Anti-Staphylococcal Biofilm Effects of Human Cathelicidin Peptides

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Supplementary information

Materials and methods

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Material and methods

Strains and media

The bacterial strains used in this study are *Staphylococcus aureus* USA200, USA300, USA400, Mu50, Newman, and UAMS-1. Transposon mutants were obtained from the Nebraska Transposon Mutant Library (NTML).[27] Tryptic soy broth (TSB) growth medium for bacterial growth was obtained from BD Bioscience MD, USA. Daptomycin and vancomycin were obtained from Sigma, USA. In all the assays for daptomycin, the medium was supplemented with 2 mM Ca^{2+}. The peptides used were synthesized chemically by F-moc solid phase synthesis and purified to >95% (GeneMed, TX).

Measurement of minimal inhibitory concentration (MIC)

The assay was performed as described previously with minor modifications.[25] In short, the bacterial strains were inoculated overnight. These cultures were then freshly inoculated and allowed to reach the exponential growth phase. The cultures were diluted accordingly to 10^6 CFU/mL in 25% TSB and 90 µL of this solution was added to a 96-well microplate (Costar, Corning, NY) containing 10 µL of serially diluted peptide solutions (in water) and incubated overnight at 37ºC for 20 h. The growth as a function of absorbance was read with a CHROMATE microplate reader at 630 nm. The wells containing sterilized water instead of peptide served as the positive control and the media was used as the negative control.

Inhibition of bacterial attachment

Overnight cultures of *S. aureus* USA300 were grown in 25% TSB media. 180 μL of this high density culture was added to each well of the microtiter plates containing 20 μL of 10× peptide solution. Media containing bacteria and water was treated as positive control while only media with water served as the negative control. The plates were then incubated at 37°C for 1 h. Media was than pipetted out and the wells were washed with normal saline to remove the non-adherent planktonic cells. Calorimetric quantitation of the inhibition of biofilm attachment was done by XTT [2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2*H*-tertazolium-5-carboxanilide] and phenazine methosulfate (PMS) assay following manufacture instructions with minor adjustments (Sigma-Aldrich, MO, USA). 180 μL of fresh TSB and 20 μL of XTT solution were added to each well and the plates were again incubated for 2 h at 37^{o}C. Absorbance at 450 nm (only media with XTT containing wells served as the blank) was obtained using a Chromate^{TM} microtiter plate reader. Percentage of biofilm growth for each case was plotted assuming 100% biofilm growth is achieved in the bacterial wells without peptide treatment.

Inhibition of biofilm formation

The potency of the peptides to inhibit the formation of biofilms was evaluated by following an established protocol with modifications.[14] In short, overnight *S. aureus* USA300 cells were inoculated in TSB and allowed to grow to the exponential phase. A bacterial density of 10^8 CFU/mL was prepared in 25% TSB medium and 180 μL was delivered to flat-bottomed 96-well polystyrene microtiter plates (Corning Costar Cat No. 3595) containing 20 μL of serially diluted 10× peptides and antibiotics solution. Media containing bacteria and water was treated as positive control while the media treated with

water served as the negative control. The plates were then incubated at 37^{o}C for 24 h. Biofilm was quantified by the same method described above in the attachment experiment.

Disruption of established biofilms

A cell density of *S. aureus* USA300 (10^5 CFU/mL) was made from logarithmic growth phase. 200 µL was placed into each well of the 96 well microtiter plates. The plates were incubated at 37^{o}C for 24 h to allow biofilm formation. Media was then pipetted out and the biofilms were washed with normal saline to remove the planktonic cells. 10X peptide solution alone or combination with antibiotics in 20 µL volume was added to 180 µL of fresh 25% TSB media. Plates were further incubated at 37^{o}C for another 24 h. Quantification of the disruption of the biofilm by the peptide was done using XTT and followed the same methods described earlier.

Live and dead staining assay of established biofilms using confocal laser scanning microscopy

S. aureus USA300 (10^5 CFU/mL) was made from the exponential phase bacteria in TSB. 2 mL of the culture was added to the chambers of cuvette (Borosilicate cover glass systems, Nunc Cat. No: 155380) and was incubated at 37^{o}C for 24 h to establish biofilms. After removing the media, the chambers were washed three times with normal saline. To disrupt the established biofilms, 200 µL of 10× peptide stocks (125 µM) was added followed by 1800 µL 25% TSB. The control cuvette was treated with water instead of peptide. The cuvettes were incubated for another 24 h at 37^{o}C. Chambers were then

washed with normal saline. For evaluation under confocal laser scanning microscope, the remaining established biofilms were stained with 10 µL of LIVE/DEAD kit (Molecular Probes, Life technologies, USA) according to the manufacturer's instructions. The samples were examined with a confocal microscope (Zeiss 710) and the data were processed using Zen 2010 software.

