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Supporting Information for

Synthetic peptides that form nanostructured micelles have potent antibiotic and anti-biofilm activity against polymicrobial infections.

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SI Materials and Methods

Bacteria strains and growth conditions. Bacterial strains (listed in Table S2) were provided by the First Affiliated Hospital of Sun Yat-sen University. *P. aeruginosa, S. aureus, E. coli, K. pneumoniae,* and *A. baumannii* were cultured in Tryptic Soy Broth (TSB) at 37°C. *S. pneumoniae* was cultured in Brain-Heart Infusion (BHI) supplemented with 5% defibrillated sheep blood at 37°C.

AMPs synthesis. Peptides were synthesized by GL Biochem Ltd. (Shanghai, China) using a solid-phase peptide synthesis strategy (SPPS). Briefly, peptide chain synthesis was performed via 9-fluorenylmethyloxycarbonyl (Fmoc)-based SPPS. Fmoc-Arg (Pbf)-OH, Fmoc-Lys(Boc)-OH, Fomc-Trp(Boc)-OH were sequentially coupled on AAM or MBHA resin. Coupled by 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU). Final deprotection was carried out with a solution of piperidine in DMF, and the resin was cleaved by a cleavage cocktail containing trifluoroacetic acid, triisopropylsilane, 1,2-ethanedithiol, and H_2O for 120 min. The solution was evaporated under nitrogen gas, and the crude product was precipitated with cold ether. The crude peptide was purified by RP-HPLC, the final purities of the peptide were >95%, and the molecular weights were analyzed by MALDI-TOF MS (Linear Scientific Inc., USA).

Circular dichroism (CD) spectroscopy. The peptides were dissolved in 10 mM PBS at a final concentration of 64 μM. A method modified from Li *et al* (1) was used to promote uniform self-assembly. Briefly, peptide samples were sonicated for 30 min and left at room temperature for 2 days to allow self-assembly to occur. CD spectra were detected on a Chirascan-plus spectrometer (Applied Photophysics, London, England) at 25°C using quartz cuvettes with a 10 mm path length. The spectrum of each AMP at 195-250 nm was recorded and scanned three times. The obtained CD spectra were then used to calculate the average residue molar ellipticity according to the formula: $\theta_M = (\theta_{obs} \times 1000)/(c \times l \times n)$, where θ_M represents the residue molar ellipticity ((deg cm2) dmol−1), θ_{obs} is the buffer-corrected ellipticity (mdeg) of the actual measurement, c is the concentration of AMP (M), l is the optical path (mm), and n is the number of amino acids.

MIC measurement. The antimicrobial activity of AMPs was measured according to a previously reported method (2). Bacterial cells were cultured at 37°C in TSB (or BHI/LB) to log-phase growth and diluted to OD_{600} = 0.4, and then diluted 1000-fold with fresh TSB (or BHI/LB), except for experiments with *E. coli* Omnimax, in which the final diluted OD_{600} = 0.02**.** 50 μL Two-fold serial dilutions of AMPs in PBS with designated concentrations were added to sterile 96-well plates, which contained 50 μL the diluted bacterial suspension. Plates were incubated at 37°C for 18 h. The MIC was defined as the lowest concentration of AMP at which no bacterial growth was observed. Fresh TSB (or BHI/LB) plus PBS was used as a negative control and TSB (or BHI/LB) plus bacteria was used as a positive control. The assays were repeated for at least three times, each with three replicates.

Determining rates and degree of AMP or gentamicin resistance. The development of bacterial resistance against gentamicin and Peptide K6 was analyzed using the sequential passaging method. MIC assay for Peptide K6 and antibiotic was performed as noted above. After 18 h of incubation, the bacterial suspension in the well with half-MIC concentration was diluted to OD_{600} = 0.05 and then diluted 10-fold with fresh TSB. The diluted bacterial suspension was used for the next passage MIC determination. Sequential passages were carried out for 24 days.

