Helical antimicrobial polypeptides with radial amphiphilicity

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α-Helical antimicrobial peptides (AMPs) generally have facially amphiphilic structures that may lead to undesired peptide interactions with blood proteins and self-aggregation due to exposed hydrophobic surfaces. Here we report the design of a class of cationic, helical homo-polypeptide antimicrobials with a hydrophobic internal helical core and a charged exterior shell, possessing unprecedented radial amphiphilicity. The radially amphiphilic structure enables the polypeptide to bind effectively to the negatively charged bacterial surface and exhibit high antimicrobial activity against both gram-positive and gram-negative bacteria. Moreover, the shielding of the hydrophobic core by the charged exterior shell decreases nonspecific interactions with eukaryotic cells, as evidenced by low hemolytic activity, and protects the polypeptide backbone from proteolytic degradation. The radially amphiphilic polypeptides can also be used as effective adjuvants, allowing improved permeation of commercial antibiotics in bacteria and enhanced antimicrobial activity by one to two orders of magnitude. Designing AMPs bearing this unprecedented, unique radially amphiphilic structure represents an alternative direction of AMP development; radially amphiphilic polypeptides may become a general platform for developing AMPs to treat drug-resistant bacteria.

antimicrobial peptide | α-helix | polypeptides | radial amphiphilicity | bacteria

Antimicrobial peptides (AMPs) typically contain ~40–60 amino acids, consisting of both cationic and hydrophobic amino acids. They adopt various secondary structures (e.g., α-helix) and can kill a range of bacteria. As AMPs target generic and necessary lipid components of bacterial membranes (1, 2) and depend less on specific bacterial metabolic status (3–6), development of resistance has been slow. Because of this feature, AMPs have attracted significant attention as potential antimicrobial agents clinically. Among all AMPs developed, the α-helical peptides are the most heavily investigated and generally are facially amphiphilic (FA) in structure with the cationic and hydrophobic amino acids separated to opposite faces of the helix. This structure correlates well to antimicrobial activity (6–10). Recent work has shown how amino acid content of AMPs enables this activity via specific types of membrane curvature generation (11–13).

Despite extensive effort, the commercial development of AMPs has seen limited success, in part due to drawbacks native to peptides. Although much has been learned from fundamental studies on AMP mechanisms (1, 2, 14, 15), precise, quantitative predictions of an AMP’s activity, therapeutic index, and antimicrobial activity compared with off-target eukaryotic cytotoxicity are currently impossible. Designing new AMP-related antibiotics relies on sequence-controlled peptide synthesis and parameter optimization, which is expensive and labor-intensive. Another drawback of most AMP-derived antibiotics is their poor stability in biological systems. LL-37 and magainin, for example, can be degraded by proteases in several minutes in blood circulation and lose their antimicrobial activities (16, 17). Finally, an important drawback of AMPs is related to their FA structure with an exposed hydrophobic helix face (Fig. 1A), which leads to undesired polypeptide interactions with blood proteins and self-aggregation. AMP helix bundles have been reported to cause substantially reduced antimicrobial activity (18, 19).

There has been significant interest in developing AMP analogs, such as β-peptides (20–23), αβ-peptides (24, 25), peptoids (26), and aromatic oligomers (15, 27–30). These compounds have demonstrated improved stability over conventional AMPs. They are in general sequence specific and often require solid-phase peptide synthesis, thereby sharing similar advantages and development drawbacks as AMPs. There has also been growing interest in synthetic polymer-based AMP mimics bearing both cationic and hydrophobic groups, which can be prepared through cost-effective polymerization processes. For example, simplified polymeric AMP analogs have been developed, including poly(methacrylamides) (31), poly(β-lactams) (14, 32–35), polypeptides (36–38), poly(norbornenes) (39, 40), and poly(carbonates) (41, 42). These compounds are considerably less expensive than peptides, and much work is being done to optimize them.

Herein, we envision a fundamentally different design of AMPs with radially amphiphilic (RA) structure (Fig. 1B) rather than FA structure (Fig. 1A). This class of antimicrobial homo-polypeptides

Significance

We developed antimicrobial polypeptides (AMPs) with unprecedented radial amphiphilicity. Unlike typical AMPs characterized by facial amphiphilicity or biomimetic antimicrobial polymers with randomly distributed charged and hydrophobic groups, this class of AMPs is made up of homo-polypeptides that feature a radially amphiphilic (RA) structure and adopt a stable α-helical conformation with a hydrophobic helical core and a charged exterior shell, which is formed by flexible hydrophobic side chains with terminal charge group. The RA polypeptides appear to offer several advantages over conventional AMPs with regard to stability against proteases and simplicity of design. They also exhibit high antibacterial activity against both gram-negative and gram-positive bacteria and low hemolytic activity. This design may become a general platform for developing AMPs to treat drug-resistant bacteria.


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features a hydrophobic helical core covered with cationic groups in all radial directions of the helix. Because the charged groups form the outer shell of the polypeptides, shielding the hydrophobic helical core, these peptides should have minimal hydrophobic force induced self-aggregation and reduced interaction with blood proteins. With such RA structure, the polypeptide backbone amide bonds should also be well protected and are expected to have improved stability against proteolytic degradation compared with a typical α-helical AMP.

**Results**

**RA Polypeptide Displays High Antibacterial Activity and Selectivity.** We developed a class of helical, charged polypeptides with long hydrophobic side chains and terminal charge groups that demonstrated remarkable helical stability against changes in pH, salts, temperature, and various denaturing conditions (43–45). We adopted this architecture to design RA polypeptides and explored their applications as AMPs. PHLG-BIm (Fig. 1C) was a specific cationic α-helical polypeptide with RA structure. It was synthesized through ring-opening polymerization of amino acid N-carboxyanhydrides (NCAs) (46), followed by amination with 1-methylbenzimidazole (SI Appendix, Scheme S1). With a separation of 11 σ-bonds between the backbone and positive charges, PHLG-BIm, with DP of 40 and 28, adopts α-helical conformation. The helical conformation of polypeptides was evident from the characteristic double minima at 208 and 222 nm in the circular dichroism (CD) spectra at a concentration of 0.40 mg/mL in water (Fig. 1D). The corresponding nonhelical PHDLG-BImα0 was synthesized through the polymerization of DL-NCAs to study the effect of helical structure on the antibacterial activity of polypeptide.

Our molecular simulations provide molecular-level theoretical support for the RA structure. A simulation snapshot in Fig. 1E illustrates the cationic side chains forming a hydrophilic shell around the hydrophobic aliphatic side chains and helical core. Simulation movies are presented in Movies S1–S3. Discounting the two terminal residues to eliminate end effects, the peptide backbone is close to the ideal helix, RMSD_helix = (0.04 ± 0.01) nm, with a radius r_helix = (0.23 ± 0.02) nm and twist γ_helix = (100 ± 1)° also nearly ideal (r_helical = 0.23 nm, γ_helical = 100°). The mean per residue molar ellipticity at 222 nm computed from our simulations using Dichro-Calc (comp.chem.nottingham.ac.uk/cgi-bin/dichroccalc/bin/getparams.cgi) (47) of [θ]_222 = (−21 ± 1) × 10^3 degrees (deg) cm^2/dmol is in excellent agreement with the experimental value of −23 × 10^3 deg cm^2/dmol for PHLG-BImα0 and −19 × 10^3 deg/cm^2/dmol for PHLG-BIm28 reported in Fig. 1D. To quantify the RA structure, we computed the probability distribution of the side chain N12 atoms after aligning the peptide backbone to a reference structure and discarding the two terminal residues to eliminate end effects, the peptide backbone is close to the ideal helix, RMSD_helix = (0.04 ± 0.01) nm, with a radius r_helix = (0.23 ± 0.02) nm and twist γ_helix = (100 ± 1)° also nearly ideal (r_helical = 0.23 nm, γ_helical = 100°). The mean per residue molar ellipticity at 222 nm computed from our simulations using Dichro-Calc (comp.chem.nottingham.ac.uk/cgi-bin/dichroccalc/bin/getparams.cgi) (47) of [θ]_222 = (−21 ± 1) × 10^3 degrees (deg) cm^2/dmol is in excellent agreement with the experimental value of −23 × 10^3 deg cm^2/dmol for PHLG-BImα0 and −19 × 10^3 deg/cm^2/dmol for PHLG-BIm28 reported in Fig. 1D. To quantify the RA structure, we computed the probability distribution of the side chain N12 atoms after aligning the peptide backbone to a reference structure and discarding the four side chains at the termini to eliminate end effects (Fig. 1F and G) (48). We also measured the probability distribution of side chain lengths from the Cα to the N12 atom. The mode of the distribution is 1.24 nm, and the mean value is 1.15 nm with a 95% CI of [0.90, 1.33] nm (SI Appendix, Fig. S3). The result was further confirmed by nuclear overhauser effect (NOE) spectroscopy, with no NOE detected between protons around the nitrogen atoms on the benzimidazole (N12) (d, b’, c, c’, e) and...
protons around Cα (h, i, g) (SI Appendix, Fig. S5). Because the positive charge in the cationic side chains resides primarily in the termini, these distributions are a proxy for the positive charge distribution, providing strong support for an RA structure.

