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SPECIAL TOPIC: DISINFECTANTS AND MICROBIAL CONTROL

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Simple oligomers as antimicrobial peptide mimics

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Abstract New approaches to antibiotic design are desperately needed. The design of simple oligomers that capture the shape and biological function of natural antimicrobial peptides could prove to be versatile and highly successful. We discuss the use of aromatic backbones to design facially amphiphilic (FA) β-sheet like structures which are potently antimicrobial. These oligomers capture the physiochemical properties of peptides like the Magainins and Defensins, which fold into specific conformations that are amphiphilic resulting in antimicrobial activity. However, natural peptides are expensive to prepare and difficult to produce on large scale. The design of polymers and oligomers that mimic the complex structures and remarkable biological properties of proteins is an important endeavor and provides attractive alternatives to the difficult synthesis of natural peptides. We therefore have designed a series of FA oligomers that are easy to prepare from inexpensive monomers. They adopt structures very reminiscent of amphiphilic β-sheets and have significant activity with minimal inhibitory concentrations at 6 h in the low microgram per ml range (μM to nM). They are active against a broad spectrum of bacteria including gram-positive and gram-negative as well as antibiotic resistant strains.

Keywords Magainin · Host defense peptides · Phenylene ethynylene · Facially amphiphilic · Antibacterial

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Introduction

Novel approaches to the development of new antimicrobial compounds and materials remains an important area of research. Many different advances are reported including tethering known antibiotics like ciprofloxacin [33, 35], as well as hydrolyzable groups such as diphenyl ether [16] or dichlorophenyl [19], or synthesizing a compound that is inherently antimicrobial. In the latter topic, cationic polymers have been the focus [12, 30, 32] including those containing ammonium [9, 14, 25] or phosphonium salts [20], polylysine [10], pyridinium salts [28, 34], or polyguanidines and polybiguanidines [1]. The cationic nature of the molecule is stressed due to the electrostatic interaction of the positive charges with the negatively charged species present in the cytoplasmic membrane [24]. Polymers often have an advantage over conventional small molecule antibacterial agents since they are essentially non-volatile and chemically stable [24]. Many of these compounds synthesized to date do not show selectivity towards bacteria over mammalian cells, rather they are biocidal.

Biocidal cationic polymers can be useful for situations where selectivity towards bacterial cells over mammalian cells is not required; however, in some instances, such as in antibiotic use, selectivity is necessary. Host defense peptides (HDPs) represent a large class of natural compounds with broad spectrum antimicrobial activity that also show selectivity between bacterial and mammalian red blood cells (RBC) [10, 31]. These peptides represent the first line of defense against bacterial infection in most eukaryotic systems. Examples of HDPs include α-helical Magainin and Cecropin as well as Defensins, which contain β-strands. They have captured the attention of many researchers and significant understanding of their essential physiochemical properties and mode of action has been determined [5, 11, 38, 39]. Most HDPs have one thing in common, the ability to adopt a facially amphiphilic (FA) structure.

The ability of HDPs to disrupt phospholipid membranes, ultimately killing the cell, is thought to come from the adoption of a FA structure [6, 10, 17, 31]. Gellman and coworkers demonstrated this concept by synthesizing a series of amphiphilic helical structures which mimic the overall structure and activity of Magainin [21, 23]. They synthesized a series of β-peptides with the same net charge yet the substitution pattern was varied [21, 23]. It was determined that the most active peptides were those that had the highest degree of FA. Therefore, they concluded the FA architecture is important for antibacterial activity and selectivity, consistent with previous findings from synthetic α-peptides [22, 23].

Another route to mimicking HDPs is the use of a peptoid backbone. Peptoids are similar to peptides with a protein-like backbone but the amino acid side chain is located on the amide nitrogen instead of the α-carbon. Due to the substitution pattern, much of the hydrogen bonding seen both intra- and inter-molecularly in natural peptides is prevented. Peptoids were recently shown to adopt stable helical structures when there are bulky α-chiral side chains [8, 36, 37]. When these helical molecules are patterned to be FA they demonstrate antibacterial properties [18]. Similar molecules have shown resistance to proteolytic degradation [15] which makes this backbone attractive to use in many biological applications.

