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Mechanism of Action of pH-Triggered, Membrane Active Peptides

Sarah Y. Kim[1], Anna Pittman[2], Gavin King[3], William C. Wimley[4], Kalina Hristova[5].
[1]Dept Molec Biophys, Johns Hopkins Univ, Baltimore, MD, USA, [2]Univ Missouri, Columbia, MO, USA, [3]Dept Phys/Biochem, Univ Missouri, Columbia, MO, USA, [4]Dept Biochem, Tulane Sch Med, New Orleans, LA, USA, [5]Dept Matl Sci/Eng, Johns Hopkins Univ, Baltimore, MD, USA.

The plasma membrane insulates a cell from its outside environment, serving as a selectively impermeable barrier across which only small or hydrophobic molecules can pass. Although the membrane is necessary for life, it is also problematic when useful macromolecules such as antibodies, peptides, polysaccharides, and imaging agents are blocked from entry. Most macromolecules can easily be uptaken by the cell through endocytosis, but remain trapped and eventually degraded within endosomes, which mature into lysosomes. To promote the escape of macromolecules from endosomes prior to their maturation into lysosomes, we used a high throughput screen to discover pH triggered, pore-forming peptides[1]. To determine their mechanism of action, we measured the peptides' membrane binding affinity, secondary structure, and induction of pore-formation in POPC membranes. We identified that at least 5 acidic residues are essential for mediating a change from a soluble, predominantly unfolded, and inactive state at pH 7 to a membrane bound, helical, and active state at pH 5. These peptides are highly potent, with significant macromolecular leakage occurring at concentrations as low as 2 peptides per 1000 lipids. We determined that the peptides behave dynamically, associating with and dissociating from membranes, and consequently form pores on multiple vesicles. By atomic force microscopy imaging, we confirmed that the peptides form macromolecular pores, with diameters as large as 50 nm. Furthermore, we developed a kinetic model to explain how a low net fraction of bound peptide can lead to significant leakage. This work yields biophysical insights that will improve the design of peptides for new biomedical applications.[1]Wiedman, G., Kim, S.Y., Zapata-Mercado, E., Wimley, W.C. and Hristova, K., 2017. J. Am. Chem. Soc., 139(2), pp.937-945.

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Discovering Novel Antimicrobial Peptides using High-Throughput Screening and Rational Variation

Jenisha Ghimire, Charles G. Starr, William C. Wimley.
Department of Biochemistry and Molecular Biology, Tulane University School of Medicine, New Orleans, LA, USA.

Antimicrobial peptides (AMPs) have long been attractive drug candidates for the next generation of clinical antibiotics due to their potent antimicrobial activity and low propensity for inducing resistance in pathogens. However, due in part to toxicity concerns and activity loss in vivo, AMPs have yet to have any impact clinically. Our lab hypothesized that the presence of host cells could cause depletion of free peptide available to target bacterial cells and showed this to be true for some AMPs using concentrated human red blood cells (RBCs) as a model eukaryotic cell. To solve this problem, we synthesized a combinatorial peptide library based on the potent AMP, ARVA, and screened the library for activity in the presence of concentrated RBCs. We isolated nine unique, but similar sequences from the screen. We designed a consensus sequence based on the nine peptides and synthesized it using only D-isomer amino acids to form D-NOGCON. D-NOGCON displays excellent antimicrobial activity against multiple human pathogens in the presence and absence of concentrated RBCs, causes very little hemolysis, and is not susceptible to cleavage by cellular or plasma proteases. D-NOGCON also has high activity against bacterial biofilms and does not readily induce leakage. In this work, we created rational variants of D-NOGCON with truncations, insertions and mutations to test various hypotheses about the basis for highly potent antimicrobial activity with low hemolysis and low toxicity against nucleated cells. In the near future, we will use this information to design a next generation combinatorial peptide library based on D-NOGCON that will be screened for i) clinically relevant antimicrobial activity in the presence of serum and concentrated red blood cells, ii) negligible hemolysis, and iii) negligible toxicity against nucleated cells.

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Toxicity and Structure of Antimicrobial Peptides Derived from the Chemokine, CXCL10

Peter Bailer[1], Amanda E. Ward[2], Matthew Crawford[3], Debra Fisher[3], Lukas K. Tamm[2], Molly Hughes[3].
[1]University of Maryland Baltimore County, Baltimore, MD, USA, [2]University of Virginia, Charlottesville, VA, USA, [3]Medicine, University of Virginia, Charlottesville, VA, USA.

