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Mechanism of a prototypical synthetic membrane-active antimicrobial: Efficient hole-punching via interaction with negative intrinsic curvature lipids

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Phenylene ethynlenes comprise a prototypical class of synthetic antimicrobial compounds that mimic antimicrobial peptides produced by eukaryotes and have broad-spectrum antimicrobial activity. We show unambiguously that bacterial membrane permeation by these antimicrobials depends on the presence of negative intrinsic curvature lipids, such as phosphatidylethanolamine (PE) lipids, found in high concentrations within bacterial membranes. Plate-killing assays indicate that a PE-knockout mutant strain of Escherichia coli drastically out-survives the wild type against the membrane-active phenylene ethynylene antimicrobials, whereas the opposite is true when challenged with traditional metabolic antibiotics. That the PE deletion is a lethal mutation in normative environments suggests that resistant bacterial strains do not evolve because a lethal mutation is required to gain immunity. PE lipids allow efficient generation of negative curvature required for the circumferential barrel of an inverted membrane pore: an inverted hexagonal HII phase, which consists of arrays of water channels, is induced by a small number of antimicrobial molecules. The estimated antimicrobial occupation in these water channels is nonlinear and jumps from ~1 to 3 per 4 nm of induced water channel length as the global antimicrobial concentration is increased. By comparing to exactly solvable 1D spin models for magnetic systems, we quantify the cooperativity of these antimicrobials.

Results and Discussion

Variations of dye leakage assays on lipid vesicles are commonly performed to study the activity of membrane active antimicrobials. To illustrate both the potential and the limitation of this approach, we performed composition-dependent dye leakage experiments using GUVs. Phenylene ethynylene-treated giant unilamellar vesicles (GUVs) with PC-rich membranes (DOPG/DOPC = 20/80) (Fig. 1A) are compared with corresponding GUVs with PE-rich membranes with similar lipid compositions to typical E. coli antibiotic resistant bacteria | host defense peptides | innate immunity | protein-membrane interactions

The recent emergence of antibiotic-resistant bacteria is a worldwide public health problem (1). Antimicrobial peptides (AMPs) from innate immunity are known to have broad spectrum and selective activity against pathogens (2–7). Despite their diversity in sequence, secondary structures, and source, most of the more than 800 different AMPs that have been identified are amphiphilic and cationic (2, 3). It is thought that electrostatic interactions facilitate association of the peptide with the anionic bacterial membrane (2, 8). Moreover, such peptides are often implicated in pore formation in the bacterial membrane. Although the amphiphilicity is important in pore formation, the exact molecular mechanism by which membrane pores are formed is still not clear. Over the last decade, synthetic molecules that mimic these features have been designed and investigated, including stereoisomers of natural AMPs (9), α-peptides (10–12), β-peptides (13–16), peptoids (17), aromatic oligomers (18, 19), and synthetic polymers (20–24), such as phenylene ethynylene. The precise molecular mechanism of activity for most of these compounds is also unknown, as is the reason why it is difficult for bacteria to evolve immunity to them.

In this paper, we dissect the membrane activity of a prototypical synthetic antimicrobial (25, 26) from the bacterium level to the molecular self-assembly level. A number of biophysical differences exist between the membranes of bacteria and eukaryotes (2, 27). We begin by showing unambiguously that

negative intrinsic curvature PE lipids, which are found in high concentrations in Gram-negative bacterial membranes, play a critical role in the activity of specifically active phenylene ethynylene antimicrobials. Plate-killing assays indicate that a PE-knockout mutant strain of Escherichia coli, AD93, drastically out-survives its WT parent strain W3899 against the membrane-active phenylene ethynylene antimicrobials, whereas the opposite is true when both strains are challenged with the traditional metabolic antibiotic tobramycin. This observed trend is consistent with the composition-dependent permeation behavior of phenylene ethynylene when using giant unilamellar vesicles (GUV) as the model bacterial cell membrane. Interestingly, this trend is different from those observed for natural antimicrobial peptides (28), which may suggest a qualitatively distinct class of antimicrobial behavior. We also show why PE lipids are important for the mechanism. PE lipids facilitate formation of negative-curvature circumferential barrels of transmembrane pores. Synchrotron x-ray scattering indicates that membranes rich in PE lipids can be induced to form an inverted hexagonal HII phase, which consists of arrays of water channels, by a surprisingly small number of antimicrobial molecules. In the induced HII phase, the antimicrobial occupation is nonlinear and jumps from ~1 to 3 per 4 nm of water channel length as the global antimicrobial concentration is increased. By comparing to the exactly solvable 1-D spin models, the 3-Potts model and the Ising model, we quantify the cooperativity of antimicrobial occupancy, and find that the system is well described by a two-level system of singly and triply occupied states separated by a free energy difference $E_{1,3}$ of $\sim 4.7$ kT.


