Transformation of Human Cathelicidin LL-37 into Selective, Stable, and Potent Antimicrobial Compounds

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*Supporting Information

ABSTRACT: This Letter reports a family of novel antimicrobial compounds obtained by combining peptide library screening with structure-based design. Library screening led to the identification of a human LL-37 peptide resistant to chymotrypsin. This D-amino-acid-containing peptide template was active against Escherichia coli but not methicillin-resistant Staphylococcus aureus (MRSA). It possesses a unique nonclassic amphipathic structure with hydrophobic defects. By repairing the hydrophobic defects, the peptide (17BIPHE2) gained activity against the ESKAPE pathogens, including Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter species. In vitro, 17BIPHE2 could disrupt bacterial membranes and bind to DNA. In vivo, the peptide prevented staphylococcal biofilm formation in a mouse model of catheter-associated infection. Meanwhile, it boosted the innate immune response to further combat the infection. Because these peptides are potent, cell-selective, and stable to several proteases, they may be utilized to combat one or more ESKAPE pathogens.

A hallmark of emerging difficult-to-treat clinical superbugs is their ability to "escape" the action of multiple traditional antibiotics, in part due to biofilm formation in the host. The ESKAPE pathogens include Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter species.1 According to the Centers for Disease Control and Prevention, the six ESKAPE bacterial species cause two-thirds of health care-associated infections, leading to 99,000 deaths annually in the United States. S. aureus infections alone cause a comparable number of deaths as human immunodeficiency virus type 1 (HIV-1).2 Antimicrobial peptides (AMPs) are host defense molecules that have maintained antimicrobial activity for millions of years by keeping pace with the evolution of bacterial resistance mechanisms. This feature makes them appealing candidates for developing new antibacterial compounds.3–5

The protective effect of human LL-37 against infection is underscored by both clinical observations and recent findings from animal models.6–8 Therefore, there is growing interest in its medical use. Interestingly, sunlight triggers the biosynthesis of dihydroxyvitamin D in skin that binds the vitamin D receptor to initiate peptide expression.6 At the moment, clinical trials of vitamin D as an anti-infective agent have not demonstrated efficacy.9 Another avenue is to administer LL-37 at infected sites. Cationic LL-37 possesses a long amphipathic helical structure covering residues 2–31 that recognizes and disrupts anionic bacterial membranes (Figure 1a).10–12 To minimize the cost for peptide synthesis, several laboratories reported the identification of active fragments, including GF-17 corresponding to the major antimicrobial region (residues 17–32).12–15 Since these natural peptides lack sufficient stability to proteases, this study intends to re-engineer human LL-37 into peptide analogues with potency, stability, and cell selectivity against superbugs.

To identify protease-resistant peptide templates, we screened 30 synthetic peptides. A standard microdilution assay
Protocol 15 was modified to include proteases into the duplicated wells with high concentrations of peptide so that both antimicrobial activity and protease stability could be evaluated simultaneously. In this setup, most of the LL-37 peptides showed bactericidal effects in the absence of the protease but became inactive in the presence of chymotrypsin after overnight incubation at 37 °C (peptide:protease molar ratio of 40:1). However, GF-17d3, a GF-17-derived peptide with incorporation of three D-amino acids at positions 20, 24, and 28 (numbered as in LL-37), retained antimicrobial activity against Escherichia coli K12 (Supplementary Table 1). In contrast, another two peptide analogues containing one to two D-amino acids at positions 20 or both 20 and 24 were not stable. Shai and colleagues also found peptide stability improvement due to the incorporation of D-amino acids into other peptides. 16 Compared to GF-17, GF-17d3 lost antibacterial activity against S. aureus USA300. 17,18 This protease-resistant template was selected for subsequent engineering of peptides for activity against S. aureus as well as other ESKAPE pathogens.

We conducted a rational design on the basis of the 3D structure of GF-17d3. NMR structural analysis revealed that the three D-amino acids distorted the regular helical backbone structure of GF-17 (Figure 1b)15 into a novel nonclassic amphipathic structure of GF-17d3 (Figure 1c). 15 The noncoherent packing of the GF-17d3 side chains caused a hydrophobic defect in the structure (Figure 1c,d, arrow), leading to reduced hydrophobicity. We hypothesized that the antimicrobial activity of this peptide 17F2 (sequence GX1KR LVRQL KDX2 LRLV-amide, see Supplementary Table 1) could be enhanced by filling in the hydrophobic cavity (Figure 1d, arrow) with a larger hydrophobic side chain.