Antibiotic-resistant bacteria are a rising public health concern. To counter this threat, new antibiotics need to be developed. Chemokines are a class of immune signaling proteins able to induce chemotaxis of immune cells to the site of infection. In addition to signaling functions, the chemokine CXCL10 exhibits direct antimicrobial activity. This project's goal is to elucidate the mechanism by which CXCL10 kills bacteria and create one or several new antibiotics derived from CXCL10 by optimizing this antimicrobial behavior. To discover the molecular determinants responsible for antimicrobial activity, our collaborative group synthesized a peptide library derived from CXCL10 and screened these peptides for bactericidal activity. Peptides found to comprise broad-spectrum antimicrobial activity represented the N- and C-terminal regions of CXCL10. These peptides were selected for further studies, along with a non-antimicrobial peptide as a negative control. As other antimicrobial peptides are known to form an alpha helix, we used circular dichroism spectroscopy to assay the ability of the three CXCL10-derived peptides to adopt a similar conformation in the presence of a Gram-negative bacterial membrane mimetic. Surprisingly, while both antimicrobial peptides became more ordered in the presence of the bacterial membrane mimetic, only the spectra of the C-terminal peptide showed features of an alpha helix. To determine the potential compatibility of these peptides with human cells, in hopes of developing a novel antimicrobial therapeutic, we assessed the ability of the peptides to lyse human red blood cells via a hemolysis assay. Even at concentrations many fold higher than those required for bacterial killing, CXCL10-derived peptides showed minimal, statistically insignificant hemolysis. These observations are encouraging for further development of antimicrobial therapeutics based on CXCL10.

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Characterization of a Histidine Containing Antimicrobial Peptide with pH Dependent Activity

Luis Santiago-Ortiz, Morgan Hitchner, Thaddeus Palmer, Gregory A. Caputo.
Department of Chemistry and Biochemistry, Rowan University, Glassboro, NJ, USA.

Antimicrobial peptides (AMPs) have been an area of great interest, due to the high selectivity of these molecules toward bacterial targets over host cells and the limited development of bacterial resistance to these molecules throughout evolution. The peptides are known to selectively bind to bacterial cell surfaces through electrostatic interactions as the cell surface is net negative charged while the peptides are positively charged. Once bound, the peptides insert into the cell membrane and cause local disruptions of membrane integrity leading to cell death. Previous experiments showed that when Histidine was incorporated into the peptide C18G it lost all antimicrobial activity. Due to the side chain pKa is near physiological pH, we wanted to investigate the role of pH on the activity of the peptide. MIC results demonstrated that decreased media pH increased antimicrobial activity. Intrinsic Trp fluorescence was used to perform binding assays to model lipid vesicles under different pH conditions while Trp quenching was used as a reporter of the local environment. TCE quenching was used to determine peptide aggregation state in solution and showed a clear pH dependence on peptide aggregation. Acrylamide quenching demonstrates that peptides are more deeply buried in the bilayer in PC:PG membranes compared to PC bilayers. Dual Quencher Analysis (DQA) confirmed that the peptides inserted more deeply in PC;PG membranes overall, but could insert into PC bilayers at pH conditions above the pKa of the His. Circular Dichroism (CD) was used to determine the secondary structure and show that in solution the peptide appears to adopt an unstructured conformation while they become helical upon binding to the bilayer. Experiments are currently being conducted to further investigate aggregation properties in solution.

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Membrane Remodeling Induced by a pH Dependant Pore Forming Peptide via Atomic Force Microscopy

Anna Pittman[1], Sarah Y. Kim[2], William C. Wimley[3], Kalina Hristova[4], Gavin King[5].
[1]Univ Missouri, Columbia, MO, USA, [2]Dept Molec Biophys, Johns Hopkins Univ, Baltimore, MD, USA, [3]Dept Biochem, Tulane Sch Med, New Orleans, LA, USA, [4]Dept Matl Sci/Eng, Johns Hopkins Univ, Baltimore, MD, USA, [5]Dept Phys/Biochem, Univ Missouri, Columbia, MO, USA.