The antibacterial activity of PHLG-BIm was evaluated by the minimal inhibitory concentration (MIC) of the polypeptide against bacteria (49). With an RA structure, PHLG-BIm40 showed strong antibacterial activity against both gram-negative bacteria, DH5α and MG1655, and gram-positive bacteria, ATCC12608 and ATCC11778, with MIC values of 3.3, 26.1, 13.1, and 13.1 μM, respectively (Fig. 1E). The helical PHLG-BIm40 showed higher antibacterial activity than the nonhelical PHDLG-BIm40, with MIC values 16, 4, 4, and 4 times lower against DH5α, MG1655, ATCC12608, and ATCC11778, respectively. Length was also found to influence the antimicrobial activity of the RA polypeptides, as PHLG-BIm28, with DP of 28, showed lower antimicrobial activity compared with PHLG-BIm40. We also tested the antimicrobial activity of RA polypeptides against other bacterial strains, including clinically isolated Helicobacter pylori strains (B107, J291, J99, J99-AF, J99-A9, and J99-A11) (50, 51) and drug-resistant strains (Methicillin-resistant Staphylococcus aureus, MRSA, and ATCC12608) in the presence of different salts (physiological concentrations of 150 mM NaCl, 1 mM MgCl2, and 2.5 mM CaCl2), and mucin, the main component of mucosa (SI Appendix, Table S2). The MIC values of RA polypeptide against DH5α and ATCC12608 decreased in the presence of human serum, fetal bovine serum (FBS), plasma, and artificial tears in comparison with RA polypeptide-only treatment. The results demonstrate that the RA polypeptides are stable in serum and plasma, and polyanionic compounds do not dramatically affect their antimicrobial activity. The decreased MIC in serum and plasma may be attributed to the serum complement system, which provides innate defense against microbial infections. PHLG-BIm40 exhibited low hemolytic activity with an HC50 (50% hemolytic concentration) value higher than 104.6 μM, which indicates a high selectivity of >32 (defined as HC50/MIC), as opposed to a selectivity of >2 for PHDLG-BIm40 against DH5α bacteria. It is important to point out that the RA polypeptide is not just bacteriostatic but bactericidal. Nearly 100% killing of all four bacterial species was observed at their respective MIC or double MIC within 2 h (SI Appendix, Fig. S6). In addition, the RA polypeptides showed concentration-dependent antimicrobial killing in medium and in conditions with NaCl, human serum, plasma, and artificial tears (SI Appendix, Fig. S7).

Although many AMPs with high antibacterial activity have been developed, the application of AMPs is usually limited by the short durations of activity due to their rapid digestion by endogenous proteases (5, 16, 17, 52, 53). The RA polypeptides, with densely packed hydrophobic side chains forming a hydrophobic cortex that can protect the amide bonds of the polypeptide backbone, in principle should be more stable against proteolysis compared with typical AMPs. We incubated PHLG-BIm40 with trypsin, pronase, and elastase from Staphylococcus aureus, DH5α and artificial tears (SI Appendix, Fig. S8). Furthermore, after 8 h of protease or tryptase treatment, the antibacterial activity of PHLG-BIm40 remained unchanged, as demonstrated by having the same MIC value against DH5α as the untreated RA polypeptide (3.3 μM).

RA Polypeptide Kills Bacteria by Directly Disrupting the Bacterial Cell Membrane. We find that the RA PHLG-BIm kills bacteria by directly disrupting the bacterial cell membrane in a manner similar to typical AMPs, through vesicle leakage, bacterial membrane permeabilization, and bacteria morphology assays. We first investigated the membrane-disruptive activity of helical PHLG-BIm40 and nonhelical PHDLG-BIm40 polypeptides on anionic liposomes 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (DOPPE1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPE) and neutral liposomes 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), which were used to model phosphatidylethanolamine (PE)-rich bacteria and eukaryotic cell membranes, respectively. At the same concentration, PHLG-BIm40 induced greater dye leakage from both anionic and neutral liposomes than PHDLG-BIm40, suggesting that the helical polypeptide has higher membrane disruption capability (Fig. 2A). PHLG-BIm40 also caused more leakage from the anionic liposomes than the neutral liposomes, which is well-correlated with the observed selectivity against bacterial over mammalian cells. The leakage results also showed the capability of PHLG-BIm to permeabilize model bacteria membranes rich in negative intrinsic curvature-forming lipids. We next used flow cytometry to evaluate permeabilization of PHLG-Bim through bacterial membranes. MG1655 bacteria were incubated with PHLG-BIm40 and propidium iodide (PI), a membrane impermeable dye. The total number of PI-containing bacteria cells was greater for those treated with

![Fig. 2. PHLG-BIm40 kills bacteria by directly disrupting the bacterial cell membrane. (A) Extent of calcein efflux in neutral vesicles (DOPC/POPC) and negatively charged vesicles (DOPE/DOPE) after treatment with PHDLG-BIm40 (nonhelical, lacking radially amphiphilic structure) or PHLG-BIm40 (helical with radially amphiphilic structure) at various concentrations for 1 h. (B) Flow cytometry analysis of propidium iodide (PI) uptake after incubation with free PI, PI with PHDLG-BIm40, or PI with PHLG-BIm40 at various concentrations. All of the data are represented as average ± SD and analyzed by Student t test (**p ≤ 0.01). (C) The fluorescence microscopy of stained Escherichia coli MG1655 in the absence and presence of PHDLG-BIm40 and PHLG-BIm40 (3.3 μM). (Scale bar, 50 μM.) (D) SEM images of MG1655 after treatment with PBS, PHDLG-BIm40 or PHLG-BIm40 (Scale bar, 1 μM.)

A | B | C | D
---|---|---|---
Control | PHDLG-BIm40 | PHLG-BIm40 | Control | PHDLG-BIm40 | PHLG-BIm40  
Dye leakage (%) | % of PI positive cells |
0 | 0 | Control | PHDLG-BIm40 | PHLG-BIm40  
20 | 50 | Control | PHDLG-BIm40 | PHLG-BIm40  
40 | 90 | Control | PHDLG-BIm40 | PHLG-BIm40  
60 | 100 | Control | PHDLG-BIm40 | PHLG-BIm40  
80 | Control | PHDLG-BIm40 | PHLG-BIm40  
100 | Control | PHDLG-BIm40 | PHLG-BIm40  

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PHLG-BIm$_{40}$ than PHDLG-BIm$_{40}$ and increased with higher concentration of RA polypeptide (Fig. 2B). An uptake study analyzed by fluorescence imaging provided additional evidence of enhanced membrane activity of PHLG-BIm$_{40}$, which permeabilized MG1655 bacterial cell membranes more effectively than PHDLG-BIm$_{40}$ when the bacterial cells were coincubated with polypeptide and dye (PI and SYTO9) (Fig. 2C). Using scanning electron microscopy, we observed drastic changes and damage of the bacterial membranes after incubation with PHLG-BIm$_{40}$, whereas PHDLG-BIm$_{40}$ minimally affected bacteria morphology (Fig. 2D). Taken together, the results indicate that membrane disruption and permeation are an important component of the antimicrobial activity of PHLG-BIm.

