrobial peptides by disulphide linked dimerization and N-terminal lipidation to increase antimicrobial activity and membrane perturbation: structural insights into lipopolysaccharide binding. J. Colloid. Interface Sci. 461, 335–345 CrossRef Medline

53. Madhusudan Makwana, K., and Malhalakshmi, R. (2015) Implications of aromatic–aromatic interactions: from protein structures to peptide models. Protein Sci. 24, 1920–1933 CrossRef Medline

54. Cabrita, L. D., Waudby, C. A., Dobson, C. M., and Christodoulou, J. (2011) Solution-state nuclear magnetic resonance spectroscopy and protein folding. Methods Mol. Biol. 752, 97–120 CrossRef Medline

55. Cavollo, L., Kleijngen, J., and Fraternali, F. (2003) POPs: a fast algorithm for solvent accessible surface areas at atomic and residue level. Nucleic Acids Res. 31, 3364–3366 CrossRef Medline

56. Bhunia, A., Saravanan, R., Mohanram, H., Mangoni, M. L., and Bhattacharya, S. (2011) NMR structures and interactions of tempolin-1Tl and tempolin-1Tb with lipopolysaccharide micelles: mechanistic insights into outer membrane permeabilization and synergistic activity. J. Biol. Chem. 286, 24394–24406 CrossRef Medline

57. Matsuzaki, K., Mitani, Y., Akada, K. Y., Murase, O., Yoneyama, S., Zasloff, M., and Miyajima, K. (1998) Mechanism of synergy between antimicrobial peptides magainin 2 and PGLa. Biochemistry 37, 15144–15153 CrossRef Medline

58. Zerweck, J., Strandberg, E., Bürck, J., Reichert, J., Wadhwani, P., Kukhareno, O., and Ulrich, A. S. (2016) Homo- and heteromeric interaction strengths of the synergistic antimicrobial peptides PGLa and magainin 2 in membranes. Eur. Biophys. J. 45, 535–547 CrossRef Medline

59. Haney, E. F., Hunter, H. N., Matsuzaki, K., and Vogel, H. I. (2009) Solution NMR studies of amphibian antimicrobial peptides: linking structure to function? Biochim. Biophys. Acta 1788, 1639–1655 CrossRef Medline

60. Zerweck, J., Strandberg, E., Kukhareno, O., Reichert, J., Bürck, J., Wadhwani, P., and Ulrich, A. S. (2017) Molecular mechanism of synergy between the antimicrobial peptides PGLa and magainin 2. Sci. Rep. 7, 13153 CrossRef Medline

61. Marquette, A., and Bechinger, B. (2018) Biophysical investigations elucidating the mechanisms of action of antimicrobial peptides and their synergism. Biomolecules 8, E18 CrossRef Medline

62. Leber, R., Pachler, M., Kabelka, I., Svoboda, I., Enkoller, D., Vácha, R., Lohnер, K., and Pabst, G. (2018) Synergism of antimicrobial frog peptides couples to membrane intrinsic curvature strain. Biochem. J. 414, 1945–1954 CrossRef Medline

63. Harmouche, N., and Bechinger, B. (2018) Lipid-mediated interactions between the antimicrobial peptides magainin 2 and PGLa in bilayers. Biophys. J. 115, 1033–1044 CrossRef Medline

64. Marquette, A., Salnikov, E. S., Glätter, E., Aisenbrey, C., and Bechinger, B. (2016) Magainin 2-PGLa interactions in membranes–two peptides that exhibit synergistic enhancement of antimicrobial activity. Curr. Top. Med. Chem. 16, 65–75 Medline

65. Chen, Y., Guarnieri, M. T., Yasil, A. I., Vasil, M. L., Mant, C. T., and Hodges, R. S. (2007) Role of peptide hydrophobicity in the mechanism of action of a-helical antimicrobial peptides. Antimicrob. Agents Chemother. 51, 1398–1406 CrossRef Medline

66. Lanzarotti, E., Biekofsky, R. R., Estrin, D. A., Marti, M. A., and Turjanski, J. (2002) Membrane active vancomycin analogs: a strategy to combat bacterial resistance. J. Chem. Inf. Model 42, 120–125 CrossRef Medline
69. Le, C. F., Yusof, M. Y., Hassan, H., and Sekaran, S. D. (2015) *In vitro* properties of designed antimicrobial peptides that exhibit potent anti-pneumococcal activity and produces synergism in combination with penicillin. Sci. Rep. 5, 9761 CrossRef Medline

70. Ruden, S., Hilpert, K., Berditsch, M., Wadhwani, P., and Ulrich, A. S. (2009) Synergistic interaction between silver nanoparticles and membrane-permeabilizing antimicrobial peptides. *Antimicrob. Agents Chemother.* 53, 3538–3540 CrossRef Medline

71. Mor, A., Hani, K., and Nicolas, P. (1994) The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. *J. Biol. Chem.* 269, 31635–31641 Medline

72. Levy, O., Ooi, C. E., Weiss, J., Lehrer, R. I., and Elsbach, P. (1994) Individual and synergistic effects of rabbit granulocyte proteins on *Escherichia coli*. *J. Clin. Invest.* 94, 672–682 CrossRef Medline

73. Brogue, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C. J., and Broglie, R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* 254, 1194–1197 CrossRef Medline

