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Design and surface immobilization of short anti-biofilm peptides

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ABSTRACT

Short antimicrobial peptides are essential to keep us healthy and their lasting potency can inspire the design of new types of antibiotics. This study reports the design of a family of eight-residue tryptophan-rich peptides (TetraF2W) obtained by converting the four phenylalanines in temporin-SHf to tryptophans. The temporin-SHf template was identified from the antimicrobial peptide database (http://aps.unmc.edu/AP). Remarkably, the double arginine variant (TetraF2W-RR) was more effective in killing methicillin-resistant *Staphylococcus aureus* (MRSA) USA300, but less cytotoxic to human skin HaCat and kidney HEK293 cells, than the lysine-containing dibasic combinations (KR, RK and KK). Killing kinetics and fluorescence spectroscopy suggest membrane targeting of TetraF2W-RR, making it more difficult for bacteria to develop resistance. Because established biofilms on medical devices are difficult to remove, we chose to covalently immobilize TetraF2W-RR onto the polyethylene terephthalate (PET) surface to prevent biofilm formation. The successful surface coating of the peptide is supported by FT-IR and XPS spectroscopies, chemical quantification, and antibacterial assays. This peptide-coated surface indeed prevented *S. aureus* biofilm formation with no cytotoxicity to human cells. In conclusion, TetraF2W-RR is a short Trp-rich peptide with demonstrated antimicrobial and anti-biofilm potency against MRSA in both the free and immobilized forms. Because these short peptides can be synthesized cost effectively, they may be developed into new antimicrobial agents or used as surface coating compounds.

Statement of Significance

It is stunning that the total deaths due to methicillin-resistant *Staphylococcus aureus* (MRSA) infection are comparable to AIDS/HIV-1, making it urgent to explore new possibilities. This study deals with this problem by two strategies. First, we have designed a family of novel antimicrobial peptides with merely eight amino acids, making it cost effective for chemical synthesis. These peptides are potent against MRSA USA300. Our study uncovers that the high potency of the tryptophan-rich short peptide is coupled with arginines, whereas these Trp- and Arg-rich peptides are less toxic to select human cells than the lysine-containing analogs. Such a combination generates a more selective peptide. As a second strategy, we also demonstrate successful covalent immobilization of this short peptide to the polyethylene terephthalate (PET) surface by first using a chitosan linker, which is easy to obtain. Because biofilms on medical devices are difficult to remove by traditional antibiotics, we also show that the peptide coated surface can prevent biofilm formation. Although rarely demonstrated, we provide evidence that both the free and immobilized peptides target bacterial membranes, rendering it difficult for bacteria to develop resistance. Collectively, the significance of our study is the design of novel antimicrobial peptides provides a useful template for developing novel antimicrobials against MRSA. In addition, orientation-specific immobilization of the same short peptide can prevent biofilm formation on the PET surface, which is widely used in making prosthetic heart valves cuffs and other bio devices.

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1. Introduction

In 1928, Alexander Fleming serendipitously discovered penicillin. This discovery opened the golden age of modern antibiotics [1]. However, a resistant staphylococcal strain was identified only four years after the large-scale production of penicillin in the 1940s. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains that are resistant to multiple antibiotics first appeared in clinical settings. Subsequently, resistant Staphylococcal strains were also isolated from the communities [2,3]. MRSA infections can cause life-threatening endocarditis, pneumonia, septicemia, septic arthritis, and osteomyelitis. The community-associated MRSA USA300 strain is responsible for the majority of the skin and soft tissue infection [4]. It is stunning that the total deaths due to MRSA infections are comparable to the deaths caused by human immunodeficiency virus type 1 (HIV-1) [5,6]. As a consequence, novel therapeutic compounds are urgently needed to control MRSA.

Natural antimicrobial peptides (AMPs) are host defense molecules identified in various organisms ranging from bacteria to humans. The majority of these molecules contain 10–50 amino acids with net charges 0–+7 and hydrophobic contents 31–70% [7,8]. AMPs can rapidly kill invading pathogens by disrupting membranes, rendering it difficult for pathogens to develop resistance. Moreover, multiple types of AMPs could be expressed in humans to control pathogens by different mechanisms [9]. In addition, these molecules can also regulate immune responses. All these properties make them appealing candidates for developing new therapies.