Bactericidal assay of mixed cultures. The ability of Peptide K6 to kill bacteria was assessed by detecting bacterial survival upon Peptide K6 treatment at various exposure times. Briefly, *Pseudomonas aeruginosa* strain PAO1 and *Staphylococcus aureus* strain SP1 from the exponential phase were suspended in PBS to OD_{600} = 0.4 then diluted 1000-fold with fresh PBS, respectively, and mixed in equal volumes. Peptide K6 or gentamicin was added to the bacterial suspensions at final concentrations of MIC and 2× MIC. At various time points (0, 5, 15, 30, 60,

120, 180, 240, 300, and 360 min), the bacterial suspension was serially diluted in PBS and plated onto TSA. After 18 h incubation at 37°C, single colonies of microorganisms were counted and CFU/mL was calculated. The experiment was repeated three times and the mean value was calculated.

Scanning electron microscopy (SEM) characterization of peptides. For the preparation of SEM samples, 10 μL of Peptide K6 concentration of 4μM in PBS were dropped on tin foil after sonicating for 30 min and sprayed with gold after drying. All samples were observed using a GiminiSEM 500.

Clearance of established biofilm in 96-well plates. The clearing effect of Peptide K6 on biofilm formed by mixed bacteria was detected by crystal violet staining. Briefly, *P. aeruginosa* strain PAO1 and *S. aureus* strain SP1 from the exponential phase were diluted to 1×10^7 CFU/mL and 1×10^6 CFU/mL, respectively, and equal volumes were mixed to prepare a mixed bacterial suspension. 200 ul of the mixed bacterial inoculum was added to polystyrene 96-well round-bottom plates and then incubated together at 37°C for 24 h to establish biofilms. The medium was then removed and the 96-well plate was washed three times with PBS to remove planktonic bacteria. Peptide K6 or gentamicin with a final concentration of 2-32 μM in PBS was added to the 96-well plate. After 24 h incubation at 37°C, the planktonic bacteria were discarded and the 96-well plate was washed 3 times with PBS. Following drying at room temperature, 200 μL/well of 0.1% crystal violet staining solution was added to the plate for 30 min at 37°C. Then the plate was washed three times with PBS. After air-drying at room temperature, 200 μL/well of 95% ethanol was added and plates were incubated at 37°C for 30 min. Solubilized crystal violet was detected spectrophotometrically at its absorbance maximum (595 nm). The biofilm percentage is calculated based on the following equation: Biofilm (%) = $[(A - A_0)/(A_{untreated} - A_0)] \times 100\%$, where A represents the absorbance of the current well, A_0 represents the absorbance of the blank well, and $A_{untreated}$ represents the absorbance of the well with established biofilm treated by PBS. At least three experiments were performed with three replicates for each experiment.

Clearance of established biofilm in 5 ml tubes. *P. aeruginosa* strain PAO1 and *S. aureus* strain SP1 from the exponential phase were diluted to 1×10^7 CFU/mL and 1×10^6 CFU/mL, respectively, and equal volumes were mixed to prepare a mixed bacterial suspension. 1 ml of the mixed bacterial suspension was added to polystyrene 5 ml round-bottom tubes and then incubated together at 37°C for 24 h to establish biofilms. The medium was then removed and the 96-well plate was washed three times with PBS to remove planktonic bacteria. Peptide K6 or gentamicin with a final concentration of 2-32 μM in PBS was added to the 96-well plate. After 24 h incubation at 37°C, the planktonic bacteria were discarded and the 96-well plate was washed 3 times with PBS. Following drying at room temperature, 1 ml of 0.1% crystal violet staining solution was added to the tube for 30 min at 37°C. Then the plate was washed three times with PBS. After air-drying at room temperature, photos were taken.

Analysis of extracellular polymeric substances present in pre-formed biofilm. The scavenging effect of Peptide K6 on the established biofilm formed by mixed bacteria was analyzed by staining extracellular polymeric substances and visualized by fluorescence microscopy. Briefly, mixed bacterial suspensions were prepared as mentioned above and incubated at 37°C for 24 h in 24-well plates with cell-climbing slices to form biofilms. Following incubation, the mixed biofilm slices were treated with Peptide K6 (16 μM in PBS), gentamicin (16 μM in PBS) or PBS at 37°C for 3 h. After washing adherent biofilms with PBS, the cell slides were incubated with Hoechst (0.5 μg/mL) for nucleic acids, SYPRO orange (Sigma-Aldrich #S5692) for proteins, and FITC-ConA (5 μg/mL) for carbohydrates, incubated in dark for 15 min and then observed with a fluorescence microscope (Eclipse Ti2-E, Nikon).