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membranes (DOPG/DOPE = 20/80) (Fig. 1B), both of which initially encapsulated fluorescently labeled (Alexa480) dextran. After treatment with phenylene ethynylene, the Alexa480 fluorescence intensity inside the PE-rich GUVs is drastically less than that inside the PC-rich GUVs, quantified by average circularly integrated fluorescence intensities in Fig. 1C. These results indicate that phenylene ethynylene induces more leakage from PE-rich GUVs, and suggest that the existence of PE lipids in the target membrane promotes phenylene ethynylene-induced membrane permeation. It can be seen, however, that there is significant nonspecific leakage. (The normalized intensity of untreated GUVs is set to one.) Moreover, bacteria are not vesicles and have, for example, more complex lipid distributions as well as lipopolysaccharides, both of which can modify results.

To evaluate directly the importance of PE lipids for antimicrobial activity, we performed bacteria plate-killing assays by using two strains of E. coli bacteria, a WT W3899 strain with a typical concentration of ~78% PE lipids in its membrane, and a mutant ‘PE knockout’ strain (AD93) with essentially no PE in its membrane. The mutant strain AD93 is accomplished by constructing an inactivated allele of the gene encoding phosphatidylethanolamine synthase, an enzyme which catalyzes the committing step to the synthesis of PE. Analysis of phospholipid composition confirms that AD93 has no measurable PE in its cell membrane (29, 30).

The results of comparative plate-killing assays in the presence of the phenylene ethynylene antimicrobial are shown in Fig. 2A. Because PE deletion is usually a lethal mutation to bacteria, growth of the PE-deficient mutant AD93 requires a supplement of certain divalent ions in the LB media (29). As a result, all comparative killing assays with phenylene ethynylene on both the mutant and WT strains are performed at the same Mg2+ concentration in sterile Hepes buffer solution supplemented with 400 mM sucrose to simulate natural molecular crowding effects in realistic environments, which osmotically stabilizes both strains of bacteria. PE-deficient AD93 cells survive at a significantly higher rate than the WT W3899 strain (~3 orders of magnitude at 4 μg/ml). In an additional set of control experiments, we challenge both strains with tobramycin, a conventional antibiotic that targets the ribosome rather than the bacterial membrane, the WT parent strain (W3899) survived at a drastically lower rate than the mutant strain (AD93) (~4 orders of magnitude at 1 μg/ml) in sterile buffer solution (10 mM Hepes, 60 mM NaCl, and 200 μM MgCl2, pH 7.5). Data points are reported as mean ± standard deviation.

AD93 now survives at a drastically lower rate than its WT parent strain W3899 (~4 orders of magnitude at 1 μg/ml), as shown in Fig. 2B (see Fig. S1 for additional information). These comparative plate-killing results using PE knockout mutants are consistent with our recent in vitro x-ray study (26), in which we show that the specificity of phenylene ethynylene antimicrobial activity is related to the PE content of the target membrane. The three basic toxicological profiles of the antimicrobials, nonactive against both bacteria and eukaryotic cells, specifically active against bacteria but not eukaryotic cells, and nonspecifically active against both bacteria and eukaryotic cells, correspond to decreasing minimal PE threshold values for inducing the inverted hexagonal phase. Moreover, that PE deletion is lethal in normative environments may help explain the unexpected absence of bacterial strains resistant to this class of antimicrobials despite repeated exposure (23): A lethal mutation is required in order for bacteria to gain immunity.

What is the role of the present prototypical phenylene ethynylene antimicrobial in the membrane permeation mechanism? In the case of natural innate immunity AMPs, it is currently thought that the peptides adopt two distinct states when membrane bound, one with induced pores and one lacking induced pores depending on the molar ratio of peptide-to-lipid. Because naturally occurring AMPs are protein-based and have electron densities that are close to those of lipids, we do not have experimental access to a more detailed picture, nor do we know how these self-assembled pore structures change as the AMP concentration is increased toward the MIC. Synthetic antimicrobials do not have this limitation. Phenylene ethynylene antimicrobials (Fig. 3A) have two high-electron-density bromines per molecule, so we can monitor the structural evolution of the induced water channels in PE-rich membranes by using high-resolution synchrotron small angle x-ray scattering (SAXS).