To examine in detail the root causes of selective membrane activity for RA polypeptides, we use synchrotron small angle X-ray scattering (SAXS) to investigate the type and quantity of membrane curvature deformations induced by PHLG-BIm. Small unilamellar vesicles (SU) were prepared with lipid compositions representative of bacterial (DOPG/DOPE 20/80) and eukaryotic (DOPS/DOPC = 20/80) membranes. Compositions DOPG/DOPE/ DOPC = 20/60/20 and 20/40/40 were also used as model systems to isolate the role of negative intrinsic curvature lipids such as PE, because eukaryotic membranes are known to have lower PE content relative to bacterial membranes. The SUVs were incubated with PHLG-BIm$_{40}$ at a peptide/lipid (PL) molar ratio of 1/400, which is equivalent to a charge ratio of 1/2, and the resulting structures were characterized using SAXS. Synchrotron SAXS spectra from the lipid vesicle solutions showed a broad characteristic feature consistent with a single lipid bilayer form factor of unilamellar vesicles. When exposed to PHLG-BIm$_{40}$, the lipid vesicles undergo a structural transition, resulting in correlation peaks with specific ratios of Q values in the diffraction data (Fig. 3).

For each membrane composition, we found a set of correlation peaks having integral Q-ratios of 1/2 consistent with a lamellar (L$_{α}$) phase, characterized by d-spacings of 6.7–8.8 nm. These lamellar phases resulting from exposure of PHLG-BIm to the SUVs indicate intermembrane attraction without the generation of significant curvature. Interestingly, for the model bacterial membrane composition, we identified a second set of correlation peaks with characteristic Q-ratios of $\sqrt{2}, \sqrt{3}, \sqrt{4}, \sqrt{6}$, which indexed to a cubic (Q2) $Pn3m$ “double-diamond” lattice having a lattice parameter of 24.8 nm. Bicontinuous cubic phases, such as the $Pn3m$, consist of two nonintersecting water channels that are separated by a lipid bilayer. The center of this bilayer traces out a minimum surface characterized by negative Gaussian curvature (NGC), also known as saddle-splay curvature, at every point. Our SAXS data showed that PHLG-BIm promotes saddle-shaped membrane deformations in model bacteria membranes to stabilize a bulk $Pn3m$ cubic phase.

NGC is the saddle-shaped curvature that manifests along the inside of pores, around the base of a bleb, and the neck of a bud, the basic membrane permeation mechanisms. Earlier studies have found a strong correlation between the formation of cubic phases and membrane permeation induced by AMPs (1, 14, 54). The ability of PHLG-BIm to generate cubic phases in bacteria-like PE-rich membranes suggests that the RA polypeptide may permeate bacteria membranes via the induction of NGC consistent with that of natural AMPs. By systematically examining a range of membranes, we determined how lipid composition affects the ability of PHLG-BIm to restructure vesicles. We observed the general trend of decreasing PE content resulting in the suppression of nonlamellar phase formation. More specifically, we found that the peptide does not disrupt membranes (no NGC generation) with a PE content of 60% and lower, which includes those representative of eukaryotes. The preference for PHLG-BIm to generate cubic phases at high PE levels of ~80% suggests a mechanism of selectivity for bacterial over animal membranes based on their specific lipid distributions, again consistent with generic AMP trends.

RA Polypeptide Enhances the Antibacterial Effects of Traditional Antibiotics. Because PHLG-BIm can cause membrane disruption and permeabilization, we explored its potential to enhance the antibacterial effect of traditional antibiotics. Synergistic-like enhancement of bacteria killing has been previously reported from coadministration of membrane-disruptive agents with commercial antibiotics (41, 55). We selected four antibiotics: streptomycin (aminoglycoside), doxycycline (tetracycline), rifampicin (rifamycin), and gentamicin (aminoglycoside). Streptomycin, doxycycline, and gentamicin are protein synthesis inhibitors, whereas rifampicin inhibits DNA-dependent RNA synthesis. We tested the antibacterial activity of these antibiotics coadministered with helical PHLG-BIm$_{40}$. Nonhelical PHDLG-BIm$_{40}$ served as the control. Bacteria were incubated with either antibiotic alone, antibiotic coadministered with PHDLG-BIm$_{40}$, or antibiotic coadministered with PHLG-BIm$_{40}$ at varying concentrations. For MG1655 bacteria, coadministration of streptomycin with PHLG-BIm$_{40}$ resulted in MIC values identical to those of streptomycin alone (Fig. 4A). However, when streptomycin was coadministered with PHLG-BIm$_{40}$ at concentrations of 1.6, 3.3, and 6.5 μM, the respective MIC values against MG1655 were 2, 133, and 400 times lower than that of streptomycin alone. Because the MIC against MG1655 with PHLG-BIm$_{40}$ alone was 26.1 μM, this result suggested a synergistic effect from combining treatments of streptomycin and PHLG-BIm$_{40}$. We also observed a similar synergistic effect of PHLG-BIm$_{40}$ and antibiotics against C101 (P. aeruginosa), a bacterium with high resistance to many antibiotics. For C101 bacteria, the MIC of PHLG-BIm$_{40}$ alone was 52.3 μM. A synergistic effect when streptomycin was coadministered with PHLG-BIm$_{40}$ (15.1 μM) was indicated by an MIC that was 128 times lower than that of streptomycin alone (Fig. 4B).

The synergistic effect of combination therapy was also detected against three other bacterial strains, DH5α, ATCC11778, and ATCC12608 (SI Appendix, Table S3) and for three other antibiotics: doxycycline, rifampicin, and gentamicin (SI Appendix, Table S4). The synergistic effect of combination therapy may be a result of enhanced antibiotic uptake due to RA polypeptide-induced membrane permeation. To test this hypothesis, bacterial

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**Fig. 3.** (A) SAXS spectra show generation of NGC by PHLG-BIm$_{40}$ in membranes rich in PE. SAXS profiles from lipid vesicle solutions after exposure to PHLG-BIm$_{40}$ at molar ratio PL = 1/400. SUV-only controls at each composition show a broad characteristic feature consistent with the form factor of the unilamellar vesicles. (B) PHLG-BIm induces a $Pn3m$ cubic phase. The measured Q positions of the diffusion peaks were plotted to show indexing of the $Pn3m$ cubic phase for DOPG/DOPE = 20/80.
cells were incubated with rifampicin without or with RA polypeptides. After 0.5- or 1-h incubation with RA polypeptide, we observed intracellular uptake of rifampicin in MG1655 and C101 cells (SI Appendix, Fig. S9). These findings suggest that the synergy from coadministering antibiotics with RA polypeptides may potentially result from increased antibiotic uptake, such as observed for peptide-induced defects, or suppressed efflux activities. Our study showed combination therapy to be a promising application for this class of membrane-active RA polypeptides, which can significantly improve the effectiveness of traditional commercial antibiotics by killing bacteria through a distinct mode of action.

Discussion

We developed a class of cationic, helical antimicrobial homo-polypeptides with unprecedented radial amphiphility. Unlike traditional AMPs characterized by facial amphiphilicity or biomimetic antimicrobial polymers with randomly distributed charged and hydrophobic groups, these AMPs are homopolypeptides with RA structure. They adopt a stable α-helical conformation with a hydrophobic helical core and a charged exterior shell, formed by long hydrophobic side chains with terminal charge group. The RA polypeptides offer various advantages over typical AMPs. They can be easily synthesized through controllable NCA polymerization followed by side chain modification. The RA structure enables the polypeptides to bind effectively to the negatively charged bacterial surface, and exhibit high antimicrobial activity against both gram-positive and gram-negative bacteria. Moreover, the shielding of the hydrophobic core by the charged exterior shell decreases nonspecific interactions with eukaryotic cells and contributes to low mammalian cytotoxicity. The RA polypeptides also demonstrate excellent stability against enzymatic degradation, potentially due to suppressed protease access to the polypeptide backbone. In addition, the antibacterial and hemolytic activities of the RA polypeptides can be tuned by varying the terminal amine group addition, the antibacterial and hemolytic activities of the RA polypeptides also demonstrate excellent stability against enzymatic degradation, potentially due to the charged exterior shell, formed by long hydrophobic side chains with terminal charge group.

