Supporting Information for

Human Defensin-Inspired Discovery of Peptidomimetic Antibiotics

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Materials and Methods

Peptide/peptidomimetics synthesis

All peptides or peptidomimetic compounds in this study were synthesized using solid phase peptide synthesis (SPPS) by Shanghai Apeptide (Shanghai, China). The molecular weight and purity for each compound were verified by ESI-MS and RP-HPLC, respectively. Results showed the successful synthesis of every compound with correct molecular weights and high purities (> 95%) (Figs. S23-S32).

Molecular modelling

Crystal structure of HD5 monomer or dimer was retrieved from Protein Data Bank (PDB code: 1ZMP). The three-dimension homologous structure of A. baumannii OmpA was modelled from the nuclear magnetic resonance (NMR) structure of K. pneumoniae OmpA (PDB code: 2K0L) using the SWISS-MODEL web server (https://swissmodel.expasy.org/) (1). Solution NMR structure of lipid II was obtained from Protein Data Bank (PDB code: 1WCO). The interactive models of HD5 monomer in complex with A. baumannii OmpA were generated by ZDOCK protein-protein docking server (http://zdock.umassmed.edu/) (2). Per residue contributions of top 10 ranked HD5/OmpA complexes for binding free energy were calculated by HawkDock MM/GBSA tool (http://cadd.zju.edu.cn/hawkdock/) based on ff02 force field, implicit solvent model and GBOBC1 model (interior dielectric constant = 1), which was minimized for 5000 steps with a cutoff distance of 12 Å for van der Waals interactions (2000 cycles of steepest descent and 3000 cycles of conjugate gradient minimizations) (3, 4). The possible docking conformations of HD5 dimer in complex with lipid II were predicted by DockThor program (https://www.dockthor.lncc.br/v2/) (5). The detailed non-bond interactions including electrostatic interactions, hydrophobic forces, H-bonds, and Van der Waal’s forces between HD5 dimer and lipid II were analyzed using Discovery Studio 4.5 Visualizer tool and displayed by PyMOL software.

Molecular dynamics (MD) simulation

The best-rank structure of HD5/lipid II complex predicted from DockThor was chosen as initial coordinates for MD simulation. Before MD simulation, the chemical structure of lipid II was optimized, and the partial atomic charges were calculated by AM1-BCC method using Antechamber module of Amber 20 (6). The general AMBER force field (GAFF) was used for lipid II molecule to generate complete library files containing the corresponding parameters of coordinates and topology (7). Then, the molecular mechanics parameters from ff19SB fore field and GAFF force field were assigned to the HD5/lipid II complex which was subsequently neutralized by adding sodium/chlorine counter ions and solvated in a cuboid box of transferable interatomic potential with three points model (TIP3P) water molecules with solvent layers 12.0 Å between solute surface and box edges, by using LEaP module of Amber 20 (7, 8).

All MD simulations were carried out by using Amber 20 (9). The SHAKE algorithm was employed to restrict all covalent bonds involving hydrogen atoms. The pmemd.MPI module was used to perform the minimization, heat, density and equilibration of the solvated HD5/lipid II system. Initially, two steps of minimization were conducted, containing a 10000-cycle minimization with main chain restrained and a 10000-cycle non-restrained minimization, thus to remove unfavorable contacts. The system was then heated from 0 K to 300 K in 80 ps and maintained 300 K for 20 ps, using Langevin dynamics at a constant volume, with a time step of 1 fs. Afterward, a 100 ps of density equilibration was applied to the system which was subsequently undergone two steps of
NPT (T = 300 K, P = 1 bar) equilibration with/without main chain restrained for total 200 ps. Finally, the system was submitted to a 30-ns NPT (T = 300 K, P = 1 bar) production MD simulation by using the pmemd.cuda module. For energy analysis, MMPBSA.py module was used to calculate the binding free energy (ΔG_{bind}) between lipid II and HD5 according to the MM/GBSA approach based on 2000 snapshots extracted from the last 20-ns trajectories, and the ΔG_{bind} was further decomposed into the contributions of each residue in HD5 (10). Besides, Chimera-1.15 Linux version was employed to visualize the MD trajectories and extract specified time-dimension structures (11). The superimposition of these time-dimension structures was achieved and visualized by PyMOL.