One hallmark of a cancer cell is the slightly acidic environment which is found in its immediate vicinity. It is of interest, therefore, to design mechanisms which can deliver cargo—drugs, nanoparticles, etc.—across membranes under acidic conditions only. Such delivery would bypass healthy cells in favor of cancerous ones. One method to deliver cargo is to create pores selectively under acidic conditions. pHD108 is a pore-forming peptide which was synthetically evolved to form pores large enough to pass macromolecules under acidic conditions, but not at neutral pH (J Am Chem Soc. **139**: 937 (2017)). Recently, we applied single molecule atomic force microscope (AFM) imaging to

characterize membrane remodeling induced by other pore forming peptides such as a mutant derivative of the bee venom peptide melittin in supported lipid bilayers (Langmuir **34:** 28 (2018)). The data revealed time dynamic interconversion between membrane-thinned and pore-like states, as well as colocalization between these different modes of membrane remodeling. Here, we use AFM imaging to characterize the lipid bilayer remodeling induced by pHD108. Studies of pHD108 conducted on supported POPC bilayers at differing pH revealed pH-dependent membrane distortions including punctate pore-like features. To provide a broader mechanistic picture, single molecule AFM imaging results are placed in context with other measurements (e.g., leakage assays) performed on the same peptide/membrane system.

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Elastic Behavior of Model Membranes with Antimicrobial Peptides Depends on Lipid Specificity and D-Enantiomers

Akari Kumagai[1], Fernando G. Dupuy[2], Zoran Arsov[3], Yasmene Elhady[1], Diamond Moody[1], Belita Opene[4], Robert K. Ernst[4], Berthony Deslouches[5], Ronald C. Montelaro[6], Y.P. Peter Di[5], **Stephanie A. Tristram-Nagle**[1].
[1]Dept Physics Biological Physics Group, Carnegie Mellon Univ, Pittsburgh, PA, USA, [2]INSIBIO, CONICET, National University of Tucumán, San Miguel de Tucumán, Argentina, [3]Condensed Matter Physics, Jozef Stefan Inst, Ljubljana, Slovenia, [4]Dept Microbiol Pathogenesis, University of Maryland, Baltimore, MD, USA, [5]Dept Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA, USA, [6]Dept Microbiology and Molecular Genetics, Univ Pittsburgh, Pittsburgh, PA, USA.

In an effort to provide new treatments for the global crisis of bacterial resistance to current antibiotics, we have used a rational approach to design several new antimicrobial peptides. The present study focuses on 24-mer **WLBU2** and its derivative **D8**. This amino acid sequence contains only R, W and V: RRWVRRVRRWVRRVVRVVRRWVRR. In D8, all of the valines are the D-enantiomer. WLBU2 and D8 have similar bactericidal activity, with low MIC values for both Gram-negative (G(-)) and Gram-positive (G(+)) bacteria, however, D8 is considerably less toxic to eukaryotic cells than WLBU2. Using low- and wide-angle X-ray diffuse scattering, we measure the elastic behavior of lipid membrane mimics with increasing concentrations of WLBU2 or D8 to probe if membrane elastic behavior and lipid chain order are correlated with bactericidal activity and toxicity. We employ membrane mimics of the outer lipopolysaccharide (LPS) membrane of G(-) bacteria, and the inner membranes of both G(-) and G(+) bacteria. LPS model membranes are very soft with disordered chains, and both WLBU2 and D8 further increase softness and chain disorder. In both G(-) and G(+) cell membrane mimics, there is a stiffening at low concentrations of both peptides, followed by a softening at higher concentrations; lipid chain order follows a similar trend. In eukaryotic mimics containing 25 mole% cholesterol, both peptides cause a general softening and disordering of chains. However, in mimics containing 50 mole% cholesterol such as in red blood cells, D8 causes a dramatic stiffening and increase in chain order, while WLBU2 causes membrane softening and chain disorder. These elastic results suggest that domain formation may play a role in killing G(-) and G(+) bacteria, and that cholesterol may play a role in protecting eukaryotic cells from antimicrobial peptides with D-enantiomers.

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Human Antibacterial Peptides Modify Lateral Structure in Lipid Monolayers Upon Interfacial Adsorption

Thomas Gutsmann[1], Beate Klösgen[2], Christian Nehls[1], Laura Paulowski[1], **Chen Shen**[3].
[1]Biophysics, Research Center Borstel, Borstel, Germany, [2]Department of Physics, Chemistry, and Pharmacy / MEMPHYS - PhyLife, University of Southern Denmark, Odense, Denmark, [3]Photon Science, Deutsches Elektronen-Synchrotron, Hamburg, Germany.