The specifically active phenylene ethynylene antimicrobial induces negative curvature by reorganizing lipids into an inverted hexagonal (HII) phase. Negative curvature is one of the structural ingredients of saddle-splay curvature (negative curvature along one principal direction and positive in the other, Fig. 3B), which is topologically required for pore formation. In fact, negative curvature can be seen in the circumferential barrel of an induced pore (Fig. 3B). SAXS data (Fig. 3C) show the self-assembled structure of the antimicrobial-membrane system
Fig. 3. The structure of induced HII phase changes with antimicrobial concentration. (A) Structure of phenylene ethynylene-based antimicrobial. (B) Top) Formation of a transmembrane pore requires both negative and positive curvatures (black and red arrows, respectively). (Bottom) Schematic representation of how this PE-dependent structural tendency may be realized on a membrane, in which the antimicrobials recruit PE lipids to achieve the negative circumferential curvature necessary for pore formation. The white and green spheres represent headgroups of zero intrinsic curvature (e.g., DOPG, DOPE) and negative intrinsic curvature lipids (e.g., DOPE) respectively. The antimicrobials are represented by blue spherocylinders. (C) Synchrotron SAXS data show that the inverted hexagonal phase induced by antimicrobial in DOPG/DOPE = 20/80 membranes evolves structurally as antimicrobial to lipid molar ratio (A/L) varies from 1/122 to 1/9.5. (D) The integrated diffraction intensities increase in a sigmoidal manner and saturate at A/L ~ 1/14.

(DOPG/DOPE = 20/80) as the antimicrobial/lipid molar ratio A/L is varied from 1/122, where the HII phase first forms, to 1/9.5, the saturation structure. At A/L = 1/122, the q positions of observed diffraction peaks have a relative ratio of 1:√3:2, and indicate the existence of a hexagonal structure with a lattice parameter a = 4π/(q100√3) = 6.43 nm. As A/L increases, the relative intensities of diffraction peaks change, and two extra diffraction peaks that index to a hexagonal structure appear (1:√3:2:√7:5), which together indicate that a significant structural change has occurred. Further, as A/L is increased, the integrated diffraction intensities increase in a nonlinear manner (Fig. 3D), and saturate at A/L ~ 1/14. We use this saturation value of antimicrobial/lipid stoichiometry to estimate an upper parameter a

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Hexanol decreases the membrane bending modulus and can be used to drive phase transitions into nonlamellar phases without changing intrinsic curvature. The addition of hexanol does not result in an HII phase without PE. However, at finite PE concentrations, addition of hexanol can produce a precursor Im3m phase with negative Gaussian curvature, which is topologically required for pore formation. All samples have A/L ~ 1/14.

Table 1. Structure of lipid-antimicrobial complexes with hexanol

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The induced generation of curvature, in agreement with earlier results (Table 1). For example, at DOPG/DOPE/DOPC = 20/00/80, the addition of hexanol does not result in HII type antimicrobial-lipid complexes. However, at PE concentrations below that required for the formation of HII phase, the addition of hexanol resulted in a transition from unilamellar vesicles to a precursor Im3m phase, a porous ‘plumber’s nightmare’ phase that is rich in negative Gaussian curvature, which is topologically necessary for pore formation (32). This shows that these antimicrobials generate curvature in a manner that depends on the local lipid composition, and that the presence of PE alone is not a sufficient condition for curvature generation. Having demonstrated the above, the next question is whether PE is a necessary condition for antimicrobial-induced curvature generation. We find that it is possible to generate the HII phase even if PE were replaced with other negative intrinsic curvature lipids such as cardiolipin in the presence of divalent ions (see SI Text and unpublished results). This is consistent with the experimental observation that the PE-knockout mutants can still be killed because their membrane compositions contain cardiolipin. However, the importance of PE is highlighted by the observation that cardiolipin is much less efficient in generating the necessary negative curvature, and the antimicrobial/lipid molar ratio required to induce the HII phase with the same diffraction intensity is increased by more than a factor of ~20 (see Fig. S2 for additional information).

To quantify how efficiently phenylene ethynylene antimicrobials generate the necessary negative curvature, we examined the structural evolution of the observed hexagonal phase as a function of A/L. The electron density ρ(x,y) of the 2-D hexagonal unit cell has been reconstructed from the x-ray data (x, y are mutually perpendicular axes in the plane perpendicular to the channel axis). Our choices for the phases are (+,−,−) and (+,−,+,+) for the induced structures below with 3 peaks and 5 peaks, respectively, which are consistent with previous assigned phases for lipid-only phases (33). Fig. 4A shows a reconstructed electron density for a unit cell at A/L = 1/14. The dark blue regions have the lowest electron density (~0.29 e/Å³) and correspond to hydrocarbon chains of the lipids, whereas the light blue circular central regions have intermediate electron density (~0.33 e/Å³) and correspond to water within the hydrophilic channels. The circular “rims” in dark red (~0.55 e/Å³) surrounding these water cores have the highest electron density, higher than those of typical phospholipid head groups (~0.41 e/Å³), suggesting that the high-electron-density bromine atoms of the antimicrobials stay close to the amphiphilic interface; this is consistent with recent experiments on phenylene ethynylene-lipid interactions at an air-water interface (34).