Table 1. Minimal Inhibitory Concentration (MIC), Cytotoxicity, and Hydrophobicity of a Series of Peptides Designed Based on Human Cathelicidin LL-37

| Peptide     | MIC (μM) | EF<sup>a</sup> | SA | KP | AB | PA | EC | HPLC (μM)<sup>b</sup> | t<sub>50</sub> (min)<sup>c</sup> |
|-------------|----------|----------------|----|----|----|----|----|--------------------------|-----------------------------|
| 17F2        | >100     | >100           | >100| 6.2–12.5 | 100 | 25 | >900                     | 9.95                        |
| 17mF-F      | 25–50    | 25             | 50 | 3.1–6.2 | 25  | 25 | >900                     | 10.32                       |
| 17F-Naph    | 3.1      | 25             | 25 | 3.1 | 12.5 | 12.5 | >900 | 10.56                   |
| 17mF-Naph   | 3.1      | 6.2            | 12.5| 3.1 | 6.2–12.5 | 6.2 | 500 | 10.99                   |
| 17Naph-mF   | 3.1      | 6.2            | 12.5| 3.1 | 6.2–12.5 | 6.2 | 500 | 10.94                   |
| 17BIPHE     | 12.5     | 12.5           | 25 | 3.1 | 12.5 | 12.5 | >900 | 10.55                   |
| 17BIPHE2    | 3.1      | 3.1            | 3.1| 3.1 | 6.2 | 3.1 | 225  | 11.26                     |

<sup>a</sup>Abbreviations used: EF, E. faecium ATCC51559; SA, S. aureus USA300 LAC; KP, K. pneumonia ATCC13883; AB, A. baumannii B2367-12; PA, P. aeruginosa PAO1; EC, E. cloacae B2366-12.  14 The peptide concentration (μM) that causes 50% lysis of human erythrocytes.  15 HPLC retention time of the peptide.
antimicrobial activities of these peptide analogues were evaluated using the standard microdilution assays, and the minimal inhibitory concentrations (MIC) against a panel of the ESKAPE pathogens are provided in Table 1. 17F2 inhibited *E. coli*, *A. baumannii*, and *E. cloacae* (MIC 6.2–25 μM) but not *S. aureus*, *E. faecium*, *K. pneumoniae*, or *P. aeruginosa* until 100 μM or higher. When X1 was replaced with 4-trifluoromethylphenylalanine (mF), the resultant peptide 17mF-F displayed MICs in the range of 25–50 μM against most of the ESKAPE strains and 3.1–6.2 μM against *A. baumannii*. We also aimed to identify a second site for activity enhancement. When X2 of 17F2 was mutated to 2-naphthylalanine (Naph), the new peptide 17F-Naph became active against *E. faecium* and *A. baumannii* (MIC 3.1 μM). When both the above substitutions were made at X1 and X2, the activity of the resultant peptide 17mF-Naph was further increased, especially against MRSA USA300 (MIC 6.2 μM). To compare the two sites, we swapped these two substitutions at positions X1 and X2 to obtain 17Naph-mF. Interestingly, 17mF-Naph and 17Naph-mF have an essentially identical antibacterial activity spectrum based on MICs (Table 1). Additional substitutions at position X1 alone or at both X1 and X2 of the 17F2 template. While 17BIPHE with one replacement was more active against *S. aureus* than 17mF-F, 17BIPHE2 with two replacements showed the highest antibacterial activity against all of the ESKAPE pathogens (MIC 3.1–6.2 μM). Killing kinetics assays revealed that 17BIPHE2 was able to eliminate *P. aeruginosa PAO1* in 30 min and *S. aureus USA300* in 90 min (Supplementary Figure 1). The killing abilities of these peptides are essentially proportional to their MIC values as well as retention times (a measure of hydrophobicity) on a reverse-phase HPLC column. Hence, our structure-based design produced a family of antimicrobials against the ESKAPE pathogens (Table 1).

In developing peptide-based antimicrobials, it is essential to minimize potential cytotoxicity to human cells. We found that 17F2 and various single substitution variants displayed poor hemolytic ability, with a 50% hemolysis concentration (HL50) greater than 900 μM (Table 1). However, the substitution swapped pair, 17mF-Naph and 17Naph-mF, gave different HL50 concentrations of 500 and 950 μM, respectively. 17BIPHE2, the most potent peptide, showed an HL50 of 225 μM. Relative to the MIC value (3.1 μM) that inhibited the majority of these ESKAPE pathogens, we obtained a cell selectivity index (i.e., the ratio between HL50 and MIC) of 72, meaning that 17BIPHE2 is not cytotoxic at the MIC. If the
same MIC of 3.1 μM was used, the cell selectivity indices for 17mF-Naph and 17Naph-mF were 161 and 306, respectively. This is extremely interesting considering that 17Naph-mF, with a better selectivity index, is also more efficient in killing MRSA than 17mF-Naph (Supplementary Figure 1). Thus, there is an excellent therapeutic window for us to utilize these novel compounds to treat infections caused by the ESKAPE pathogens.