Growth curves measurement of LamB-peptide expressing *E. coli*. Growth kinetics were essentially performed as previously described (3, 4). Briefly, E. coli Omnimax expressing peptides of interest as part of LamB were grown in a pre-culture for ~16 h in LB, 0.2% glucose with

aeration and shaking at 37°C. Pre-cultures were adjusted to OD600 = 0.05 in LB with 0.2% glucose or IPTG (0.01 mM, 0.1 mM, 1 mM). The OD600 was monitored every 10 min in a microplate reader at 37°C with shaking. For the presentation of data, the mean of at least four independent growth curves was plotted and area under curve (AUC) values were calculated by GraphPad prism version 9.4.1.

Microscopy assay on LamB-peptide expressing *E. coli*. O/N cultures of *E. coli* Omnimax were grown in LB. Bacteria were diluted 1:100 in LB and grown to early-log phase. Bacteria were mixed with 0.1 mM of peptides and after 10 minutes at 37°C transferred onto 1.5% agarose pads made in 0.5x D-PBS, Propidium Iodide (Life Technologies) was added directly into the melted agarose at 55 °C immediately before preparing the pads at a final concentration of 1 μg/ml. Images were recorded using a Nikon Ti-E inverted motorized microscope equipped with a Nikon Plan Apo 100X/1.4 NA Ph3 DM oil immersion objective lens with Type B immersion oil (Cargille Laboratories), Lumencor SPECTRA X3 light engine and a Zyla 4.2 CL 10 sCMOS camera. Phase and Propodium iodide fluorescence was collected by ET-mCherry (Chroma 49008) using Nikon NIS Elements 4.0. Further image analysis was performed in Fiji v 2.0.0-rc-54/1.51g (5).

Inhibition of biofilm formation measurement. The inhibitory effect of Peptide K6 on the biofilm formation of mixed bacteria was detected by crystal violet staining. Briefly, bacterial mixed inoculums were prepared as described above. 100 μL serial two-fold dilutions of Peptide K6 in PBS or gentamicin were added into a polystyrene 96-well round-bottom plate, containing 100 μL of the mixed bacterial suspension to a final concentration of 2-32 μM. After 24 h incubation at 37°C, crystal violet staining and absorbance were measured according to the method described above.

Swimming, swarming, and twitching motility assay. *P. aeruginosa* strain PAO1 in the logarithmic growth phase was diluted to 1×10^8 CFU/mL. For the swimming experiment, different concentrations of Peptide K6 (4 μM, 8 μM or 16 μM) were added to LB medium containing 0.3% agar, and the medium was added to a 6-well plate in 10mL/well for natural solidification. Subsequently, the bacterial suspension was acupuncture inoculated on the center of the LB agar plate and cultured at 37°C for 15 h. For the swarming experiment, different concentrations of Peptide K6 were added to LB medium containing 0.5% agar and added to a 6-well plate at 10 ml/well. After natural solidification, 1 μL of the bacterial suspension was inoculated onto the agar surface and incubated at 37°C for 15 h. For the twitching experiment, different concentrations of Peptide K6 were added to the LB medium containing 1% agar. After the medium was solidified, the LB agar-plastic interface was pierced with a toothpick dipped in bacterial suspension, and the plate was incubated at 37°C for 24 h. The diffusion diameter of bacterial cells was measured to assess their motilities. At least three experiments were performed and each experiment consisted of three replicates.

Scanning electron microscopy (SEM) characterization of biofilms. The pre-formed mixed biofilms were prepared as mentioned above and treated with PBS or Peptide K6 with final concentrations of 8 μM or 16 μM in PBS at 37°C for 3 h. Then, the following steps were modified by the previously reported method (6). Briefly, cell slides were washed three times with PBS to remove unattached bacteria. 2.5% (w/v) glutaraldehyde was added to fix the cells overnight at 4°C and the cell slides were washed three times with PBS to remove 2.5% (w/v) glutaraldehyde. Then the slides were dehydrated in a gradient of 30%, 50%, 70%, and 90% ethanol for 15 min, and dehydrated in anhydrous ethanol for 3 consecutive times, 15 min each time. The slides were dried by the critical-point method, fixed to the plate with conductive adhesive, and sputter-coated with gold. All samples were observed using a GiminiSEM 500.