Based on the reconstructed electron density profile, we can estimate the size of the water core, d, by measuring the diameter.
of the “rim” with highest electron density (Fig. 4B). From these measurements, we can see that the water core diameter \(d\) changes abruptly: For \(A/L < 1/18\), \(d\) stays almost constant at \(30.3 \pm 0.2\) Å. At \(A/L = 1/18\), \(d\) abruptly increases to \(\approx 33.5\) Å and stays nearly constant at \(33.4 \pm 0.2\) Å for larger \(A/L\). Another way to consider these changes in the water channel diameter is membrane thinning induced by increases in \(A/L\), which is reminiscent of the behavior of multimellar stacks of lipids with antimicrobial peptides (35–37). In contrast with natural antimicrobial peptides, where membrane thinning may generate pores, the most pronounced membrane thinning induced by these synthetic antimicrobials occur at \(A/L\) ratios much higher than those required to form \(H_2\) water channels. This membrane thinning correlates with changes in the highest electron density (\(\rho_{\text{head/antimicrobial}}\)) at the ‘rim’ around the water channel near the phospholipid head groups. Fig. 4C shows the average value of the high electron density rims (\(\rho_{\text{head/antimicrobial}}\)) of the 2D hexagonal unit cell at different \(A/L\). When \(A/L < 1/18\), \(\rho_{\text{head/antimicrobial}}\) stays approximately constant at \(\approx 0.44\) e/Å\(^2\); when \(A/L > 1/18\), \(\rho_{\text{head/antimicrobial}}\) increases to \(\approx 0.54 \pm 0.01\) e/Å\(^2\) and saturates. Based on the extent of enhancement in \(\rho_{\text{head/antimicrobial}}\) and the electron density of the Br-rich antimicrobial, we estimate a saturation value of \(\approx 3.4 \pm 1.3\) antimicrobials per 4 nm of water channel. This is in good agreement with our previous independent, stoichiometry-based estimate above. This corresponds to one antimicrobial per \(\approx 1.227\) Å\(^2\) (\(\approx 20\) lipid molecules). Given that the effective surface area of a curved transmembrane pore is larger than that for a schematic cylinder, the number of antimicrobials in an actual transmembrane pore is expected to be larger (\(\approx 7.8 \pm 3\) antimicrobials per pore if the surface coverage density were constant. In this estimate, we approximate the edge of an induced pore by using a rim with constant radius and assume a constant antimicrobial surface density. The precise value of the number of antimicrobials will depend on how the head group area per lipid changes in this geometric organization).

We use our antimicrobial occupancy data on the induced \(H_2\) phase to deduce thermodynamic parameters governing antimicrobial cooperativity in the generation of the necessary negative membrane curvature, by analogy to exactly solvable 1D spin systems. Electron density reconstructions indicate that each 4-nm segment of an \(H_2\) channel is occupied by 1, 2, or 3 antimicrobials. The antimicrobials interact with each other on a 4-nm segment, but also with their neighboring segments because it is part of an \(H_2\) channel. To understand trends in the behavior of antimicrobial occupancy within an \(H_2\) channel, we first use a general 1D 3-Potts model (a 1D chain of sites 4 nm apart, each with three possible states) with a maximum of five adjustable parameters. The measured data indicate that sites essentially change from singly to triply occupied (Fig. 4D). This suggests that we can describe the results by using a simpler Ising model with only two states (and described by two adjustable parameters). Each model contains a single parameter for the difference in free energy of triply and singly occupied states, \(E_{1,3}\), and \(E_{0,1}\). In the Ising model, \(E_{1,3} = kT\log(c_{\text{mid}}/c_0)\), where \(c_{\text{mid}}\) is the antimicrobial concentration at the transition between single and triple occupation, and \(c_0 = 46.1\) mM is the concentration at which one antimicrobial is expected per site without any interactions. Fig. 4D shows the result of Bayesian sampling of model fits, and the range of predictions from different 3-Potts and Ising model fits. An initial set of 3-Potts model parameters are fit by using a maximum likelihood estimate with a normal distribution; these parameters are used to define a uniform prior distribution within 5 kT. Both models give excellent fits with essentially identical values for \(E_{1,3}\) near 4.68 kT which justifies the simpler Ising approximation. In fact, the posterior distribution estimates a narrow range of good fit values for this parameter (Fig. 4E). It is interesting to note that the present model implicates two different types of cooperativity, intrasite and intersite. The former describes antimicrobial interactions within a site, and is usually described by the Hill analysis used to quantify cooperativity in biological systems (25), and the latter describes interactions between such sites, which likely involves strong coupling to the membrane elasticity. Both are necessary for a satisfactory fit to the data (See SI Text and Fig. S3).

