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Two interdependent mechanisms of antimicrobial activity allow for efficient killing in nylon-3-based polymeric mimics of innate immunity peptides ☆

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ABSTRACT

Novel synthetic mimics of antimicrobial peptides have been developed to exhibit structural properties and antimicrobial activity similar to those of natural antimicrobial peptides (AMPs) of the innate immune system. These molecules have a number of potential advantages over conventional antibiotics, including reduced bacterial resistance, cost-effective preparation, and customizable designs. In this study, we investigate a family of nylon-3 polymer-based antimicrobials. By combining vesicle dye leakage, bacterial permeation, and bactericidal assays with small-angle X-ray scattering (SAXS), we find that these polymers are capable of two interdependent mechanisms of action: permeation of bacterial membranes and binding to intracellular targets such as DNA, with the latter necessarily dependent on the former. We systemically examine polymer-induced membrane deformation modes across a range of lipid compositions that mimic both bacteria and mammalian cell membranes. The results show that the polymers' ability to generate negative Gaussian curvature (NGC), a topological requirement for membrane permeation and cellular entry, in model *Escherichia coli* membranes correlates with their ability to permeate membranes without complete membrane disruption and kill *E. coli* cells. Our findings suggest that these polymers operate with a concentration-dependent mechanism of action: at low concentrations permeation and DNA binding occur without membrane disruption, while at high concentrations complete disruption of the membrane occurs. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

1. Introduction

Over the past decade, the development and spread of antibiotic resistance has become a major global health risk. Each year in the United States, antibiotic-resistant infections affect over 2 million people and result in more than 23,000 deaths. The expenditures associated with these infections in terms of annual health care costs and productivity losses are estimated to be as high as $20 billion and $35 billion, respectively [1]. Most antibiotics in clinical use kill or inhibit the growth of metabolically active bacteria by targeting various biosynthetic processes in growing bacteria, including the synthesis of proteins, RNA, DNA, peptidoglycan, and folic acid [2–5]. For instance, the β-lactam class of antibiotics, which includes penicillins, cephalosporins, and carbapenems, inhibit cell wall synthesis in cells undergoing division. Aminoglycoside, macrolide, tetracycline, and other antibiotics target the bacterial ribosome to inhibit protein synthesis. Resistance can develop in at least two general ways: biomacromolecules targeted by an antibiotic can mutate to minimize or eliminate susceptibility (genetic antibiotic resistance), or bacteria can also adapt physiologically to become quiescent, or slow-growing. These persisting bacteria are able to evade deleterious effects through the down-regulation of biosynthetic processes that are often targeted by conventional antibiotics. Persisting bacteria are found in chronic infections, such as endocarditis, cystic fibrosis, and tuberculosis, which require prolonged treatment periods. Hence, there is a critical need for new structural classes of antibiotic

Abbreviations: AMP, antimicrobial peptide; SAXS, small-angle X-ray scattering; NGC, negative Gaussian curvature; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; GUV, giant unilamellar vesicle; SUV, small unilamellar vesicle; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; 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)]; CL, cardiolipin; PE, phosphoethanolamine or phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; ONP, *o*-nitrophenol; P/L, polymer to lipid molar ratio; P/D, polymer to λDNA charge ratio; L_α, lamellar phase; H_{II}, inverted hexagonal phase; Q_{II}, cubic phase.

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therapeutics that are effective against slow-growing cells, and are not impeded by mechanisms of antibiotic tolerance.

Antimicrobial peptides (AMPs) constitute a critical component of the eukaryotic innate immune system. Collectively, AMPs have broad-spectrum antimicrobial activity [6–11]. These host-defense peptides are diverse in sequence and structure, but two common features are cationic charge and hydrophobicity [8,9,12–15]. Some AMPs, such as cecropin and magainin, adopt an amphipathic α-helical secondary structure, in which cationic and hydrophobic side chains are spatially segregated from one another, upon interaction with membranes [8]. Other AMPs, such as bactenecin and defensins, feature anti-parallel β-sheet structure, around which cationic and hydrophobic regions are segregated. The combination of hydrophobic and cationic subunits is believed to play a key role in the antimicrobial activity of AMPs, enabling the disruption of bacterial membranes through a combination of electrostatic interactions involving the cationic side chains with the anionic membrane along with the insertion of hydrophobic side chains into the nonpolar interior of the lipid membrane bilayer [8–10,16–18]. AMPs can destabilize membranes through a variety of processes, including pore formation, blebbing, budding, and formation of mixed peptide–lipid micellar assemblies ("carpet mechanism" [10]). Antimicrobial agents that mimic natural AMPs by targeting generic aspects of bacterial membranes may have potential for treating both antibiotic-resistant and slow-growing dormant infections. Membrane-disruptive antimicrobial agents that directly interact with the bacterial membrane bilayer can destabilize and compromise the physical integrity of the membrane. In general, membrane-targeted approaches have been shown to be clinically effective with newer antibiotics such as daptomycin, which is currently used to treat *Staphylococcus aureus* infections [2].