Fluorescence staining assay for detection of live and dead bacterial cells. The killing effect of Peptide K6 on bacterial cultures was detected by Hoechst and PI dyes. Briefly, *P. aeruginosa* strain PAO1 and *S. aureus* strain SP1 from log-phase were diluted to 1×10^7 CFU/mL and 1×10^6 CFU/mL, respectively. And they were incubated at 37°C for 24 h (48h for *S. aureus*) in 24-well plates with cell-climbing slices to form monomicrobial biofilms. Then, the slides were washed

with PBS to remove the unattached bacteria, and treated with PBS, gentamicin, or Peptide K6 with final concentrations of 16 μM for 1h at 37°C, individually. Then, cell slides were washed with PBS and incubated with Hoechst (0.5 μg/mL) and PI (500 nM) in the dark for 15 min. The treated biofilms were observed under a fluorescence microscope (Eclipse Ti2-E, Nikon).

Hemolysis assay. The hemolytic activity of Peptide K1-K6 was tested using fresh murine red blood cells. Fresh blood samples were taken from C57BL/6 mice and then erythrocytes were collected by centrifugation at 1000 ×*g* for 5 min, re-suspended, and then washed twice more with PBS. Washed erythrocytes, and then about 1% (v/v) erythrocyte suspension was prepared in PBS. 50 μL of red blood cell suspension and an equal volume of serially diluted Peptide K6 dissolved in PBS were added to a 96-well microtiter plate and incubated at 37°C for 1 h. The final concentration of peptide was 4-64 μM. Then the intact erythrocytes were pelleted by centrifugation at 1000 ×*g* for 5 min, the hemolyzed supernatant was transferred to a new 96-well plate. Subsequently, the amount of hemoglobin released was detected by measuring the OD at 570 nm. Erythrocytes treated with PBS and 0.1% Triton X-100 served as negative and positive controls, respectively. Three replicates were included in each experiment, and at least three experiments were repeated.

Cytotoxicity measurement. The cytotoxicity of Peptide K1-K6 on murine macrophage cell line RAW264.7 was assessed by MTT dye reduction assay (1). Briefly, (1.0-2.0) × 10^5 cells/well in Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) were plated in a 96-well plate and incubated for 24 h at 37°C in 5% CO_2. Then, Peptide K1-K6 was respectively added to the cell cultures at a final concentration of 4-64 μM for 3 h. PBS-treated cell cultures served as controls. After 3 h, the culture was mixed with MTT (50 μL, 0.5 mg/mL), and incubated at 37°C for 3 h, centrifuged at 1000 ×*g* for 5 min, and the supernatants were discarded. Subsequently, 150 μL of DMSO was added to dissolve the formazan crystals, and the absorbance at 570 nm was measured. The experiment was repeated at least three times, and each experiment consisted of three technical replicates.

***In vivo* safety assay.** Twelve six-week-old female athymic NU/NU nude mice (Crl:NU-*Foxn1*^{nu}) purchased from Charles River Laboratories (Beijing, China) were randomly divided into three groups (4 mice in each group). After one week of adaptive feeding, PBS, 10 mg/kg, or 20 mg/kg of Peptide K6 in PBS was injected subcutaneously into the back of nude mice. At 24 h after the injection, euthanasia was performed under isoflurane anesthesia. Blood was collected to detect the levels of ALT, AST, Urea, CREA, and UA in serum. Mouse organs (heart, liver, spleen, lung, and kidney) were stained with hematoxylin and eosin (H&E) for histological analysis.

Subcutaneous-implanted-catheter-infection model. A model for biofilm-contaminated catheters was developed based on the observations of the catheter-biofilm model infected by *S. aureus* (7). This animal study was performed according to protocols and guidelines approved by the Laboratory Animal Welfare and Ethics Committee of Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences (N2022002). 24 six-week-old female athymic NU/NU nude mice (Crl:NU-*Foxn1*^{nu}) purchased from Charles River Laboratories (Beijing, China) were randomly divided into three groups (8 mice in each group). Mice were fed commercial standard food and drinking water, living in a ventilated environment with a temperature of 22-25°C, a humidity of 50%-60%, and a day-night cycle of 12/12 h.