The β-peptides and peptoids demonstrate the power of coupling design with function. However, these peptides still require costly and time intensive synthetic methods. At the same time, both are structurally similar to natural α-peptides and mimic the helical nature of HPDs. The extension of design principles to simpler oligomers and polymers will provide fundamental understanding of structure–activity relationships and lead to new biologically active materials [2, 3, 4, 13, 29]. Here, we describe our efforts at designing simple aromatic oligomers that capture the biochemical properties of the HDPs.

Materials and methods

Compounds

The synthesis of these compounds is reported elsewhere [3, 27].

Antimicrobial testing

The compounds were dissolved in either DMSO or buffer to make a stock solution. The stock solution was then diluted into 96-well plates and diluted with Mueller Hinton (M-H) medium to a constant volume. *Escherichia coli* D31 and *Bacillus subtilis* ATCC 8037 were taken from stock glycerol solutions, diluted into M-H medium, and grown overnight at 37°C. Subsamples of these cultures were grown for 3 h at 37°C with agitation, the OD_{600} was measured and then the cells were diluted to 0.001 OD_{600}. The diluted cell solutions (approximately 10^5 cells/ml were then added to the 96-well plate and incubated at 37°C for 6 h. The MIC values reported in Table 1 are the minimum concentration necessary to inhibit 50% of the cell growth. This was determined by measuring cell growth at OD_{600} after 6 h in twofold serial dilutions of the abiogenic polymer following standard protocols. All reported values represent a minimum of quadruplicate experiments.

Hemolysis assay

Hemolysis experiments were performed by incubating a 0.35% (v/v) suspension of fresh human erythrocytes in 10 mM TRIS buffer containing 150 mM NaCl at pH 7.0 with varying amounts of polymer. Hemolysis samples were prepared by combining 80 μl of the washed RBC suspension and 20 μl total of buffer and polymer solution in 96-well plates. After incubation for 30 min at 37°C, the suspensions were concentrated at 1,000x *g* for

Table 1 Antimicrobial activities and selectivity of the arylamide and PE oligomers

Bacterial species	Arylamide (μg/ml) MIC_{50}	PE (μg/ml) MIC_{50}	HC_{50}/ MIC_{50} Arylamide	HC_{50}/ MIC_{50} PE
Gram-negative				
E. coli D31[a]	0.64[c]	0.42[c]	117	179
Klebsiella pneumoniae ATCC 13883	1.28	1.68	59	45
Proteus vulgaris ATCC 13315	1.68	1.68	45	45
Pseudomonas aeruginosa ATCC 10145	3.36	1.68	22	45
Gram-positive				
B. subtilis ATCC 8037	0.42	1.68	117	45
Bacillus cereus var. *mycoides*	1.28	<0.42	59	>179
Staphylococcus aureus ATCC 25923	0.42	0.84	179	89

[a] Clinical isolate, uropathogenic strain, University of Pennsylvania culture collection
[b] The HC_{50} was determined as 75 μg/ml by extrapolating a best-fitted curve to 50% cell lysis
[c] MSI-78, a magainin derivative, has MIC of 12 μg/ml against *E. coli* D31 and selectivity of 10

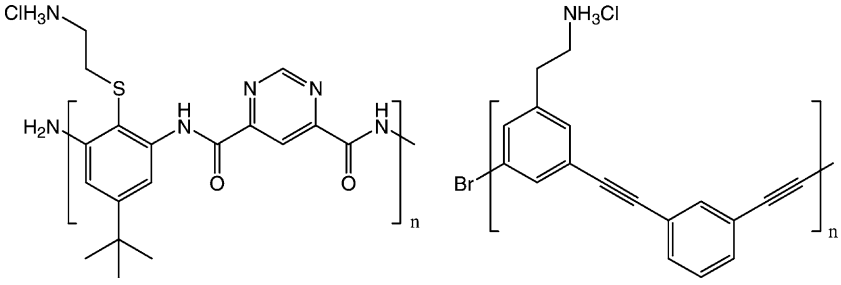

Fig. 1 Chemical structure of (*left*) arylamide and (*right*) PE oligomers designed to be FA