To validate the stability of the engineered peptides, we followed the degradation kinetics of 17BIPHE2 in the presence of chymotrypsin by SDS-PAGE. While a GF-17 analogue was rapidly digested in 4 h (t1/2 < 0.5 h), the level of intact 17BIPHE2 remained constant during this time period (Figure 1e). Furthermore, 17BIPHE2, but not GF-17, was also stable for at least 24 h in the presence of S. aureus V8 protease or fungal protease K (Supplementary Figure 2). We conclude that 17BIPHE2 is stable to the action of several proteases.

To shed light on the killing mechanism of 17BIPHE2, we conducted flow cytometry studies. When bacterial membranes are compromised, flow cytometry can follow the cellular entry of the nonpermeable dye TO-PRO3, which upon binding bacterial DNA leads to a rapid increase in fluorescence. At 80 μM, this was indeed the case with 17BIPHE and 17BIPHE2 (Figure 1f), which were comparable to GF-17, a peptide known to target bacterial membranes. At a lower peptide concentration (20 μM), the killing ability of peptides was found to be inversely proportional to the MIC (Supplementary Figure 3). To validate membrane damage, we used transmission electron microscopy (TEM) to directly visualize possible damage to bacteria. While untreated bacteria possessed a uniform membrane architecture (Figure 1g), the addition of 17BIPHE2 caused bacterial lysis (Figure 1h).

Bacterial membrane damage raised the possibility that 17BIPHE2 might also enter cells and bind DNA. To provide evidence for this, we used gel retardation experiments. While 17F2 did not bind to DNA in this assay, 17BIPHE2 at 12 μM was trapped in the wells (Figure 1i). At the same concentration, both 17BIPHE and 17F-Naph, with a single substitution, retarded DNA to a similar degree. 17mF-Naph and 17Naph-mF, with the two substitutions swapped, behaved similarly. Thus, this family of peptide analogues possesses a wide range of DNA retarding abilities. Interestingly, the extent of DNA retardation (presumably the amount of peptide associated with DNA) is proportional to bacterial killing ability, with 17BIPHE2 being the strongest and 17F2 the poorest. A similar trend was observed using the DNA purified from P. aeruginosa PAO1 (Supplementary Figure 4). These correlations suggest that DNA binding could be part of the mechanism of bacterial killing.

The in vivo efficacy of 17BIPHE2 against S. aureus USA300 LAC was examined using a mouse model of catheter-associated biofilm infection as previously described. Animals used in this study were cared for by following institutional guidelines. Peptide treatment was initiated at the time of infection (time 0), by directly injecting 200 μg peptide, dissolved in PBS, into the catheter followed by additional administration of 200 μg peptide injected subcutaneously at four different sites surrounding the catheter at 24 and 48 h postinfection. Bacterial titers associated with the catheter and surrounding host tissue were evaluated at day 3 (Figure 2a–c) and day 14 postinfection (Figure 2c–e) to determine the impact of peptide treatment on bacterial burdens. Mice treated with 17BIPHE2 exhibited a significant decrease in bacterial titers on the catheter surfaces (Figure 2a,d) as well as surrounding tissues compared to vehicle-treated animals (Figure 2b,e). Importantly, early peptide treatment was key to preventing S. aureus biofilm establishment, since minimal bacterial growth was detected at day 14 following infection even though the last dosing occurred at 48 h (Figure 2b,e). As a negative control, LL-23V9, a peptide designed based on the N-terminal 23 residues of LL-37, which is inactive against S. aureus USA300 in vitro, was unable to decrease bacterial burdens on biofilm-infected catheters in vivo (Figure 2c).

We were also interested in determining whether the peptide had any impact on the host immune response by measuring chemokine levels with and without peptide treatment. 17BIPHE2 significantly increased CCL2 (MCP-1) and CXCL10 (IP-10) expression and reduced IL-10 at day 3 postinfection (Figure 2f–h). This observation is remarkable given our previous findings that bacterial biofilm formation strongly suppressed CCL2 and increased IL-10 in the same animal model. Increased CCL2 expression coincided with significantly heightened monocyte recruitment at day 3, which returned to baseline at day 14 postinfection (Figure 2i). Thus, one mechanism whereby 17BIPHE2 treatment may deter S. aureus biofilm development is through rapid monocyte recruitment. In addition, the expression of CXCL10 might also directly inhibit MRSA based on its known antimicrobial activity.