**Bacterial culture medium**

Luria-Bertani (LB) medium was composed of 10 g tryptone (Shanghai Biological Technology Co., Ltd, LP0042), 5 g yeast extract (Shanghai Biological Technology Co., Ltd, LP0021) and 10 g NaCl (Sinopharm Chemical Reagent Co., Ltd, 10019318) per 1000 mL double distilled water (ddH2O). To generate solid LB agar plates, additional 15 g agar powder (Sinopharm Chemical Reagent Co., Ltd, 10000561) was added into the above formula. All bacteriological media were sterilized under high pressure steam to remove possible biological contamination before use.

**Bacterial strains**

Laboratory strains used in this study were obtained from American Type Culture Collection (ATCC), including *Staphylococcus aureus* (ATCC 25923), Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 27853), and *Acinetobacter baumannii* (ATCC 17978). Clinical isolates including *S. aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, *Salmonella typhimurium*, carbapenem-resistant *P. aeruginosa*, carbapenem-resistant *K. pneumoniae*, pandrug-resistant *A. baumannii*, and pandrug-resistant *Providencia rettgeri*, were retrieved from The First Affiliated Hospital, Zhejiang University School of Medicine. OmpA-knockout *E. coli* ATCC25922 (*E. coli*ΔOmpA) was purchased from Abiocenter (Beijing, China).

**In vitro antibacterial assay**

A virtual colony-count assay was employed to measure the bacterial-killing efficacy of compounds as previously described (12, 13). Two-fold serial dilutions of compounds with sterile ddH2O containing 0.5% dimethyl sulfoxide (DMSO, Sinopharm Chemical Reagent Co., Ltd, 30072428) were performed in sterile 96-well plates (Corning Life Sciences, 3599) with 0.05 mL/well to generate the initial concentration gradient from 0.39 μM to 200 μM. Meanwhile, the wells with pure 0.05 mL sterile ddH2O containing 0.5% DMSO were set as the vehicle group. 0.05 mL of bacterial suspension with 5×10^5 colony-forming units (CFU)/mL was then added in each well containing compound solution or vehicle to a total volume of 0.1 mL per well with the final concentrations of compounds ranging from 0.195 μM to 100 μM. Plates were then incubated at 37 °C for 2 h, and 0.1 mL of 2× LB added into the plate. Growth of bacteria was determined by a dynamic monitoring of optical density at 600 nm (OD_{600}) every 5 minutes overnight, using a Molecular Devices SPECTRA MAX 190 microplate reader (Sunnyvale, CA, USA.) with SoftMax Pro software (version 6.5) running. Each experiment was performed in triplicate. The bactericidal rates of each compound were calculated according to the resultant growth curves of detected bacteria strains. The effective concentration that kills 50% of detected bacteria (EC50) of tested
compounds was calculated from dose-efficacy curves by GraphPad Prism 8 using nonlinear regression [agonist] versus response, constraining the bottom to 0, fitting by using a four-parameter dose-response model. Additionally, the lowest concentration that kills 99% of detected bacteria (LC99) of each tested compound was also recorded.

Scanning electron microscopy (SEM)

6×10^8 CFU of *E. coli* ATCC 25922 were incubated with 12.5 μM of tested compounds (10, HD5, PMB, or kanamycin) for 2 h. *E. coli* suspension was centrifuged at 4000 rpm for 10 min and washed with phosphate buffered saline (PBS, Biological Industries, 02-024-1A) three times. The resultant *E. coli* pellets were resuspended in 2.5% glutaraldehyde, and were incubated at 4 °C for 24 h. Afterwards, the bacterial cells were washed with PBS three times, fixed with 1% OsO₄ for 1.5 h, and post-dehydrated by a graded ethanol series (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 15 min at each concentration. The 100% ethanol step was repeated three times. The samples were then dried by a LEICA EM CPD300 critical point dryer (LEICA, Germany) before coating with platinum, and were finally visualized by using a Nova Nano 450 field-emission SEM (Thermo FEI, USA).