An equal volume mixture of *P. aeruginosa* strain PAO1 (1 × 10^5 CFU/mL) and *S. aureus* strain SP1 (1 × 10^5 CFU/mL) in logarithmic growth phase were inoculated into a 24-well plate with a 1 cm central venous catheter and incubated at 37°C for 24 h to form biofilm. The mice were anesthetized with isoflurane, then the catheters described above were implanted into the skin on the back of the mice, and the wounds were sutured. After 24 h, PBS or Peptide K6 (10 mg/kg) was injected at the site of the implanted catheter in the back of the mice for five consecutive days. Mice were euthanized on the sixth day, and the catheter, EDTA anticoagulant blood, and skin tissue from the infection site were collected. Samples were processed in four parallel pipelines. 1) Bacteria were removed from the catheters by sonicating using an ultrasound device namely

SONICS VCX 150 (150 w, 20 kHz) for 14 min in PBS at 20% amplitude. Viable bacteria released into PBS were measured as CFU counts after quantitative serial dilution and plating of the sonicated catheter suspension on Tryptic Soy Agar plates (TSA); 2) The level of leukocytes in EDTA anticoagulant blood were collected by orbital venous plexus; 3) The skin at the infected site was quickly frozen in liquid nitrogen and stored at -80°C. After the skin was minced and fully homogenized, the supernatant fluid was collected after centrifugation at 3000 ×*g* for 20 min at 4°C. The expression level of IL-6 in the supernatant was detected by an IL-6 enzyme-linked immunosorbent assay (ELISA) kit. 4) The skin of the infected site was stained with hematoxylin and eosin (H&E) for histological observation.

Statistical analysis. Quantitative data were presented as the mean ± standard deviation (SD) and plotted with GraphPad Prism version 9.0 (San Diego California, USA). Significance was analyzed by independent sample t-test between two groups or one-way ANOVA among multiple groups with IBM SPSS Statistics 25 software, followed by Tukey post hoc test. $P < 0.05$ was considered a significant difference.

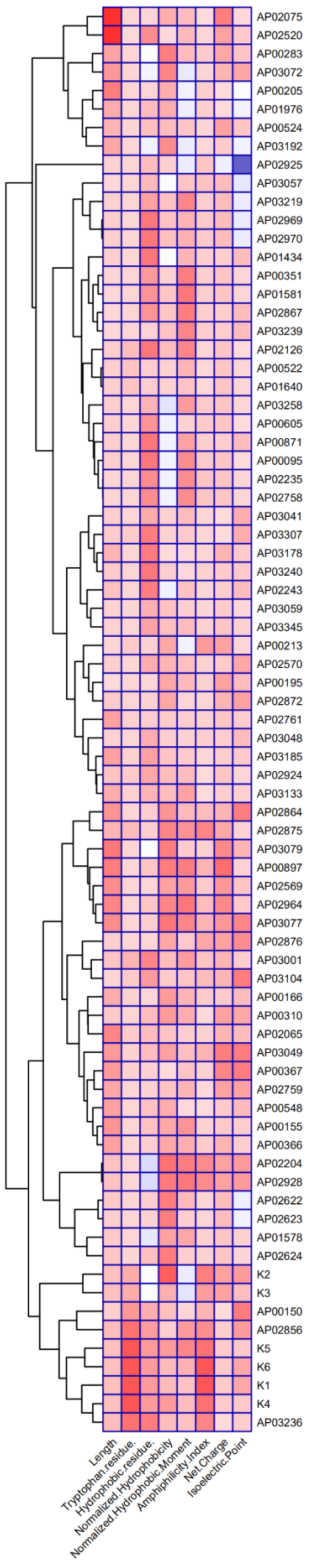

Fig. S1. The cluster analysis of the designed peptides and the peptides from the ADP. Peptide properties were calculated using the Property Calculation tool on the website of the Database of Antimicrobial Activity and Structure of Peptides (DBAASP.org) (8) in the Moon and Fleming hydrophobic scale (9)