74. Alexander, D., Goodman, R. M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Abl-Goy, P., Luntz, T., and Ward, E. (1993) Increased tolerance to two oomycete pathogens in transgenic potato plants expressing pathogenesis-related protein 1a. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7327–7331 CrossRef Medline

75. Zhu, B., Chen, T. H., and Li, P. H. (1996) Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. *Planta* 185, 37–46 CrossRef Medline

76. Jongedijk, E., Tigelaar, H., van Roekel, J. S. C., Bres-Vloemans, S. A., Dekker, L., van den Elzen, P. J. M., Cornelissen, B. J. C., and Melchers, L. S. (1993) Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* 85, 173–180 CrossRef Medline

77. Peschen, D., Li, H. P., Fischer, R., Kreuzaler, F., and Liao, Y. C. (2004) Fusion proteins comprising a *Fusarium*-specific antibody linked to antifungal peptides protect plants against a fungal pathogen. *Nat. Biotechnol.* 22, 732–738 CrossRef Medline

78. Bhattacharyya, D., Kim, M., Mroute, K. H., Park, M., Tiwari, A., Saleem, M., Lee, D., and Bhunia, A. (2019) Role of non-electrostatic forces in antimicrobial potency of a dengue-virus derived fusion peptide VG16KRP: mechanistic insight into the interfacial peptide-lipid interactions. *Biochim. Biophys. Acta Biomembr.* 1861, 798–809 CrossRef Medline

80. Odds, F. C. (2003) Synergy, antagonism, and what the chequerboard puts between them. *J. Antimicrob. Chemother.* 52, 1 CrossRef Medline

81. Li, Q., Lawrence, C. B., Maelor Davies, H., and Everett, N. P. (2002) A tridecapeptide possesses both antimicrobial and protease-inhibitory activities. *Peptides* 23, 1–6 CrossRef Medline

83. Twomey, S. (2002) *Introduction to the Mathematics of Inversion of Remote Sensing and Indirect Measurements*. Dover Publications, Mineola, NY

84. Sklenar, V., Piotto, M., Leppik, R., and Saudek, V. (1993) Gradient-tailored water suppression for 1H-15N HSQC experiments optimized to retain full sensitivity. *J. Magn. Reson.* 102, 241–245 CrossRef

85. Goddard, T. D., and Kneller, D. G. (2008) *Sparky3*, University of California, San Francisco

86. Koradi, R., Billeter, M., and Wüthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* 14, 51–55. 29–32 CrossRef Medline

87. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* 8, 477–486 Medline

88. Schrödinger, LLC. (2017) The PyMOL Molecular Graphics System, Version 2.0, Schrödinger, LLC, New York

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**Structural basis of peptide synergism**

74. Alexander, D., Goodman, R. M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Abl-Goy, P., Luntz, T., and Ward, E. (1993) Increased tolerance to two oomycete pathogens in transgenic potato plants expressing pathogenesis-related protein 1a. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7327–7331 CrossRef Medline

75. Zhu, B., Chen, T. H., and Li, P. H. (1996) Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. *Planta* 185, 37–46 CrossRef Medline

76. Jongedijk, E., Tigelaar, H., van Roekel, J. S. C., Bres-Vloemans, S. A., Dekker, L., van den Elzen, P. J. M., Cornelissen, B. J. C., and Melchers, L. S. (1993) Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* 85, 173–180 CrossRef Medline

77. Peschen, D., Li, H. P., Fischer, R., Kreuzaler, F., and Liao, Y. C. (2004) Fusion proteins comprising a *Fusarium*-specific antibody linked to antifungal peptides protect plants against a fungal pathogen. *Nat. Biotechnol.* 22, 732–738 CrossRef Medline

78. Bhattacharyya, D., Kim, M., Mroute, K. H., Park, M., Tiwari, A., Saleem, M., Lee, D., and Bhunia, A. (2019) Role of non-electrostatic forces in antimicrobial potency of a dengue-virus derived fusion peptide VG16KRP: mechanistic insight into the interfacial peptide-lipid interactions. *Biochim. Biophys. Acta Biomembr.* 1861, 798–809 CrossRef Medline

79. Blazyk, J., Wiegand, R., Klein, J., Hammer, J., Epand, R. M., Epand, R. F., Maloy, W. L., and Kari, U. P. (2001) A novel linear amphipathic β-sheet cationic antimicrobial peptide with enhanced selectivity for bacterial lipids. *J. Biol. Chem.* 276, 27899–27906 CrossRef Medline

80. Odds, F. C. (2003) Synergy, antagonism, and what the chequerboard puts between them. *J. Antimicrob. Chemother.* 52, 1 CrossRef Medline

81. Li, Q., Lawrence, C. B., Maelor Davies, H., and Everett, N. P. (2002) A tridecapeptide possesses both antimicrobial and protease-inhibitory activities. *Peptides* 23, 1–6 CrossRef Medline

82. Schmidtchen, A., Ringstad, L., Kasetty, G., Mizuno, H., Rutland, M. W., and Malmsten, M. (2011) Membrane selectivity by W-tagging of antimicrobial peptides. *Biochim. Biophys. Acta* 1808, 1081–1091 CrossRef Medline

83. Twomey, S. (2002) *Introduction to the Mathematics of Inversion of Remote Sensing and Indirect Measurements*. Dover Publications, Mineola, NY