There is a high interest in linear AMPs because such peptides can be readily synthesized. Previously, we attempted to define the minimal sequence requirements for helical AMPs. We found it possible to convert a 10-residue non-toxic bacterial membrane anchor to a 13-residue antibacterial peptide, by extending the helix [10]. We also identified KR-12, which corresponds to the minimal antimicrobial region of human cathelicidin LL-37 based on NMR structural studies [11,12]. Interestingly, we also obtained 13-residue peptides against methicillin-resistant *S. aureus* (MRSA) with *in vitro* and *in vivo* efficacy based on the antimicrobial peptide database (APD) [13,14]. These results imply that approximately 12–13 residues are required for helical AMPs.

This study intends to design even shorter peptides (<10 amino acids), as a template for developing alternative antibiotics or antibiofilm surfaces. This is because a short peptide can be made cost effectively. In addition, shorter peptides contain less units to optimize, reducing the R & D cost for industry. We first identified a short peptide template with the aid of the APD database [8]. The temporin-SHf template possesses the highest phenylalanine content with moderate activity against MRSA [15]. To enhance the peptide activity, we hypothesize that replacements of four phenylalanines (F) in temporin-SHf with tryptophans (W) will achieve this goal because tryptophans are known to prefer membrane interfaces [16–18]. Based on these four replacements, the new peptide family is named as TetraF2W. Among the series of peptides designed, TetraF2W-RR with a pair of arginines, shows higher activity against MRSA USA300 than other lysine-containing analogs. Interestingly, the double arginine peptide is less cytotoxic to human skin and kidney cells. In addition, it is possible to covalently immobilize the most potent peptide to the polyethylene terephthalate (PET) surface, opening another possible use of these short peptides in preventing biofilm formation on medical devices, where different microbes may exist in the same community.

2. Materials and methods

2.1. Chemicals, peptides and surfaces

All the peptides in this study were chemically synthesized and purified to >95% (Genemed Synthesis, TX). The peptide used for surface immobilization was >90% pure. Peptides were solubilized in distilled water and their concentrations were determined by UV spectroscopy based on the Trp absorbance at 280 nm [19]. The PET surfaces were purchased from Goodfellow Corporation (PA, USA). All chemicals used in the immobilization were of analytical grade and purchased from Sigma (MO, USA) unless specified.

2.2. HPLC retention time measurements

The retention time of the peptide was measured on a Waters HPLC system equipped with an analytical reverse-phase Vydac C18 column (4.6 × 250 mm). The peptide detected at 220 and 280 nm was eluted with a gradient of acetonitrile (containing 0.1% TFA) from 5% to 95% at a flow rate of 1 mL/min [14].

2.3. Peptide parameter calculations

Peptide molecular weight, net charge, hydrophobic content, and Boman index were calculated using the prediction tool of the APD [8].

2.4. Bacterial strains

The bacterial strains used here included Gram-positive bacteria *Staphylococcus aureus* USA300 LAC, *Staphylococcus epidermidis* 1457, *Bacillus subtilis* 168, and Gram-negative *Escherichia coli* K12. Clinical strains of *S. aureus*, including Mu50, UAMS-1 and Newman, were also evaluated.

2.5. Antibacterial assays

The antimicrobial activity of peptides was evaluated using a standard broth microdilution protocol as described previously [12]. In brief, exponential phase bacteria at 10^6 CFU were incubated with serially diluted peptides overnight for ~20 h at 37 °C. The plates were read on a ChroMate 4300 Microplate Reader at 630 nm (GMI, Ramsey, MN). The minimal inhibitory concentration (MIC) was defined as the lowest peptide concentration that fully inhibited bacterial growth. In addition, the effects of human serum (up to 10%), pH (6.8, 7.4 and 8.0) and 150 mM sodium chloride (NaCl) on MICs against *S. aureus* USA300 were also evaluated as described [20].

The minimal bactericidal concentration (MBC) was determined by plating 50 μL of the content in the clear wells of the above MIC assays. MBC was determined on the petri dishes without bacterial colony formation after overnight growth [21].

2.6. Killing kinetics

Killing kinetic experiments were conducted similar to antibacterial assays described above with the following modifications [22]. Aliquots of cultures (10^5 CFU) treated with TetraF2W peptides were taken at 15, 30, 50, 90, and 120 min, diluted 100-fold, and plated on Luria-Bertani (LB) agar plates. Colonies were counted after overnight incubation at 37 °C.