Recent work [7,19,20] has shown that the existence of negative intrinsic curvature lipids, such as those with phosphoethanolamine (PE) head groups, in the target membrane is an important determining factor in whether such membranes are permeated by AMPs: bacterial membranes with high PE concentrations are vulnerable to AMP-induced permeation, while eukaryotic membranes with low PE concentrations are not. Because many AMPs and synthetic compounds inspired by AMPs interact directly with membranes, the development of bacterial resistance against these agents is more difficult to achieve than against conventional antibiotics [21–24]. Nonetheless, bacterial resistance, in the form of reduced susceptibility to AMPs, is still possible through the modification of the membrane composition. Previous work has shown that bacteria can actively detect AMPs through two-component signal transduction systems, such as PhoQP in Gram-negative bacteria and GraSR in Gram-positive bacteria, and respond by altering their membranes [21,25–33]. However, a PE deletion, which would essentially confer immunity against membrane-active antibiotics, is found to be lethal in bacteria. This may help explain the unexpected absence of bacterial strains resistant to AMPs despite repeated exposure [19]. Thus, with increased clinical prevalence of bacterial resistance to conventional antibiotics, interest has grown in the prospect of using antimicrobial peptides as therapeutic agents, and in designing new antibiotics inspired by AMPs.

The attractive properties of natural AMPs have inspired extensive effort to develop synthetic analogues. Such efforts have included both oligomers of α-amino acids (α-peptides) [34–36] and oligomers that contain unnatural subunits, such as β-peptides [37–40], α/β-peptides [41,42], peptoids [43], and aromatic oligomers [44–48]. These AMP analogs have been demonstrated to provide various potential advantages over conventional antibiotics: (1) tunable, custom designs, (2) ease in preparation, (3) cost-effectiveness, (4) antibacterial potency with reduced likelihood of resistance, and (5) allowance for additional new built-in functions. In fact, recent work has highlighted quantitative differences between natural AMPs and their present synthetic analogs in terms of their hydrophobic content and cationic charge [49]. However, all unnatural AMP-mimetic oligomers have specific sequences of subunits, which require solid-phase synthesis, a technique that is costly and therefore not practical for many applications [6]. This situation has prompted a number of groups to explore synthetic polymers as a novel source of AMP mimics over the past decade. In contrast to α-peptides and other sequence-specific oligomers, for which every molecule in a given sample is in principle identical, materials generated via polymerization reactions are mixtures, with variations in chain length and, for co-polymers, in subunit sequence. However, polymer production is much less expensive than production of sequence-specific oligomers. Early studies revealed that polymers with high intrinsic hydrophobicity could be effective against bacteria but not cell-type selective, since these materials are hemolytic [44,47,48,50]. More recently, polymers with carefully tuned hydrophobic-hydrophilic balance have been shown to match the generic AMP activity profile: inhibiting bacterial growth at low concentrations but causing hemolysis only at much higher concentrations [51–55]. Nylon-3 polymers have proven to be quite promising in this regard [51,53]. The nylon-3 backbone, comprised of β-amino acid residues, should be similar to the polyamide backbone of proteins in terms of physicochemical properties. Binary hydrophobic-cationic nylon-3 materials are readily prepared via anionic ring-opening polymerization of appropriate pairs of β-lactams [56].

In recent work, we have found that the bactericidal activity of a broad range of peptidic antimicrobials is correlated with the induction of negative Gaussian membrane curvature (NGC), also known as saddle-splay curvature, which enables membrane destabilizing processes such as pore formation, blebbing, and budding. All the membrane-active antimicrobials examined induce negative Gaussian curvature when the target membrane lipid compositions mimic those of bacterial membranes, but not when the lipid compositions are more representative of mammalian membranes. A key parameter for activity in this broad range of compounds is the concentration of negative intrinsic curvature ($C_0 < 0$) lipids, such as those with PE head groups, which exist at significantly higher concentrations in bacterial cytoplasmic membranes compared to eukaryotic membranes. Existence of homologous behavior in synthetic antimicrobials [19,20,57] suggests a common root mechanism for selective membrane permeation. In fact, the trends observed for antimicrobial-lipid interactions are consistent with killing assays using *Escherichia coli* mutants engineered to have different amounts of PE lipids in their cytoplasmic membranes [19]. Importantly, we have deduced a criterion for amino acid compositions of AMPs based on the requirement for generating saddle-splay membrane curvature, and we have shown that this criterion is consistent with trends in amino acid composition of 1080 known cationic AMPs [7].

Most biophysical studies of interactions between AMPs and membranes examine membrane behavior at a single lipid composition. However, bacterial membranes are known to exhibit different membrane compositions, which can be modulated in response to antimicrobials [58–65]. To complicate matters further, natural AMPs are intrinsically multifunctional. While many AMPs have membrane activity, it is known they also can bind intracellular targets [66–68] and have immunomodulating activities [6,69,70]. Due to these complications and others, it has been hard to correlate biophysical parameters, such as vesicle leakage and permeability, with antibacterial potency in general [9].

In this work, we examine a more circumscribed problem. We study the antimicrobial activity and membrane permeation activity of a set of nylon-3 polymers, including some that show AMP-like activity profiles. We show that this family of antimicrobial polymers is capable of two interdependent mechanisms of activity, one based on membrane permeation and one on DNA binding. Interestingly, at the minimal bactericidal concentration (MBC), the extent of bacterial membrane permeation, as measured by a β-galactosidase-based colorimetric assay on an *E. coli* ML-35 strain, is modest and clearly not enough to solubilize the entire membrane. All members of this family of antimicrobial polymers have sufficient local surface charge density to bind efficiently to intracellular DNA, in a manner similar to AMPs indolicidin [67] and buforin [68];