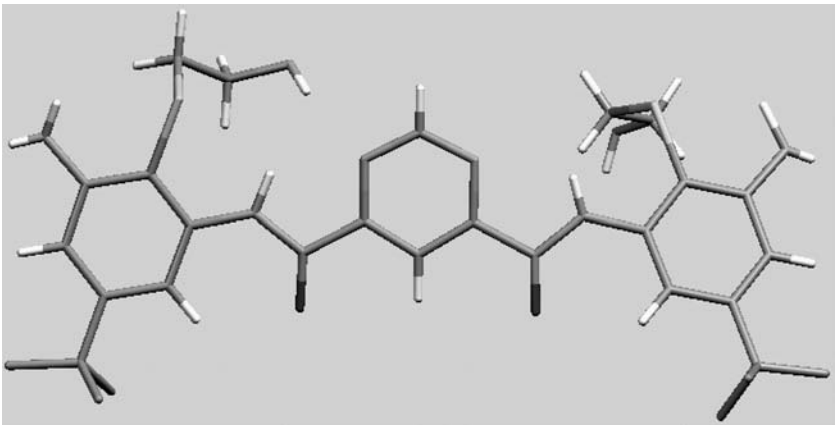

Fig. 2 Single crystal structure of an arylamide model compound showing the FA conformation

5 min. An aliquot of the supernatant was diluted with buffer and the OD_{414} of the solution was measured to quantitate released hemoglobin. Complete hemolysis was measured by adding 1% Triton X-100 to the RBCs and measuring OD_{414}. Non-linear exponential curve-fitting plots of OD_{414} versus polymer concentration resulted in HC_{50}, the hemolytic dose required to lyse 50% of the RBCs.

Results

Designing FA structures of non-natural oligomers requires adoption of low energy conformations in which polar and nonpolar groups extend from opposite sides of the structure [4, 3, 29]. Figure 1 highlights molecules which represent the two extremes in our design. The arylamide has multiple internal hydrogen bonds that severely limit the conformational freedom [7] while, in contrast, the phenylene ethynylene (PE) structure is free to rotate. However, both of these structures should adopt FA conformations at the water–lipid interface in the presence of bacterial cells [31].

The overall conformation of the arylamide oligomer was studied in both solution and in the solid state. Solution characterization included nuclear magnetic resonance (NMR) investigation of both the amide proton chemical shifts and nuclear overhauser effect (NOE) measures [27]. The X-ray single crystal structure of a model oligomer is shown in Fig. 2 and confirms the expected overall conformation in which the cationic and nonpolar side chains segregate to opposite sides of the molecular backbone.

Having successfully synthesized these two different oligomers, the antibacterial activity was screened using *E. coli* and *B. subtilis* as model Gram-negative and Gram-positive bacteria, respectively. All experiments were performed in 96-well microtiter plates using optical density at 600 nm after 6 h to determine cell count. A typical experimental result is shown in Fig. 3.

These oligomers proved to be potent antimicrobial agents with activity against several bacteria as listed in Table 1. The minimal inhibitory concentration (MIC) fluctuated depending on the exact bacterial species

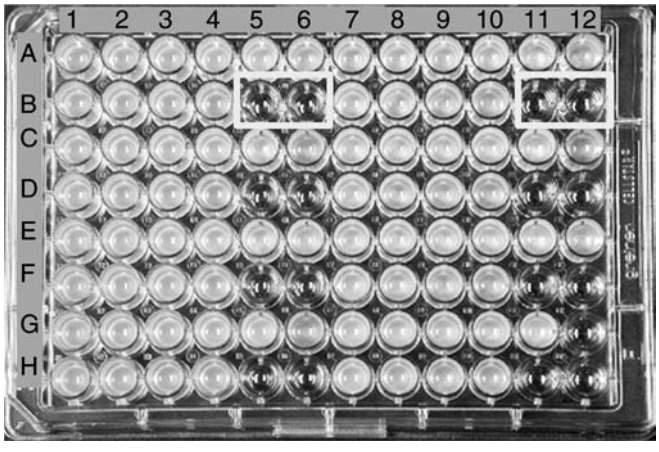

Fig. 3 A 96-well microtiter plate with rows A–H containing different antibacterial oligomers as well as a control while columns 1–12 vary the concentration of the active agent. Optical density, or turbidity, measurements are used to determine growth. For example, A1 has full growth while B5 and B6 have no growth

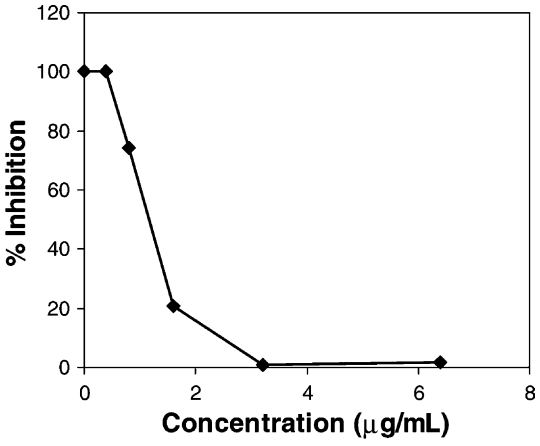

Fig. 4 Inhibition curve for S. aureus showing potent activity of PE

which is a common trait of HDPs. Compared to a control, MSI-78, these oligomers were significantly more potent and displayed an equal or superior selectivity. A typical inhibition curve is shown in Fig. 4.