Transcriptomic analysis

To prepare bacterial samples for transcriptomic analysis, 1 mL of *E. coli* ATCC 25922 at 6×10^8 CFU was exposed to 12.5-μM tested compound (10, HD5, or PMB) for 2 h in biological triplicate. Afterwards, bacterial cells were immediately pelleted by centrifuging at 4000 rpm for 5 min at 4 °C. Supernatants were removed and the bacterial samples were immediately frozen in liquid nitrogen and stored at -80 °C until they were processed for RNA isolation and extraction. Total RNA was extracted from above bacterial samples using TRIzol® Reagent according to the manufacturer’s instructions (Invitrogen) and genomic DNA was removed using DNase I (TaKara). RNA quality was determined using 2100 Bioanalyser (Agilent) and quantified by the ND-2000 (NanoDrop Technologies). High-quality RNA samples (OD260/280 = 1.8~2.2, OD260/230 ≥ 2.0, RIN ≥ 6.5, 28S:18S ≥ 1.0, >10μg) were used to construct sequencing library. rRNAs were removed from total RNA to yield only mRNA for library construction by employing RiboZero rRNA removal kit (Epicenter). After rRNA removal, total amount of 5 μg rRNA-free samples were subjected as input materials to construct each cDNA library for RNA sequencing by using TruSeq RNA sample preparation Kit from Illumina (San Diego, CA). After quantity checking, libraries were quantified and normalized by qPCR, and then paired-end libraries were sequenced by Illumina NovaSeq 6000 sequencing (150bp*2, Shanghai BIOZERON Co., Ltd). The raw paired-end reads were trimmed and quality controlled by Trimomatic with parameters (SLIDINGWINDOW:4:15 MINLEN:75). Then clean reads were separately aligned to reference genome with orientation mode using Rockhopper (http://cs.wellesley.edu/~btjaden/Rockhopper/) software. As input, Rockhopper takes RNA sequencing reads generated by high-throughput sequencing technology. This software was used to calculate gene expression levels with default parameters. Differential expression between different samples was computed using the fragments per kilobase of read per million mapped reads (RPKM) method. An adjusted *P* value < 0.05 (Student’s *t*-test with a Benjamini–Hochberg false discovery rate adjustment) was identified as the cut-off for significantly differentially expressed genes (DEGs). Hierarchical clustering analysis of DEGs and volcano plots were performed by using R program and Heatmap package.

Peritoneal macrophage isolation
Primary murine peritoneal macrophages were extracted as previously described (14). Briefly, 3 mL of 3% thioglycollate broth (Millipore, 108190) in ddH2O was injected intraperitoneally into mice. Three days later, mice were euthanatized and the murine abdomen was cleaned thoroughly with 70% ethanol. A gentle incision using a sterile scalpel was performed to expose the peritoneum. Then the abdomen was massaged softly and the peritoneal cavity was pierced by a 5 mL syringe with a 25-gauge needle, and 15 mL PBS used to obtain the peritoneal lavage. Lavage was centrifuged at 350 g for 5 minutes at 4 °C, the cell pellet resuspended and cultured in the complete DMEM comprising Dulbecco’s modified Eagle’s medium (DMEM, Gibco, C11995500BT) supplemented with 10% fetal calf serum (FBS, Gibco, 10099-141) with 1% penicillin–streptomycin (P/S) (Gibco, 15140-122) in an incubator at 37 °C with 5% CO2. The adherent cells were regarded as peritoneal macrophages after incubation for 3 h.

**Cell lines**
Mouse normal hepatocyte BNL CL.2 cell line and BALB/c clone fibroblast 3T3 cell line were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China and cultured in the complete DMEM medium in a humidified incubator with 5% CO2 at 37 °C.