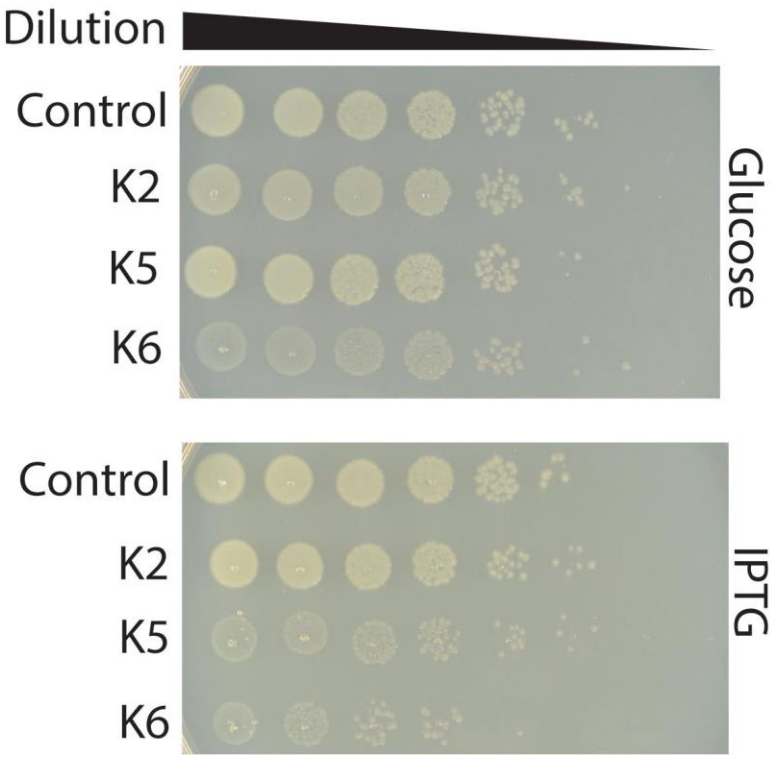

Fig. S2. Expression of Peptide K6 as part of the LamB construct reduces viability. 10-fold dilutions of *E. coli* OMNIMAX expressing a random control peptide or K2, K5, or K6 peptides as part of LamB were plated on LB subsequent to 120 minutes of repression with 0.2% glucose or induction with 1 mM IPTG in LB at 37 °C.

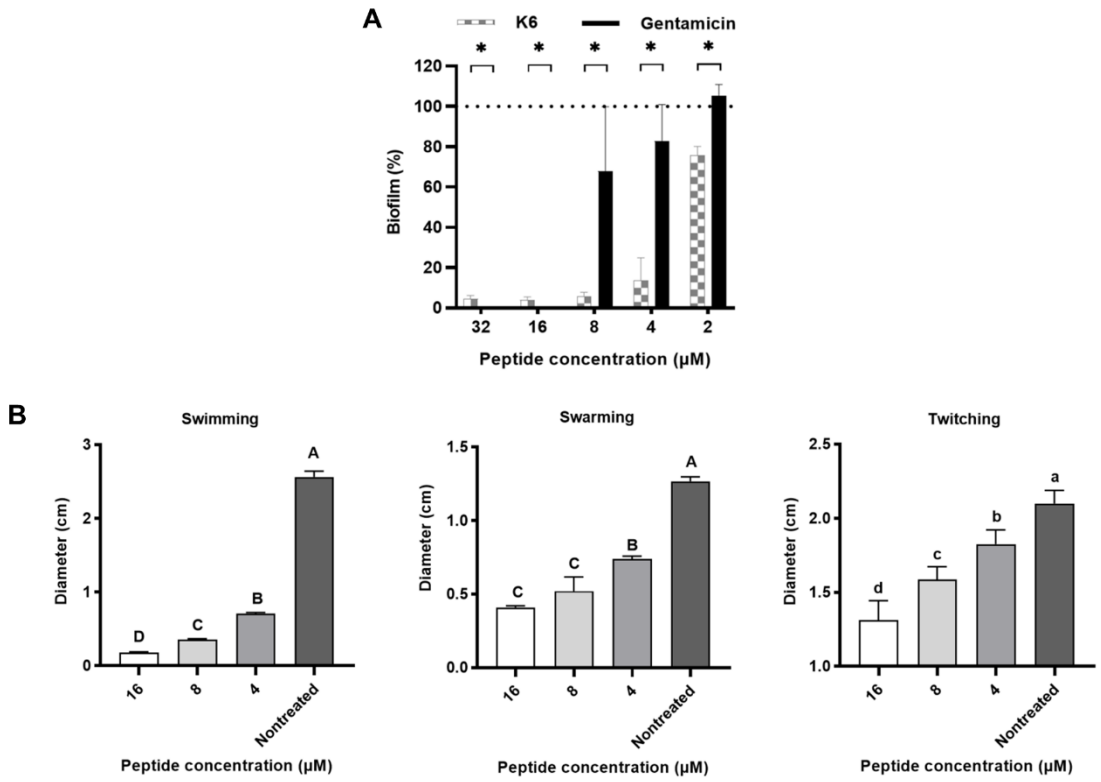

Fig. S3. (A) Peptide K6 inhibited the de-novo formation of biofilm; (B) Peptide K6 inhibited swimming, swarming, and twitching motilities.