The antibacterial activity of these RA polypeptides likely results from electrostatic interactions between their cationic groups and anionic bacteria cell membranes, followed by the disruption of the bacteria cell membranes by the membrane-active polypeptide helix. Additional factors are involved in bacterial membrane destabilization, as suggested by our SAXS studies on model bacterial membranes in which helical polypeptide PHLG-BIm promoted saddle-shaped membrane deformations (NGC), a topological requirement for membrane destabilizing events such as pore formation. All tested model membranes had a fixed anionic charge, yet we observed selective generation of NGC in the PE-rich bacteria model membrane. This result is consistent with previous work, which showed that membranes with greater amounts of negative intrinsic curvature lipids promote destabilization and are more susceptible to pore formation (2). Moreover, both anionic and negative intrinsic curvature lipids are necessary for activity, whereas neither alone is sufficient. Therefore, the preference for the helical RA polypeptides to generate NGC at high PE content points to a mechanism of selectivity that involves membrane curvature effects.

The observed synergistic bacterial effect may result from increased cellular penetration of antibiotics that is facilitated by the membrane permeabilization activity of the RA polypeptides. Induced NGC is broadly enabling in the context of membrane permeation mechanisms, such as transmembrane pores, blebbing, budding, and scission. It is likely that permeation can involve a hierarchy of mechanisms. We observe NGC in the form of a cubic phase; the size of the defect on a 2D membrane will depend on the type of defect it is. The Peptide cubic phase induced by the RA polypeptide PHLG-BIm at 0.2 μM has an average Gaussian curvature (K) value of ~0.01065 nm⁻². For example, this is the amount of NGC found in a transmembrane pore with a size of ~40 nm, if we ignore all other effects. If on the other hand, the destabilization mechanism is that of a budding event followed by scission, then we can estimate the size of the defect using a catenoid surface, which is an approximate representation of the surface of a scission pore (56, 57). The Gaussian curvature of a minimal catenoid surface with a neck radius of c along its z axis is defined by K(z) = −(sech²(z))/c². The measured curvature value corresponds to a catenoid neck diameter of ~15.4 nm. If we account for the approximate bilayer membrane thickness of 4 nm, this diameter translates to a pore size of ~15.4 nm. In both of the above cases, the size of the defect is significantly larger than the size of a typical antibiotic.

In conclusion, designing AMPs bearing the unique RA structure represents an alternative direction of AMP development; the RA polypeptide may become a general platform for developing AMPs to treat drug-resistant bacteria. Although outside the scope of this investigation, specifics pertaining to the mechanism and selectivity of these polypeptides can aid in the design and optimization of future synthetic AMPs based on a RA structure. We suggest further studies on this class of polypeptides using ellipsometry, electrochemistry, NMR, and atomic force microscopy to examine these defects (58–61). Future studies to determine the toxicity of the degradation products will also be necessary to assess the potential applicability of the peptide as a clinical therapeutic.

Materials and Methods

PHLG-Bim was synthesized by mixing PCHLG, NaI, and 1-methylbenzimidazole in DMF and acetonitrile in a 25-mL Schlenk tube. The mixture was stirred at 80 °C for 48 h. Molecular dynamics simulations of PHLG-Bim with a DP of 20 follow a procedure similar to that we previously detailed in ref. 62. Details describing preparation and characterization of PHLG-Bim; simulation methods; antibacterial assays and hemolytic assay; and small-angle X-ray scattering experiments can be found in SI Appendix.

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Supporting Information

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**Movie S1.** Movie of the 68-ns production simulation with frames rendered every 150 ps. For clarity of viewing, the peptide backbones in each configuration have been mutually aligned to a single reference configuration and water molecules removed. The peptide backbone is colored in red and the side chains in gray. This movie was produced using VMD (14).
Movie S2. A 360° rotation around a representative peptide configuration. For clarity of viewing, the water molecules have been removed and the backbone and side chains colored in red and gray, respectively. This movie was produced using VMD (14).

Movie S2
**Movie S3.** Fly-around of the 3D probability distribution of the side chain N$_{12}$ atoms around the $\alpha$-helical backbone. Histograms were compiled over the simulation trajectory using a grid comprising cubic cells of size 0.1 x 0.1 x 0.1 nm, and the probability density function computed by normalizing by the total count and the cell volume. The value of the probability density in any one cell ranged from 0 to 3.7 nm$^{-3}$. Four contours in the probability density are plotted at 0.05, 0.1, 0.2, and 0.3 nm$^{-3}$. Because the positive charge in the cationic side chains resides primarily in the termini, this distribution may be considered a proxy for that of the positive charge. This movie was produced using MATLAB (15).

**Movie S3**

**Other Supporting Information Files**

*SI Appendix (PDF)*
Supporting Information

Helical Antimicrobial Polypeptides with Radial Amphiphilicity

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Experimental section:

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. Anhydrous dimethylformamide (DMF), ethyl acetate (EtOAc), and hexane were dried by passing them through alumina columns and kept anhydrous by storing them in the presence of molecular sieves in a glove box. Hexamethyldisilazane (HMDS) was dissolved in DMF in a glovebox. SiliaFlash P60 silica gel (particle size 40-63 μm) was purchased from SiliCycle Inc. (Quebec City, Quebec, Canada) and heated to 150 °C for 48 h before use. Spectra/Por® dialysis tubing with a molecular weight cut-off (MWCO) of 1 kDa was purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). Gram-negative bacteria, DH5α (Escherichia coli), MG1655 (E. coli), C101 (Pseudomonas aeruginosa), and Gram-positive bacteria, ATCC12608 (Staphylococcus aureus) and ATCC11778 (Bacillus toyonensis) were grown in luria broth (LB) medium at 37 °C. Clinical isolated Helicobacter pylori strains, B107, J291, J99, J99-AF, J99-A9 and J99-A11, supplied by Dr. Peek and Dr. Chen, were incubated in brucella broth (BB) with 10% fetal bovine serum (FBS) supplemented with vancomycin (5 μg/mL) at 37 °C. Among them, J99-AF, J99-A9 and J99-A11 are drug resistant bacteria. All lipids were obtained from Avanti Polar Lipids, Inc. Propidium iodide (PI) and BacLight™ Kit L-7012 was purchased from Thermo Fisher Scientific Inc. Artificial tears were obtained from Boss Safety Products. Pseudomonas Aeruginosa Elastase was purchased from Elastin Products Company, Inc. LL-37, LLGDFRFRKSKKEKIGKEFKRIVQRIKDRLNLVPRTES, was obtained from
AnaSpec, Inc. Elastase from human leukocytes, plasma from human, human serum was obtained from Sigma-Aldrich.

**Characterization.** $^1$H NMR spectra were recorded on a Varian U500 MHz or a VXR-500 MHz spectrometer. $^{13}$C-$^1$H HSQC (heteronuclear single quantum coherence) NMR, $^1$H-$^1$H TOCSY (total correlation spectroscopy) NMR, and NOESY (nuclear overhauser effect spectroscopy, the mixing time is 150 ms) NMR were recorded on a VNS750NB spectrometer. Chemical shifts were reported in ppm and referenced to the solvent proton impurities. The molecular weights of prepared polypeptides were determined by gel permeation chromatography (GPC) equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS multi-angle laser light scattering detector (MALLS) detector (Wyatt Technology, Santa Barbara, CA, USA), and an OptilabrEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The detection wavelength of HELEOS was set at 658 nm. Separations were performed using serially connected size exclusion columns ($100 \text{"Å}, 500 \text{"Å}, 10^3\text{"Å} and 10^4\text{"Å}$ Phenogel columns, 5 µm, 300 × 7.8 mm, Phenomenex, Torrance, CA, USA) at 60 °C using DMF containing 0.1 mol/L LiBr as the mobile phase. The MALLS detector was calibrated using pure toluene and can be used for determination of the absolute molecular weights (MWs). The MWs of polymers were determined based on the dn/dc value of each polymer sample calculated offline by using the internal calibration system processed by the ASTRA V software (version 5.1.7.3, Wyatt Technology, Santa Barbara, CA, USA).
Circular dichroism (CD) measurements were carried out on a JASCO J-815 CD spectrometer. The polypeptides samples were prepared at concentrations of 0.40 mg/mL in water, and placed in a quartz cell with a pathlength of 0.10 cm prior to measurements. Infrared spectra were recorded on a Perkin Elmer 100 serial FTIR spectrophotometer calibrated with polystyrene film. Lyophilization was conducted in a FreeZone lyophilizer (Labconco, Kansas City, MO, USA). HPLC analyses were performed on a Shimadzu CBM-20A system (Shimadzu, Kyoto, Japan) equipped with SPD20A PDA detector (190 nm-800 nm) and RF10Axl fluorescence detector, and an analytical C18 column (Shimadzu, 3 µm, 50*4.6 mm, Kyoto, Japan).