In conclusion, we succeeded in engineering human cathelicidin LL-37 into potent compounds by combining peptide library screening with structure-based design (Figure 1). Our engineered peptides have multiple desired features: in vitro and in vivo efficacy against community-associated MRSA USA300, stability to the action of multiple proteases, and good cell selectivity indices (72–306). The most potent compound 17BIPHE2 appears to kill bacteria by damaging bacterial membranes, which may also involve the access and binding to the intracellular bacterial DNA. Apart from bacterial killing in vivo, 17BIPHE2 can augment host innate immunity to further combat bacterial infection. Combined, these properties make 17BIPHE2 an attractive candidate for the treatment of complicated clinical infections involving biofilms of one or more ESKAPE pathogens, especially in implanted medical devices that cost millions of dollars per year in the USA alone.

### METHODS

#### Peptides and Hydrophobicity Measurements

All peptides were chemically synthesized at purity of >95% (Genemed Synthesis Inc.). Peptide hydrophobicity was estimated by measuring its retention time on a Waters HPLC system equipped with an analytical reverse-phase VydaC C18 column (250 mm × 4.6 mm) as described.

#### Antibacterial Activity Assays

The antibacterial activities of the peptides were determined using the standard broth microdilution method as described.

#### Hemolytic Assays

Peptide hemolysis was assayed using an established protocol.

#### Transmission Electron Microscopy

S. aureus USA300 were grown in LB medium at 37 °C to the mid-logarithmic phase. The samples were prepared as described previously and observed in an FEI Tecnai G2 TEM operated at 80 kV accelerating voltage at the University of Nebraska Medical Center (UNMC).

#### Flow Cytometry

Membrane permeation analysis was performed by using the BacLight bacterial membrane potential kit (Invitrogen) as described on a FACSSAna flow cytometer. In short, logarithmic growth-phase cultures of S. aureus USA300 (OD₆₀₀ 0.6–1.0), after being washed twice and resuspended in PBS (0.2 μM filter-sterilized) (∼5 × 10⁶ CFU/mL), were treated at 80 μM concentration of the
peptides in Figure 1f and 20 μM in Supplementary Figure 3. Bacteria were then treated for 10 min with 3,3′-diethyloxocarbocyanine iodide [DiOC(3) (10 μM)]/TO-PRO-3 (100 nM) dye mixture. Data were analyzed with FlowJo software.

Peptide Stability to the Action of Proteases. Peptide stability was evaluated based on our published protocol17 with one modification. Aliquots (10 μL) of the reaction solutions (peptide/protease molar ratio, 40:1) in 10 mM PBS buffer (pH 8) at 37 °C were taken at 0, 6, and 24 h and immediately mixed with 10 μL of 2× SDS loading buffer followed by boiling for 5 min to stop the reaction. Samples were analyzed using 5% stacking/18% resolving tricine gel.

*S. aureus* Biofilm Infection Model. Male C57BL/6 mice, 6–8 weeks old, were anesthetized with tribromoethanol (avertin), and the skin was shaved and scrubbed with povidone-iodine. A small subcutaneous (sc) incision was made in the left flank and a blunt probe was used to create a pocket for insertion of a sterile 14-gauge Teflon catheter 1 cm in length. The incision was sealed and 1,000 CFU USA300 LAC::lux in 20 μL of sterile PBS was slowly injected through the skin into the infected catheter lumen. Animals were initially treated with 200 μg peptide, dissolved in PBS, injected into the catheter at the time of infection (time 0) followed by 200 μg peptide injected subcutaneously at four different sites surrounding the catheter at 24 and 48 h postinfection. At days 3 and 14 postinfection catheters were removed in order to quantitate bacterial burdens. Catheters were sonicated in 1 mL of PBS to dissociate the biofilm from the intra- and extraluminal surfaces. The tissue surrounding the catheter was also removed, weighed, and homogenized in 500 μL homogenization buffer. Bacterial titers associated with catheters and surrounding tissues were quantified by plating on blood agar plates. To compare the expression of inflammatory mediators associated with biofilm-infected tissues treated with our novel antimicrobial peptides, a mouse microbead array or ELISA detection kit was utilized according to the manufacturer’s instructions. Results were normalized to the amount of total protein recovered to correct for differences in tissue sampling size.

■ ASSOCIATED CONTENT

Supporting Information

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Notes

The authors declare no competing financial interest.

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