The increasing bacterial resistance against antibiotic treatment is a challenge of high socio-economic impact. An improved insight into the mechanisms of the natural biological antibacterial defense systems may trigger the development of alternative medical approaches for the treatment of infective diseases. Here we report results on a study of the interaction of the human antimicrobial peptide LL-37, and its fragments LL-32 and LL-20, with lipid interfaces. As a starting point, lipid monolayers at the air/water interface were chosen as a tool to observe the impact of the peptide when adsorbing to and into the lipid film from an aqueous subphase. During interaction, the surface pressure of the monolayer was monitored, and the initial Langmuir isotherm and its variation upon to the peptide insertion was acquired. At selected isotherm points, the lateral structure of (A) neutral monolayers (DPPC) and (B) negatively charged monolayers (90% DPPC, 10% DPPS, mol/mol) were studied by grazing incidence X-ray diffraction (GIXD). The results show the changes in the crystallographic order of the model systems upon their interaction with the peptides. Complimentary studies on lipid bilayer systems are on their way.

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Can Molecular Dynamics Simulations Predict the Effect of Truncating Histone-Derived Antimicrobial Peptides?

Kerry Gao[1], Donald E. Elmore[2].
[1]Department of Biological Sciences, Wellesley College, Wellesley, MA, USA, [2]Department of Chemistry and Biochemistry Program, Wellesley College, Wellesley, MA, USA.

Antimicrobial peptides (AMPs) represent a potential source of therapeutics to address the continued evolution of bacteria and viruses to resist existing antibiotics. Since the cost of producing AMPs increases for longer peptides, we evaluated the effect of removing residues from the N and C termini of the histone-derived antimicrobial peptides DesHDAP1 and DesHDAP3. Like many AMPs, DesHDAP1 and DesHDAP3 are cationic peptides that have electrostatic interactions with the negatively charged head groups of bacteria. Thus, we have focused on exploring the effects of removing arginine and lysine residues from either end of the peptide sequences. Bacterial assays showed that truncating five amino acids from the N-terminus of DesHDAP1 had no effect on the activity of the peptide but appeared to alter its mechanism of action from a membrane translocating to a membrane permeabilizing peptide. Further N-terminal truncations and any C-terminal truncations dramatically decreased DesHDAP1 activity, and DesHDAP3 was also more sensitive to truncation with only a one residue C-terminal truncation maintaining full activity. To provide a molecular-level interpretation for these findings, we have performed MD (molecular dynamics) simulations of both full-length peptides interacting with a mixed POPE-POPG membrane. In particular, we have considered whether one could have predicted the effect of truncations on DesHDAP1 and DesHDAP3 activity from simulations of the full-length peptides. To this end, we have measured whether there is a correlation between the interactions a particular amino acid has with the lipid membrane and whether it that residue must be present for full peptide activity. Future work will use MD to further investigate the effect of specific truncations on peptide mechanisms and consider how truncations might affect other AMP properties, such as DNA binding.

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Effects of Cholesterol on Fengycin, an Antimicrobial Lipopeptide Using Weighted Ensemble Path Sampling Method

Sreyoshi Sur[1], Alan Grossfield[2].
[1]Dept Chemistry, Univ Rochester, Rochester, NY, USA, [2]Dept of Biochemistry and Biophysics, Univ Rochester, Rochester, NY, USA.

Fengycin is an antimicrobial lipopeptide synthesized by the bacteria *Bacillus subtilis* and commercially available as an agricultural fungicide. One of the ways fengycin kills fungal cells is by binding and damaging their cell membranes. Previous all-atom simulations suggested aggregation of lipopeptides is the first step that leads to membrane deformation. Here we attempt to explain fengycin's selectivity for fungal over mammalian cells by examining the effects of cholesterol on its aggregation. Using weighted ensemble path sampling, we found that fengycin causes membrane demixing to some extent in the membrane consisting of POPC and cholesterol, with the latter being clustered around the lipopeptides. We also found that the free energy of a single fengycin binding to a membrane is independent of the presence of cholesterol. We are currently generalizing these calculations to examine fengycin aggregates in solution and their affinity for membranes. The results will help us understand the implications of cholesterol on fengycin's fungicidal properties and possibly indicate whether fengycin can be a potential drug candidate.

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Effect of Biopolymer Tethers on Antimicrobial Peptide Activity in Biomembranes

Fathima T. Doole[1], Abhishek Singharoy[2], Michael F. Brown[1,3], Minkyu Kim[4,5].
[1]Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA, [2]School of Molecular Sciences, Arizona State University, Tempe, AZ, USA, [3]Department of Physics, University of Arizona, Tucson, AZ, USA, [4]Department of Material Science and Engineering, University of Arizona, Tucson, AZ, USA, [5]Department of Biomedical Engineering, University of Arizona, Tucson, AZ, USA.

Remedies for health-associated infectious diseases entails innovations in cost-effective alternatives, industrial-scale synthesis, non-cytotoxicity, and non-biodegradability. We propose a smart biopolymer composed of an