**Synthesis of γ-(6-chlorohexyl)-L-glutamate (CH-L-Glu).** L-Glutamic acid (10.0 g, 68.0 mmol) and 6-chlorohexanol (15 mL, 112.4 mmol) were mixed and stirred at 0 °C, followed by the dropwise addition of H₂SO₄ (4 mL). The reaction was allowed to warm up to the room temperature and kept stirred for 24 h. Saturated Na₂CO₃ solution was then added to adjust the pH value to 7. The resulting precipitate was collected by filtration and purified by recrystallization from isopropanol/H₂O (1:1, v/v). Isopropanol was removed under vacuum and water was removed via lyophilization to obtain a white solid powder (6.92 g, yield: 38%). ¹H NMR (DMSO:DCI-D₂O, 9:1, v/v): δ 3.91 (t, 2H, -CH₂OOC-), 3.82 (t, 1H, α-H), 3.52 (t, 2H, -CH₂Cl), 2.53-2.32 (m, 2H, -CH₂CH₂COO-), 1.98 (m, 2H, -CH₂CH₂COO-), 1.64-1.17 (m, 8H, ClCH₂(CH₂)₄CH₂O-).
CH-DL-Glu was synthesized similarly using DL-glutamic acid as the starting material (Yield: 36 %). $^1$H NMR (DMSO-DCl-D$_2$O, 9:1, v/v): 3.91 (t, 2H, -CH$_2$OOC-), 3.82 (t, 1H, α-H), 3.52 (t, 2H, -CH$_2$Cl), 2.53-2.32 (m, 2H, -CH$_2$CH$_2$COO-), 1.98 (m, 2H, -CH$_2$CH$_2$COO-), 1.64-1.17 (m, 8H, ClCH$_2$(CH$_2$)$_4$CH$_2$O-).

Synthesis of CH-L-Glu based N-carboxyanhydride (CH-L-Glu-NCA). A round-bottomed flask (100 mL) was charged with CH-L-Glu (7.3 g, 27.4 mmol) and dried under vacuum for 2 h. Anhydrous tetrahydrofuran (THF, 60 mL) and phosgene (15wt% in toluene, 39.2 mL, 54.9mmol) were added successively with the protection of nitrogen. The mixture was stirred at 50 °C for 2 h. The solvent was removed under vacuum to yield an oily liquid. The product was purified by silica gel column chromatography using EtOAC/hexane (from 100 % to 60 % hexanes) as the eluent (6.6 g, yield: 83 %). $^1$H NMR (CDCl$_3$, ppm): δ 7.16 (s, 1H, -NH), 4.43 (t, 1H, -CHNH), 4.05 (t, 2H, -CH$_2$OOC-), 3.51 (t, 2H, -CH$_2$Cl), 2.51 (t, 2H, -CH$_2$CH$_2$COO-), 2.25-2.07 (m, 2H, -CH$_2$CH$_2$COO-), 1.79-1.30 (m, 8H, ClCH$_2$(CH$_2$)$_4$CH$_2$O-). $^{13}$C NMR (CDCl$_3$, ppm): δ 172.8, 170.0, 152.6, 65.4, 57.1, 45.2, 32.6, 29.7, 28.5, 27.0, 26.6, 25.4.

CH-DL-Glu-NCA was synthesized similarly using CH-DL-Glu as the starting material (Yield: 79 %). $^1$H NMR (CDCl$_3$, ppm): δ 7.31 (s, 1H, -NH), 4.41 (s, 1H, -CHNH), 3.99 (s, 2H, -CH$_2$OOC-), 3.45 (s, 2H, -CH$_2$Cl), 2.45 (s, 2H, -CH$_2$CH$_2$COO-), 2.10 (d, 2H, -CH$_2$CH$_2$COO-), 1.75-1.22 (m, 8H, ClCH$_2$(CH$_2$)$_4$CH$_2$O-). $^{13}$C NMR (CDCl$_3$, ppm): δ
Synthesis of poly(γ-6-chlorohexyl-L-glutamate) (PCHLG). In a glovebox, CH-L-Glu NCA (100 mg, 0.34 mmol) was dissolved in DMF (1.5 mL), followed by the addition of 85 µL or 136 µL HMDS (0.1 M) in DMF to obtain PCHLG$_{40}$ and PCHLG$_{28}$. The mixture was stirred at room temperature for 48 h. The polymer was then obtained by precipitation in cold methanol and dried under vacuum at 40 °C for 8 h (Yield: PCHLG$_{40}$: 66 %, PCHLG$_{28}$: 56 %). $^1$H NMR (CDCl$_3$-TFA-d, 85:15, v/v): DP=40: δ 4.60 (m, 1H, -CHNH), 4.09 (m, 2H, -CH$_2$OOC-), 3.52 (t, 2H, -CH$_2$Cl), 2.50 (m, 2H, -CH$_2$CH$_2$COO-), 2.19-1.90 (m, 2H, -CH$_2$CH$_2$COO-), 1.81-1.30 (m, 8H, ClCH$_2$(CH$_2$)$_4$CH$_2$O-). DP=25 δ 4.60 (m, 1H, -CHNH), 4.09 (m, 2H, -CH$_2$OOC-), 3.52 (t, 2H, -CH$_2$Cl), 2.50 (m, 2H, -CH$_2$CH$_2$COO-), 2.19-1.90 (m, 2H, -CH$_2$CH$_2$COO-), 1.81-1.30 (m, 8H, ClCH$_2$(CH$_2$)$_4$CH$_2$O-).

PCHDLG$_{40}$ was synthesized similarly using CH-DL-Glu-NCA as the monomer (M/I=40) (Yield: 72 %). $^1$H NMR (CDCl$_3$-TFA-d, 85:15, v/v): δ 4.60 (m, 1H, -CHNH), 4.10 (m, 2H, -CH$_2$OOC-), 3.52 (t, 2H, -CH$_2$Cl), 2.48 (s, 2H, -CH$_2$CH$_2$COO-), 2.28-1.90 (m, 2H, -CH$_2$CH$_2$COO-), 1.81-1.30 (m, 8H, ClCH$_2$(CH$_2$)$_4$CH$_2$O-).

Synthesis of PHLG-BIm polypeptides. PCHLG$_{40}$ (86.5 mg, 0.35 mmol of Cl groups) in DMF (2 mL) and NaI (157 mg, 1.05 mmol) in acetonitrile (2 mL) was mixed and added to 1-methylbenzimidazole (92.4 mg, 0.70 mmol) in a 25 mL Schlenk tube. The mixture
was stirred at 80 °C for 48 h. After most solvent was removed under vacuum, NaCl aqueous solution (1.0 M, 3 mL) was added. The solution was then stirred at room temperature for 3 h to promote ion exchange. The product was purified by dialysis (MWCO = 1 kDa) against distilled water for 3 days. White solid powder was obtained after lyophilization (yield: 62%). $^1$H NMR (TFA-d): δ 9.02 (s, 1H, -NCHN-), 7.86 (m, 4H, Ar-H), 4.86 (s, 1H, α-H), 4.36 (m, 2H, -COOCH$_2$(CH$_2$)$_4$CH$_2$N-), 4.29 (s, 2H, -COOCH$_2$(CH$_2$)$_4$CH$_2$N-), 4.24 (s, 3H, -NCH$_3$), 2.72 (s, 2H, -CH$_2$CH$_2$COO-), 2.46-1.75 (m, 6H, -CH$_2$CH$_2$COO- and -COOCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$N-), 1.59 (s, 4H, -COOCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$N-).

PHDLG-$\text{BIm}_{40}$ was synthesized similarly using PCHDLG$_{40}$ as the starting material. $^1$H NMR (D$_2$O): δ 7.71 (s, 1H, -NCHN-), 7.52 (m, 4H, Ar-H), 4.66 (s, 1H, α-H), 4.36 (m, 2H, -COOCH$_2$(CH$_2$)$_4$CH$_2$N-), 3.89 (s, 3H, -NCH$_3$), 3.74 (s, 2H, -COOCH$_2$(CH$_2$)$_4$CH$_2$N-), 2.32 (s, 2H, -CH$_2$CH$_2$COO-), 2.16-1.75 (m, 4H, -CH$_2$CH$_2$COO- and -COOCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$N-), 1.49-0.89 (s, 6H, -COOCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$N-).