Measurement of peptide hemolysis

In brief, human blood was obtained from the UNMC Blood Bank and washed three times (800 g, 10 min) with normal saline to remove plasma. A 2% human red blood cell (hRBC) suspension was then prepared in normal saline. 90 µL of the 2% hRBC was added to 10 µL of serially diluted peptide solutions and was incubated at 37ºC for one hour. After centrifugation on an Eppendorf bench-top centrifuge 5415D at 13,000 rpm for 5 min, 80 µl of the supernatant was carefully transferred to a fresh 96-well microplate (Costar, Corning, NY). The absorbance of the plate was read at 545 nm to detect the amount of hemoglobin released. Percentage of cell lysis was calculated based on the extent of hemoglobin released, where 100% release is assumed due to the treatment of 1% Triton X-100 and 0% release is assumed in saline.

Cellular cytotoxicity assessment

HaCaT cells from American Type Culture Collection (ATCC) were maintained in DMEM High Glucose media containing 4 mM L-Glutamine (NyClone), 100 U/mL penicillin, 100 µg/mL streptomycin (pen/strep) (Life Technologies), and 10% (v/v) inactivated fetal bovine serum (FBS) (NyClone). Cells were grown in at 37 °C in the

atmosphere of 5% CO_2. At 80% confluency, cells were detached from the culturing dish by treating with 0.025% trypsin-EDTA (NyClone). The effect of peptide on the cell viability was estimated by using the MTS assay according to manufacturer's protocol (MTS, CellTiter96 AQ One Solution Cell Proliferation Assay, Promega) with minor modifications. In short, cells were cultured in flat bottomed 96-well microtiter plates (Corning Life Science) at a seeding density of 20,000 cells/well. A confluence of 70–80% was achieved after 20–24 h of cultivation. 10 µL of the peptide solution was then added to achieve the final concentration range of 10-80 µM. After incubation at 37°C for 1 h, 10 µL MTS, CellTiter96 was further added to the wells and the plates were incubated for another 2 h at 37°C. Finally, the absorbance was measured on a ChroMate reader (Awareness Technology) at 492 nm. Culture media and 0.2% SDS treated wells were used as negative and positive controls, respectively.

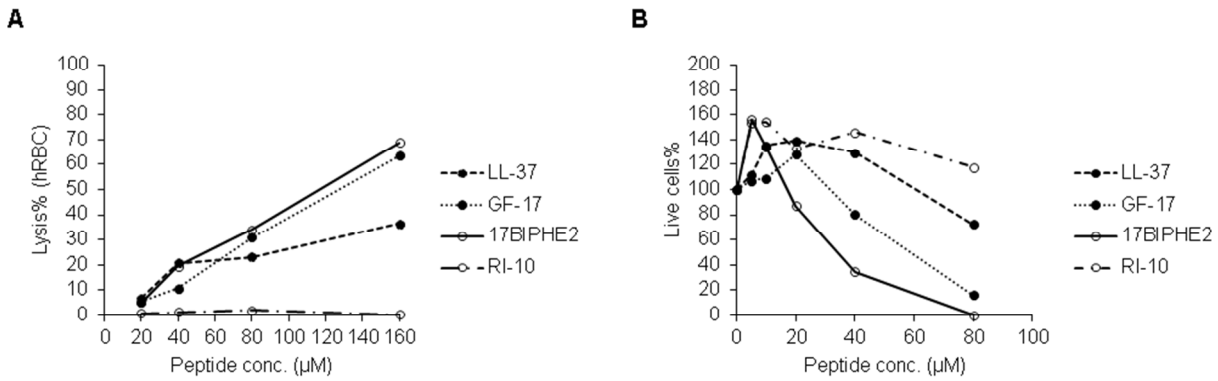

Figure S1. Cell cytotoxicity assays of LL-37, GF-17, 17BIPHE2 and RI-10 to (A) 2% v/v human red blood cells (hRBCs) and (B) human HaCat cells. **These figures indicate that the cytotoxicity of these peptides is in the following order: RI-10 < LL-37 < GF-17 < 17BIPHE2. However, 17BIPHE2 is not toxic to human cells when treated at the MIC (3.1 μM) that kills *S. aureus***.