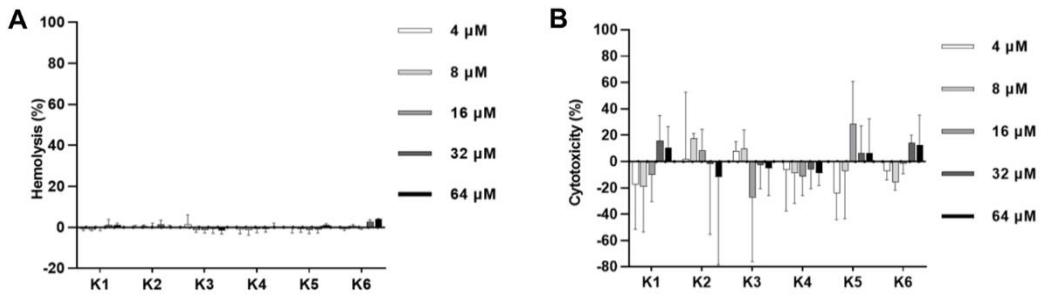

Fig. S4. (A) Hemolytic activity of peptide K1-K6 against human red blood cells (hRBCs). (B) Cytotoxicity of peptide K1-K6 to RAW264.7.

Table S1. Peptide design and their key physicochemical parameters were calculated using the Property Calculation tool on the website of the Database of Antimicrobial Activity and Structure of Peptides (DBAASP.org) (8) in the Moon and Fleming hydrophobic scale (9)

Peptide	Sequence	Theoretical MW	Measured MW	Net charge residue	Hydro-phobic residue %	Hydro-phobicity	Hydro-phobic Moment
K1	KWKWWWKWK-NH2	1461.75	1460.81	+4	55.50%	0.61	0.51
K2	KKKWWWKKK-NH2	1345.67	1344.73	+6	33.33%	1.9	0.14
K3	RRRWWWRRR-NH2	1513.76	1512.81	+6	33.33%	0.78	0.01
K4	RWRWWWRWR-NH2	1573.8	1572.86	+4	55.50%	-0.13	0.44
K5	WWRRWRRWW-NH2	1573.8	1572.86	+4	55.50%	-0.13	1.29
K6	WWKKWKKWW-NH2	1461.75	1460.81	+4	55.50%	0.61	1.71

Table S2. Bacterial strains used in this study

Strain name	Description	Reference
Pseudomonas aeruginosa PAO1	Isolated from a wound	(10)
Staphylococcus aureus SP1	Isolated from sputum; ICU	this study
Pseudomonas aeruginosa PL1	Isolated from pleural drainage; Gastrointestinal surgery	this study
Pseudomonas aeruginosa SP1	Isolated from bronchial sputum; Emergency department	this study
Staphylococcus aureus 544	Isolated from venous blood; PICU; MRSA	this study
Staphylococcus aureus 103	Isolated from bronchial sputum; EICU; MRSA	this study
Escherichia coli 729	Isolated from venous blood; MICU; ESBL	this study
Escherichia coli 112	Isolated from venous blood; EICU; ESBL	this study
Klebsiella Pneumoniae 106	Isolated from venous blood; ICU of cardiothoracic surgery department; Carbapenem resistance	this study
Klebsiella Pneumoniae 727	Isolated from bronchoalveolar lavage fluid; SICU; ESBL	this study
Streptococcus pneumonia 383	Isolated from bronchoalveolar lavage fluid	this study
Acinetobacter baumannii 946	Isolated from venous blood; SICU; Carbapenem sensitivity	this study
Acinetobacter baumannii 780	Isolated from venous blood; SICU; Carbapenem resistance	this study

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