PHLG-$\text{BIm}_{28}$ was synthesized similarly using PCHLG$_{28}$ as the starting material. $^1$H NMR (TFA-d): δ 9.02 (s, 1H, -NCHN-), 7.86 (m, 4H, Ar-H), 4.86 (s, 1H, α-H), 4.36 (m, 2H, -COOCH$_2$(CH$_2$)$_4$CH$_2$N-), 4.29 (s, 2H, -COOCH$_2$(CH$_2$)$_4$CH$_2$N-), 4.24 (s, 3H, -NCH$_3$), 2.72 (s, 2H, -CH$_2$CH$_2$COO-), 2.46-1.75 (m, 6H, -CH$_2$CH$_2$COO- and
Simulation Methods. We conducted molecular dynamics simulations of PHLG-BIm with a DP of 20 following a procedure similar to that we previously detailed in Ref. (1). The non-natural peptide side chains were constructed using the Automated Topology Builder (ATB) server (http://compbio.biosci.uq.edu.au/atb/) (2,3,4) to define the structure, partial charges, and bonded and non-bonded interactions within the GROMOS 54A7 force field (5). The peptide backbone was initialized in a $\alpha$-helical conformation with the assistance of the Bax Group PDB Utility Server (http://spin.niddk.nih.gov/bax/nmrserver/pdbutil). An in-house code was used to graft the side chains to the backbone to synthesize the initial peptide structure. The peptide was prepared as a zwitterion, and the terminal benzimidazole groups' protonated, such that the peptide carries a net formal charge of (+20). The peptide was placed in a $9 \times 9 \times 9$ nm cubic simulation box with periodic boundary conditions, and solvated by SPC water molecules (6) to a density of 1.0 g/cm$^3$ along with 20 Cl$^-$ counter ions such that the system carried no net charge. The size of the simulation box was specified such that with a 1.2 nm real-space cutoff each solvent molecule was able to interact with at most one periodic image of the peptide, even in its fully extended conformation.

Molecular dynamics simulations were conducted using the GROMACS 4.6 simulation
suite (7). High-energy overlaps in the initial configuration were eliminated by steepest
descent energy minimization to remove forces exceeding 1000 kJ/mol.nm. Simulations
were performed in the NPT ensemble at 298 K and 1 bar, employing a Nosé-Hoover
thermostat (8) and Parrinello-Rahman barostat (9). Initial atom velocities were randomly
assigned from a Maxwell distribution at 298 K. The equations of motion were
numerically integrated using a leap-frog algorithm (10) with a 2 fs time step, and bond
lengths fixed using the LINCS algorithm to improve efficiency (11). Electrostatic
interactions were treated using Particle Mesh Ewald (PME) with a real-space cutoff of 1.2
nm and a 0.12 nm Fourier grid spacing (12). Lennard-Jones interactions were shifted
smoothly to zero at a 1.2 nm, and Lorentz-Berthelot combining rules used to determine
interaction parameters between unlike atoms (13). A 1.5 ns equilibration run was
conducted, at which time the temperature, pressure, energy, and peptide radius of gyration
had attained stable values. We then performed a 68 ns production run, harvesting
snapshots of the system configuration for analysis every 5 ps. The peptide remained fully
alpha-helical over the entire course of the simulation trajectory. We present in the
Supplementary Information a movie of the simulation trajectory showing the dynamical
evolution of the side chains around the α-helical core (Movie S1), and a 360° rotation
around a representative peptide configuration (Movie S2).

**Minimal inhibition concentration (MIC).** Gram-negative bacteria, DH5α (*E. coli*),
MG1655 (E. coli), C101 (Pseudomonas aeruginosa), and Gram-positive bacteria, ATCC12608 (S. aureus), ATCC11778 (Bacillus toyonensis), and methicillin-resistant S. aureus (MRSA) strains, NRS382, NRS383, NRS384 were grown in LB medium at 37 °C. Clinical isolated Helicobacter pylori strains, B107, J291, J99, J99-AF, J99-A9 and J99-A11 were incubated in BB with 10% FBS supplemented with vancomycin (5 µg/mL) at 37 °C. Among them, J99-AF, J99-A9 and J99-A11 are drug resistant bacteria. For determination of the MIC, polypeptides were dissolved in media using serial dilutions from a stock solution. Into each well of a 96-well plate was added 200 µL of each concentration and 2 µL of bacteria (1 × 10^8 CFU (colony forming units)) in medium. The plate was incubated at 37 °C. The optical density readings of microorganism solutions were measured after 24 h incubation. The MIC was considered as the lowest concentration of peptide where no visual growth of bacteria was detected.

The stability of polypeptide was tested in the MIC assay in different environments, including salts, serum, plasma, tear fluid, and mucin. 1 ×10^6 CFU/ml of E. coli DH5α and S. aureus ATCC12608 were treated with peptides, while different conditions were added to LB medium, and the final concentrations of physiological conditions were as follows: 150 mM NaCl, 1 mM MgCl₂, 2.5mM CaCl₂, 2% human serum, 5% FBS, 10% FBS, 2% plasma, 5% plasma, 2% artificial tear (from Boss Safety Products) and 1 mg/mL mucin. After these treatments, the procedures were same as MIC assay described above.
**Killing kinetics.** The killing kinetics and killing efficiency of PHLG-BIm were measured against the microbes by counting the colony forming units of live bacteria with agar plating. The bacteria were prepared using the same procedure described in the MIC measurement. The samples were treated with PHLG-BIm$_{40}$ at MIC or double of the MIC and incubated at 37 °C under constant shaking (100 rpm). Samples were taken for a series of ten-fold dilutions, and plated out in LB agar plates at predetermined time intervals (1 h, 2 h, 8h and 24h). The plates were incubated over night at 37 °C and the bacteria were counted by CFU. The bacteria (DH5α, ATCC12608) were also incubated with PHLG-BIm$_{40}$ at various concentrations without or with NaCl (150 mM), 2% human serum, 2% plasma, 2% artificial tear. The bacteria were prepared using the same procedure described in the MIC measurement. After 8 h incubation, Samples were taken for a series of ten-fold dilutions, and plated out in LB agar plates, and the bacteria were counted by CFU after overnight incubation at 37 °C.

**Hemolytic assay.** Fresh rabbit blood was obtained and subjected to 25-fold dilution with PBS buffer to reach a concentration of approximately 4% (in volume) of the blood cells. 300 μL of PBS solution containing a polymer at various concentrations was placed in a 1.5 mL microfuge tube, followed by the addition of an equal volume (300 μL) of red blood cell suspension. The mixture was incubated at 37 °C for 1 h to allow for the hemolysis process to take place. At the end of the incubation time, the non-hemolysed red blood cells were separated by centrifugation at 1000 rpm for 5 min. Aliquots (100 μL) of
the supernatant were transferred to a 96-well plate, and hemoglobin release was measured by UV-absorbance at 576 nm using a microplate reader (TECAN, Switzerland). Two controls were provided in this assay: an untreated red blood cell suspension in PBS solution was used as the negative control; a solution containing red blood cells lysed with 1% Triton-X was used as the positive control. Percentage of hemolysis was calculated using the following formula: Hemolysis (%) = [(O.D. 576 nm of the treated sample-O.D. 576 nm of the negative control)/(O.D. 576 nm of positive control- O.D. 576 nm of negative control)]×100%.