These results show that phenylene ethynylene antimicrobials are extremely efficient in generating negative curvature in PE-rich membranes; only 1 antimicrobial per \(\approx 20\) lipid molecules are needed to initiate formation of an \(H_2\) phase of water channels. We find that the antimicrobial occupation as a function
of increasing global antimicrobial concentration is highly non-linear, but is well described by a surprisingly simple two-level system of singly and triply occupied states separated by a well defined free energy difference $E_{1,3} \approx -4.7$ kcal/mol which quantifies the extent of cooperativity in the membrane-active antimicrobial activity of the phenylene ethynylene antimicrobial. These observations coincide with reorganization of the PE-rich membrane, and results in observed membrane thinning and resultant increases in the water channel diameter.

**Materials and Methods**

**Phenylene Ethynylene Synthesis.** The phenylene ethynylene molecule, referred to as antimicrobial oligomer 2 (AMO 2) (26), was synthesized by coupling tert-buty-3,5-dibromophenylcarbamate with 1,3-diethylenbenzene by using Sonogashira coupling in a 4 to 1 stoichiometry. The compound was then purified to homogeneity by silica gel chromatography to yield a white solid (yield 45%).

$^1$H NMR (CDCl$_3$): 7.68 (t, 1H, phenyl H), 7.51 (t, 1H, phenyl H), 7.48 (d, 1H, phenyl H), 7.37 (d, 1H, phenyl H), 7.34 (m, 2H, phenyl H), 7.31 (m, 2H, phenyl H), 4.58 (s, 2H, 2NH), 3.38 (m, 4H, 2CH$_2$), 2.79 (t, 4H, 2CH$_2$) ppm.

$^{13}$C NMR (CDCl$_3$): 156.1, 141.7, 137.5, 132.8, 132.5, 132.0, 131.1, 129.0, 125.4, 126.6, 126.7, 122.9, 122.6, 89.5, 87.9, 60.3, 58.2, 48.0, 41.6, 40.4, 32.9 ppm.

**Bacteria Plate-Killing Assay.** The PE-deficient strain AD93 was constructed from the WT E. coli strain W3899 (29). Both AD93 and W3899 were grown in LB medium (10 g of Bacto-tryptone, 5 g of Bacto-yeast, and 5 g of NaCl per liter) supplemented with 20 mM MgCl$_2$ at pH 7.5. Cells were first grown to stationary phase at 37°C. A 25-μl culture was diluted with fresh media by one hundredfold and regrown at 37°C to midlog growth phase (OD$_{600}$ = 0.5–0.7). The cells were harvested and washed twice with a sterile buffer solution (10 mM Hepes, 60 mM NaCl, 400 mM sucrose, and 100 μM MgCl$_2$, pH 7.5) via centrifugation at 10,000 rpm for 5 min, and within 15 min were adjusted with sterile buffer solution to ~5 × 10$^6$ CFU/ml before being incorporated into wells of a polystyrene 96-well microplate.

Serial 2-fold dilutions of phenylene ethynylene antimicrobial stock solutions in DMSO were first made with buffer solution in Eppendorf centrifuge cups. We inoculated 20 μl of adjusted bacteria suspensions into each zero-dilution well in preset microplates to achieve ~5 × 10$^6$ CFU/ml in each well (200 μl). A 100 μl sample was loaded with 100 μl of each PE-deficient E. coli mutant. X-ray work was performed at the Stanford Synchrotron Radiation Lab, the Advanced Photon Source, and the Frederick Seitz Materials Research Lab. This work was supported in part by National Science Foundation Grants DMR08–04363 and CBET08–27293, the Center of Advanced Materials for Purification of Water with Systems Science and Technology Centers, and the Rensselaer Polytechnic Institute–University of Illinois at Urbana-Champaign Nanoscale Science and Engineering Center, National Institutes of Health Grants R01-AI-074866 and R01-AI15650, and Office of Naval Research Grant N00014–05–1–0003.

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