**MTT assay**
In order to improve reliability of the safety evaluation of compounds, hepatocyte BNL CL.2, fibroblast 3T3, and peritoneal macrophages were all subjected to cytotoxicity assays. Considering the differences in size and replication rate, 5×10³ 3T3 cells, 5×10³ peritoneal macrophages, and 3.5×10³ BNL CL.2 cells were plated per well in 96-well plates overnight, respectively. Tested compounds with concentration gradients were put into cell media, while the untreated cells cultured in fresh media were set as the control group. Exactly 24 h later, 10% volume of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Aladdin, T100896) solution (5 mg/mL) was added into each well and incubated for 4 h at 37 °C. Next, 150 μL DMSO was added per well and blended adequately by pipets after removal of supernatants. Plates were wobbled gently using a horizontal rotator for 10 minutes at room temperature. The optical density for each well was determined by the microplate reader at 490 nm (OD490). The assay was repeated three times. Cell viability was calculated relative to the control groups.

**Antimicrobial block assay**
Antimicrobial block assay was performed based on the virtual colony-count assay. For OmpA-mediated block assay, aliquots of *E. coli* ATCC25922 with recombinant OmpA β-barrel or periplasmic protein (Detai Biologics Co., Ltd, Nanjing, China) and compound 10 were added into a 96-well plate. The bactericidal activity of tested compounds against *E. coli* ATCC25922 were determined in the absence or presence of OmpA protein with final concentration of 125, 250, or 500 nM. For LPS-mediated block assay, aliquots of *E. coli* ATCC25922 with purified LPS from *E. coli* O111:B4 (Sigma, USA) and tested compound (10, PMB, or kanamycin) were added into a 96-well plate. The bactericidal activity of tested compounds against *E. coli* ATCC25922 were determined in the absence or presence of LPS with final concentration of 0.25, 2.5, or 25 μg/mL. For bacterial ribosome-mediated block assay, aliquots of *E. coli* ATCC25922 with purified *E. coli* 70S ribosome (NEB, USA) and tested compound (10, PMB, or kanamycin) were added into a 96-well plate. The bactericidal activity of tested compounds against *E. coli* ATCC25922 were determined in the absence or presence of 70S ribosome with final concentration of 12.5 nM.
Additionally, the minimal concentration that kills 100.0% of detected bacteria (minimal bactericidal concentration, MBC) of each tested compound was also recorded.

**Outer membrane permeability assay**
Fluorescent probe N-Phenyl-1-naphthylamine (NPN) (Aladdin, Shanghai, China) was used to characterize the outer membrane integrity of *E. coli* ATCC25922 or *E. coli*ΔOmpA challenged by tested compounds, as described previously (15). Briefly, a single colony was picked and inoculated into 5 ml of LB medium and cultured overnight at 37 °C with shaking at 200 rpm. Then the cultures were centrifugated at 4000 rpm and washed for three times. After removing the supernatant, the bacterial pellets were suspended with 5 mM of HEPES (pH 7.0, + 5 mM of glucose). The OD600 value of obtained bacterial suspension was adjusted to 0.5 using above HEPES buffer. Afterward, the dye NPN was added to bacterial suspension with a final concentration of 10 μM, and incubated at 37 °C for 30 min. The resultant NPN-bacteria mixture was transferred to a 96-well plate at 190 μL per well, following addition of 10-μL tested compounds (final concentration 0.195–100 μM). After incubation for 1 h, fluorescence was measured on an SpectraMax® iD3 and iD5 Multi-Mode Microplate Readers (Molecular Devices, USA) with the excitation wavelength at 350 nm and the emission wavelength at 420 nm.

**Surface Plasmon Resonance (SPR)**
SPR binding assay data were collected and processed by a Biacore 3000 system (BLAcore Inc., Piscataway, NY) using the instrument’s integrated software. Compound 10 or HD5 was immobilized on a Sensor Chip CM5 using the amine-coupling chemistry kit recommended by the manufacturer, at 20 μg/mL in immobilizing buffer (10 mM NaAc, pH 4.5). Running buffer adopted in this assay composed of 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20. Analyte substances were diluted from concentrated stocks into running buffer, thus generating tested samples with different concentrations. For initiation of SPR assay, 40 μl of each sample was injected into the flow-channels in the running buffer at a flow velocity of 20 μl/min. After 2 min of association step, running buffer was introduced into the channels of chip to initiate the dissociation process for total 120 s. After each analysis, the CM5 chip was regenerated with 10 mM Gly•HCl solution (pH 2.5), and equilibrated with the running buffer prior to next injection. The resonance signals were corrected for nonspecific binding through subtracting background noises of the blank flow-channels. BIAevaluation Software was applied in fitting of signal curves and calculation of association constant (Ks), dissociation constant (Kb), and affinity constant (KD).