The stability of polypeptides against protease. PHLG-BIm (1.0 mg/mL) was incubated with trypsin (1.0 mg/mL) or pronase (0.12 mg/mL) or Pseudomonas Aeruginosa elastase (6.25 mg/L) in Tris buffer (pH 7.4) at 37 °C. PHLG-BIm (1.0 mg/mL) was incubated with elastase from human leukocytes (25 mg/L) in sodium acetate buffer (0.05 M, pH 5.5, with 0.6 M NaCl) at 37 °C. As a positive control, LL-37 (0.1 mg/mL) was also incubated with trypsin (0.1 mg/mL) or pronase (12 mg/L) or Pseudomonas Aeruginosa elastase (0.6 mg/L) in Tris buffer (pH 7.4) at 37 °C. LL-37 (0.1 mg/mL) was incubated with elastase from human leukocytes (2.5 mg/L) in sodium acetate buffer at 37 °C. After 8 h of incubation, the samples were taken out for HPLC analyses. In a separate experiment, PHLG-BIm (1.0 mg/mL) was incubated with trypsin (1.0 mg/mL) or pronase (0.12 mg/mL) in Tris buffer (pH 7.4) at 37 °C for 8 h and MIC measurement was conducted.
Flow cytometry analysis of pore-forming activities. For membrane permeability assay, 1 × 10^6 CFU MG1655 cells were combined with propidium iodide (PI) (final concentration 25 μM), HEPES (1 mM), glucose (1 mM) and polypeptide, and incubated for 15 min at room temperature. Changes in cell-associated dye fluorescence were measured with a BD Biosciences LSR II flow cytometer, using excitation at 488 nm with an argon laser and measurement of emission through a band-pass filter at 695/40 nm for PI. A minimum of 25,000 events were detected for each sample. Calculation the geometric mean fluorescence intensity (MFI) of each population was performed using FCS Express 3.00.0311 V Lite Stand-alone software.

Fluorescence microscopy of stained bacterial cells. A Zeiss XBO 75 Fluorescence Microscope (Carl Zeiss) was used for fluorescence studies. A BacLight™ Kit L-7012 was used as the fluorescence dye in a mixture of propidium iodide : SYTO9 to examine bacteria in the presence of polypeptides. It is important to mention that an initial bacterial concentration of ~10^8 cells/mL was used for microscopy for ease of visualization. The dye mixture was incubated with the bacteria at room temperature for 15 min prior to the addition of polypeptide solution. Solution of cells, dye, and polymer were allowed to stand for 30 min, and 50 μL of the solution was placed on a slide, mounted with a coverslip, and visualized under fluorescence microscope. Bacteria were viewed under a green filter (excitation/emission, 420-480 nm/520-800 nm) or a red filter (480-550 nm/590-800 nm).
**Liposome dye leakage assay.** Calcein dye was dissolved in Tris buffer (pH = 7) to achieve a concentration of 40 mM. To a clean round-bottom flask, appropriate volumes of lipid stocks were added to make up 1 mL of CHCl$_3$ (For 3:1 POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine)/POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt)) vesicles, POPE (130 µL, 25 mg/mL CHCl$_3$) and POPG (115 µL, 10 mg/mL CHCl$_3$) were used; For DOPC (1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine)/POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) vesicles, DOPC (100 µL, 25 mg/mL CHCl$_3$) and POPC (1 mL, 10 mg/mL CHCl$_3$) were used). The solvent was removed by a stream of nitrogen gas to obtain a thin lipid film, which was then hydrated by 1 mL of calcein solution. The mixture was left to stir for 1 h, after which it was subjected to 10 freeze–thaw cycles (using dry ice/acetone to freeze and warm water to thaw). The suspension was extruded 20 times through a polycarbonate membrane with 400 nm pore diameter. The excess dye was removed using Sephadex G-50 column as the eluent. The dye-filled vesicle fractions were diluted 200 times with Tris buffer. This suspension (90 µL) was subsequently mixed with polypeptide stock solutions (10 µL) on a 96-well black microplate (Greiner, flat bottom). Tris buffer (10 µL) and Triton-X (0.1% v/v, 10 µL) were employed as the negative and positive controls, respectively. After 30 min, the fluorescence intensity in each well was recorded using the microplate reader with excitation and emission wavelengths of 490 and 515 nm, respectively. The percentage of
leaked calcein dye in each well was determined as follows: leakage (%) = 
\[ \frac{(F - F_0)}{(F_{TX} - F_0)} \times 100\% \], where F is the fluorescence intensity recorded in the well, F_0 is the intensity in the negative control well, and F_{TX} is the intensity in the positive control well.

**SEM analysis.** MG1655 bacterial cells grown in LB with or without polypeptides treatment were performed using a similar protocol as MIC measurements but with a 30 min incubation time. All the samples were collected into a microfuge tube and pelleted at 4000 rpm for 5 min, and then washed twice with phosphate-buffered saline. Subsequently, bacteria were fixed with paraformaldehyde solution (4%) for 1 h before proceeding, followed by washing with DI water. Dehydration was performed with a series of graded ethanol solution (10%, 25%, 50%, 75%, 95%, and 100%). The dehydrated samples were dried under vacuum overnight before being mounted on carbon tape and coated with gold-platinum for imaging using a Hitachi S-4700 High Solution SEM (Japan).

**Liposome preparation for X-ray measurements.** DOPS

(1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt)), DOPE

(1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOPC

(1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPG

(1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt)), and CL (bovine heart cardiolipin (sodium salt)), lyophilized lipids from Avanti Polar Lipids, were used.
without further purification to form small unilamellar vesicles (SUVs). Individual lipid stock solutions of DOPS, DOPE, DOPC, DOPG, and CL were prepared in chloroform at a concentration of 20 mg/mL. Mixtures of these lipids were prepared at molar ratios to yield each model membrane composition. The lipid solution mixtures were placed under N₂ to evaporate chloroform, and were further dried by overnight desiccation under vacuum. The dried lipid mixtures were solubilized the following day in 100 mM NaCl, 10 mM HEPES at pH 7.4 to a concentration of 20 mg/mL. These aqueous lipid solutions were incubated at 37 °C overnight and then sonicated until clear. SUVs were obtained by extrusion of sonicated lipid solution through a 0.2 µm pore Nucleopore filter (Whatman).

**Small-angle X-ray scattering experiments.** Polypeptide and SUVs were mixed at specific P/L molar ratios. Samples were prepared in 100 mM NaCl, 10 mM HEPES at pH 7.4 and hermetically sealed in quartz capillaries (Hilgenberg GmbH, Mark-tubes). Small-angle X-ray scattering (SAXS) experiments were conducted at the Stanford Synchrotron Radiation Light source (SSRL, BL 4-2) using monochromatic X-rays with an energy of 9 keV. The scattered radiation was collected using a Rayonix MX225-HE detector (pixel size of 73.2 µm). No radiation damage was observed for the incident beam intensities and the exposure times used. 2D SAXS powder patterns were integrated using the Nika 1.50 package for Igor Pro 6.21 and FIT2D.

**SAXS Data Fits.** Q positions of the diffraction peaks were obtained by visual inspection.
of the integrated I(Q) vs. Q SAXS data graphed in Origin Lab software. The ratios between the measured peak positions (Q_{(hkl)meas}) were compared with those of permitted reflections for different crystal phases to determine the phases present in each sample. After identifying each crystal phase, a linear regression was fit through the set of points corresponding to the reflections, with each of these points having coordinates of its Q-position, Q_{(hkl)meas}, and the associated reflection in terms of Miller indices, h, k, l. For a powder-averaged cubic phase, Q_{(hkl)meas} = (2\pi/a)\sqrt{(h^2 + k^2 + l^2)}. As such, with a linear regression of Q_{(hkl)meas} vs. \sqrt{(h^2 + k^2 + l^2)} for cubic phases, we can calculate the cubic lattice parameter from the slope (m = 2\pi/a).

The intracellular uptake of rifampicin. Bacterial strains MG1655 and C101 (1 \times 10^6 CFU) were incubated with rifampicin (4.85 \mu M and 38.9 \mu M, respectively) without polypeptide or with PHLG-BIm_{40} (3.3 \mu M) or with PHDLG-BIm_{40} (3.3 \mu M). After 0.5 h or 1 h incubation, the supernatant was obtained by filtration through 0.2 \mu m membrane and used for HPLC analysis.
**Scheme S1.** Synthesis of PHLG-BIm polypeptide.

(i) phosgene  (ii) HDMS/DMF  (iii) 1-methylbenzimidazole, NaI, DMF/MeCN
Table S1. Synthesis of Polypeptides with Chloroalkyl Side Chains.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Monomer</th>
<th>M/I</th>
<th>$M_n$ (kDa)\textsuperscript{b}</th>
<th>$M_w/M_n$\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCHLG\textsubscript{40}</td>
<td>CH-L-glut-NCA</td>
<td>40/1</td>
<td>10.0</td>
<td>1.02</td>
</tr>
<tr>
<td>PCHDLG\textsubscript{40}</td>
<td>CH-DL-glut-NCA</td>
<td>40/1</td>
<td>9.7</td>
<td>1.10</td>
</tr>
<tr>
<td>PCHLG\textsubscript{28}</td>
<td>CH-L-glut-NCA</td>
<td>25/1</td>
<td>7.0</td>
<td>1.21</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Polymerizations were carried at room temperature for 48 h. Monomer conversions were all above 99%.  \textsuperscript{b}Determined by GPC.