Hospital infections remain a tremendous problem and those related to polymeric materials are rising at an alarming rate [26]. The discovery of these simple oligomers with potent and selective activity enables the formulation of polymeric materials with non-fouling characteristics. To explore the possibility that these oligomers would retain their activity after incorporation into a polymeric thin film, we examined a polyurethane coating. Figure 5 shows a control sample of polyurethane along with one that was pretreated with the PE oligomer before exposure to *E. coli*. Both samples were placed next to each other and statically immersed in bacterial growth media for 72 hours. It is clear from the results presented in this figure that the ability of the treated material to resist bacterial fouling is quite high.

Discussion

The FA structure and antibacterial activity of these oligomers against both Gram-negative and Gram-positive bacteria suggests they exhibit many of the properties central to the HDPs. They show potent antibacterial activity ranging from 0.4 μg/ml to 4 μg/ml. Although the compounds are more hemolytic than would be ideal with HC_{50} values of 75 μg/ml their potent action against bacteria leads to a reasonable therapeutic window described in the table as the ratio of HC_{50}/MIC_{50}. The ability to discriminate between bacterial and mammalian RBCs is another hallmark of many HDPs. The broad spectrum activity suggests these oligomers do not act against a specific protein or molecular target but, instead, are active against the plasma membrane [3, 4]. As a result, the key to obtaining selectivity appears to be the overall charge density and hydrophobicity of the structure since both the rigid arylamide and more flexible PE oligomer are highly active and selective.

Incorporation in a polyurethane coating generated excellent inhibition of growth on the surface. Both samples were immersed in rich media with *E. coli* for 72 h, imaged directly, and the difference between them is readily apparent. Although the direct method of inhibition remains under investigation in our laboratories, these results clearly indicate the potential of these oligomers to generate novel coatings for medical applications. The current hypothesis assumes the active oligomers slowly leach out of the film over the course of the experiment. This is the most likely scenario since the molecules are quite small and it would be unlikely that they could interact with both the coating and cell membrane at the same time; however, until further experiments are finished no direct conclusions about the mode of action can be stated. If the oligomer leaches out of the coating, the selectivity of these molecules becomes even more important. Therefore, regardless of how they prevent growth on the surface, these novel biomimetic materials represent excellent candidates for antifouling materials.

Novel biomimetic oligomers of HDPs have been generated and the diversity of the design is illustrated with two examples. These new molecules represent a unique approach to antibiotics and capture the biochemical activity of complex proteins in much simpler structures. As a result, they enable material applications and the ability to generate polyurethane coatings that resist bacterial growth on the surface was demonstrated.

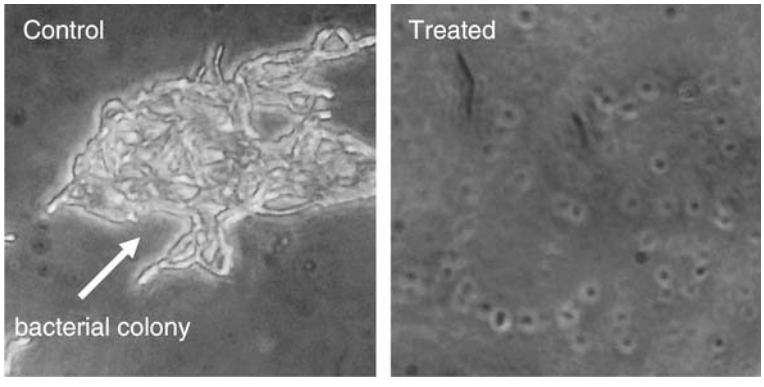

Fig. 5 Untreated (*left*) and treated (*right*) polyurethane film. The treated sample does not support *E. coli* growth while the untreated sample is significantly colonized. Film roughness on the untreated sample is visible but also present in the control under the bacterial colony

This biomimetic approach will prove to be extremely versatile.

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