**Fluorescent quenching assay**
Binding affinity between compound 10 and LPS/*E. coli* 70S ribosome was determined by a fluorescent quenching assay (16). Briefly, compound 10 with LPS/70S ribosome were concomitantly added into 96-well plates to give a final volume of 100 μl per well. In this experiment, the concentration of 10 was stabilized at 250 μM, while the concentration of LPS varied from 0 to 1000 nM, or the concentration of 70S ribosome ranged from 0 to 100 nM. After incubation for 30 min at 37 °C, the plates were transferred to a Molecular Devices SPECTRA MAX M5 microplate reader (Molecular Devices, USA), and then detected the fluorescent intensities using spectrum module at the excitation wavelength of 260 nm. There fluorescence data were then used to calculate interactive parameters including Ksv, Ka, Kb, KD, according to the following equations (16):

\[
\frac{I_0}{I} = 1 + K_{sv}[Q]
\]
\[
\frac{I_0}{I_0 - I} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a}
\]

\[
\log \left( \frac{I_0 - I}{I} \right) = n \log [Q] + \log K_b
\]

\[
K_D = \frac{K_b}{K_a}
\]

Where, \( I_0 \) and \( I \) represent the steady-state fluorescent intensities in the absence and presence of quenchers (LPS or 70S ribosome), respectively; \([Q]\) denotes the concentrations of quenchers; \( K_{sv} \) is the Stern-Volmer quenching constant; \( f_a \) denotes the fraction of accessible fluorescence; \( K_a \) is the association constant; \( K_b \) is the dissociation constant; and \( K_D \) represents the affinity constant; \( n \) is the complex ratio.

**Limulus amebocyte lysate (LAL) assay**

Neutralization effect of compound 10 to LPS was determined by using a Pierce™ Chromogenic Endotoxin Quant kit (Thermo Scientific, USA). All compounds used in this study were dissolved in endotoxin-free water. Compound 10 and LPS were concomitantly added into a 96-well plate to give a final concentration of 0.25 EU/mL for LPS and a final concentration of 25, 50, 100 μM for 10. Then, the plate was placed at 37 °C for 30-min incubation. Following steps were performed according to manufacturer’s protocols. After stopping the chromogenic reaction, the plate was transferred to a Molecular Devices SPECTRA MAX 190 microplate reader (Sunnyvale, CA, USA.) with SoftMax Pro software (version 6.5) running for detection of optical density (OD) values at 405 nm. The LPS-neutralization activity was quantified as the following formula: LPS neutralization (%) = (OD\(_{10}\) - OD\(_{\text{blank}}\))/(OD\(_{\text{ctrl}}\) - OD\(_{\text{blank}}\) × 100%.

**In vitro translation assay**

Inhibitory activity of 10 against *E. coli* 70S ribosome was determined using the PURExpress ΔRibosome kit (NEB, USA) for cell-free protein synthesis. Reaction system was constructed by adding 2 μl solution A, 0.6 μl Factor Mix, 0.9 μl *E. coli* 70S ribosome, and 0.8 μl DNA plasmid, and other components such as tested compounds, for a final reaction volume of 5 μl per system. Compound 10 was tested in a concentration gradient of 0.01, 0.1, 1, 10, 100 μM (final concentration) in this reaction system. Translation reactions were performed in triplicate at 37 °C for 4 h. Afterward, reaction systems were transferred into 0 °C ice bath to terminate reaction. Finally, the protein contents of resultant reaction samples were quantified by BCA kit, and were then corrected through subtracting background proteins of control samples without adding DNA plasmid. Raw data were processed using Excel software and visualized by GraphPad Prism 8. The lowest concentration inhibiting 50% of ribosome activity (IC50) was calculated using nonlinear regression [inhibitor] versus response, and fitting by a four-parameter dose-response model.