Table S2. MIC values of PHLG-BIm\textsubscript{40} in the presence of salts (150 mM NaCl, 1mM MgCl\textsubscript{2}, 8 µM CaCl\textsubscript{2}), human serum (2%), fetal bovine serum (FBS, 5% or 10%), plasma from human (2% or 5%), artificial tear (2%) or mucin (1 mg/mL) against \textit{E. coli} DH5\textalpha{} and \textit{S. aureus} ATCC12608. Control represents treated with polypeptide only.

<table>
<thead>
<tr>
<th>Condition</th>
<th>MIC (µM)</th>
<th>NaCl</th>
<th>MgCl\textsubscript{2}</th>
<th>CaCl\textsubscript{2}</th>
<th>2% Human Serum</th>
<th>5% FBS</th>
<th>10% FBS</th>
<th>2% Plasma</th>
<th>5% Plasma</th>
<th>2% Artificial Tear</th>
<th>Mucin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
<td>1.5</td>
<td>1.5</td>
<td>0.7</td>
<td>1.5</td>
<td>0.7</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>DH5\textalpha{}</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
<td>1.5</td>
<td>1.5</td>
<td>0.7</td>
<td>1.5</td>
<td>0.7</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>ATCC12608</td>
<td></td>
<td>13.1</td>
<td>13.1</td>
<td>6.6</td>
<td>6.6</td>
<td>1.5</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
<td>6.6</td>
<td>13.1</td>
</tr>
</tbody>
</table>
Figure S1. The $^1$H NMR spectra of PCHLG$_{40}$ (a), PCHDLG$_{40}$ (b) and PCHLG$_{28}$ (c) in CDCl$_3$/d-TFA (7/1, v/v).
Figure S2. The $^1$H NMR spectra of PHLG-BIm$_{40}$ (a, TFA-d), PHDLG-BIm$_{40}$ (b, D$_2$O), and PHLG-BIm$_{28}$ (c, TFA-d).
**Figure S3.** The probability distribution of side chain lengths measured from the $C_\alpha$ to the $N_{12}$ atom extracted from the molecular simulation trajectory and averaged over all 20 side chains with a bin resolution of 0.005 nm$^{-1}$. The mode of the distribution is 1.24 nm, and the mean value is 1.15 nm with a 95% confidence interval of [0.90, 1.33] nm.
Figure S4. $^1$H-$^1$H TOCSY NMR (a, CH$_2$, blue; CH, red) and $^{13}$C-$^1$H HSQC NMR experiments (b) for PHLG-BIm$_{40}$ in D$_2$O.
Figure S5. One-dimensional Proton NOE spectra of PHLG-BIm₄₀. A) ¹H spectrum; B) NOESY1D from b¹ and b¹'; C) NOESY1D from c₁ and c₁'; D) NOESY1D from d₁; E) NOESY1D from h₁. A mixing time of 150 ms was used for the NOESY1D experiments.
Figure S6. The killing kinetics of PHLG-BIm₄₀ against DH5α (a), MG1655 (b), ATCC12608 (c) and ATCC11778 (d).
Figure S7. Percentage of CFU of DH5α (a) and ATCC12608 (b) in the samples treated with PHLG-BIm40 only, PHLG-BIm40 with NaCl (150 mM), artificial tear (2%), human serum (2%), or plasma (2%), at various concentrations for 8 h as compared to the control sample without any treatment.

Figure S8. The stability of LL-37 when incubated with trypsin or pronase, or elastase from P. aeuginosa, or elastase from human leukocytes for 8 h.
**Table S3.** The Antimicrobial Activity of Streptomycin Co-administered with PHLG-BIm or PHDLG-BIm at Various Concentrations against DH5α (MIC of PHLG-BIm: 3.3 µM), ATCC11778 (MIC PHLG-BIm: 26.1 µM) and ATCC12608 (MIC PHLG-BIm: 26.1 µM).

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>MIC (DH5α, µM, ΣFIC(^a))</th>
<th>MIC (ATCC11778, µM, ΣFIC(^a))</th>
<th>MIC (ATCC12608, µM, ΣFIC(^a))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without polypeptide</td>
<td>6.9</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td>With PHDLG-BIm(_{40}) (1.6 µM)</td>
<td>6.9 (1.5)</td>
<td>6.9 (1.1)</td>
<td>6.9 (1.1)</td>
</tr>
<tr>
<td>With PHLG-BIm(_{40}) (0.8 µM)</td>
<td>1.7 (0.5)</td>
<td>1.7 (0.3)</td>
<td>3.4 (0.6)</td>
</tr>
<tr>
<td>With PHLG-BIm(_{40}) (1.6 µM)</td>
<td>0.05 (0.5)</td>
<td>0.4 (0.2)</td>
<td>0.05 (0.1)</td>
</tr>
</tbody>
</table>

\(^a\) ΣFIC = MIC\(_{A,combination}\)/MIC\(_{A,alone}\) + MIC\(_{B,combination}/MIC_{B,alone}\). Synergy is defined as ΣFIC index ≤0.5. Indifference was defined as ΣFIC index of >0.5 but ≤4. Antagonism was defined as ΣFIC index >4.0.
**Table S4.** The Antimicrobial Activity of Various Antibiotics Co-administered with PHLG-BIm (3.3 µM) or PHDLG-BIm (3.3 µM) against MG1655 (MIC of PHLG-BIm: 26.1 µM) and C101 (MIC PHLG-BIm: 52.3 µM).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Polypeptide</th>
<th>MIC (Doxycycline, µM, ∑FIC)</th>
<th>MIC (Rifampicin, µM, ∑FIC)</th>
<th>MIC (Gentamicin, µM, ∑FIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>Without polypeptide</td>
<td>4.5</td>
<td>9.7</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>With PHLG-BIm₄₀</td>
<td>0.6 (0.2)</td>
<td>0.125 (0.1)</td>
<td>0.5 (0.4)</td>
</tr>
<tr>
<td></td>
<td>With PHDLG-BIm₄₀</td>
<td>4.5 (1.1)</td>
<td>0.2 (1.1)</td>
<td>2.1 (1.1)</td>
</tr>
<tr>
<td>C101</td>
<td>Without polypeptide</td>
<td>288.0</td>
<td>77.8</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>With PHLG-BIm₄₀</td>
<td>3.6 (0.2)</td>
<td>2.4 (0.2)</td>
<td>1.0 (0.2)</td>
</tr>
<tr>
<td></td>
<td>With PHDLG-BIm₄₀</td>
<td>288.0 (1.1)</td>
<td>77.8 (1.1)</td>
<td>8.4 (1.1)</td>
</tr>
</tbody>
</table>
Figure S9. The intracellular uptake of rifampicin in MG1655 (a) and C101 (b) after incubated with PHDLG-BIm₄₀ (3.3 µM) or PHLG-BIm₄₀ (3.3 µM) for 0.5 h and 1 h.

Movie S1. Movie of the 68 ns production simulation with frames rendered every 150 ps. For clarity of viewing, the peptide backbones in each configuration have been mutually aligned to a single reference configuration and water molecules removed. The peptide backbone is colored in red and the side chains in grey. This movie was produced using VMD (14).

Movie S2. A 360° rotation around a representative peptide configuration. For clarity of viewing, the water molecules have been removed and the backbone and side chains colored in red and grey, respectively. This movie was produced using VMD (14).

Movie S3. Fly-around of the 3D probability distribution of the side chain N12 atoms
around the α-helical backbone. Histograms were compiled over the simulation trajectory using a grid comprising cubic cells of size 0.1×0.1×0.1 nm, and the probability density function computed by normalizing by the total count and the cell volume. The value of the probability density in any one cell ranged from 0-3.7 nm$^{-3}$. Four contours in the probability density are plotted at 0.05, 0.1, 0.2, and 0.3 nm$^{-3}$. Since the positive charge in the cationic side chains resides primarily in the termini, this distribution may be considered a proxy for that of the positive charge. This movie was produced using MATLAB (15).

Reference