**Animals**

C57BL/6 mice were purchased from Charles River, which were used for experimental models including peritoneal sepsis and bacterial pneumonia. ICR mice from Charles River were used for pharmacokinetic analysis. Mice were housed in a specific pathogen-free (SPF) facility in a 12-h light-dark cycle, under the condition with *ad libitum* access to irradiation sterilized rodent diet and
aseptic water. All animal tests were performed on experiment-naïve female mice ranging from 6-8 weeks old and conducted under the authorization of the Animal Advisory Committee at Zhejiang University.

In vivo efficacy on peritoneal sepsis model
Therapeutic effect of compounds on sepsis was tested according to previously reported methodology (13). Briefly, mice were divided into four groups randomly and infected with 7×10^6 CFU/mouse of *E. coli* (ATCC 25922) intraperitoneally, followed 1 h by intraperitoneal administration of peptidomimetic 10 at 2 mg/kg or 4 mg/kg (given as 3 doses every 2 h) or PMB at 4 mg/kg (given as 3 doses every 2 h). At 8 h post treatment, mice were injected intraperitoneally with ketamine/xylazine (100 µg/kg and 20 µg/kg body weight) to anesthetize them, then blood and organs including liver, spleen, lung and kidney were collected. Organs were split into three parts and placed in sterile PBS for bacterial burden analysis, 4% formaldehyde in aqueous solution for histopathological evaluation and liquid nitrogen for other assays.

In vivo efficacy on pulmonary bacterial infectious model
To examine the broad-spectrum antibacterial efficacy of compounds *in vivo*, two types of bacteria-induced pneumonia were established by intratracheal instillations of 9×10^7 CFU of *S. aureus* (ATCC 25923) or 5×10^7 CFU of *K. pneumoniae* (ATCC 13883) per mouse after intraperitoneal anesthesia with ketamine/xylazine. Mice were divided randomly for each type of bacterial pneumonia. After infection, antibiotics 10, PMB, or vancomycin (2 mg/kg) were intratracheally instilled into these infectious mice. Bronchoalveolar lavage fluid (BALF) of mice was isolated for further study at 6 h post intratracheal instillation.

Murine BALF extraction and its estimation
BALF was collected according to published methods (17). Pulmonary lavage was performed by intratracheal recovery of sterile PBS with ice-water three times to obtain the final volume of 1.5 mL fluids. BALF of each mouse was separated into three parts to prepare for further evaluation of total cell content, total protein concentration, and bacterial burden analysis. The two first indicators are positively related to the degree of destruction of blood-air barrier, and the latter two reflect inflammatory damage and infectious status respectively.

Enumeration of total cells in BALF of each mouse was determined using a blood cell counting plate by two examiners, and the results were corrected by averaging after three replications.

Total protein in BALF was quantified through the bicinchoninic acid (BCA) assay. Specifically, BALF was centrifuged at 2000 rpm, at 4 °C for 5 min to remove cellular debris, and the supernatant was used for measurement. Then 200 µL working reagent made by mixing reagent A (sodium bicinchoninate) and reagent B (4% CuSO₄•5H₂O aqueous solution) provided by Pierce™ BCA Protein Assay Kit (Thermo, 23227), was added into 25 µL of samples/standard. Standard curve was generated from bovine serum albumin (BSA) resuspended in PBS at a serial two-fold range of concentrations from 0 mg/mL to 1 mg/mL. Samples were composed of 20 µL PBS and 5 µL BALF from each mouse in 96-well plates. After incubation at 37 °C for 30 minutes, plates were measured at 562 nm by the microplate reader. The protein content of murine BALF were calculated according to standard curve.

Bacterial burden assay
Organs from peritoneal sepsis models were weighed and homogenized thoroughly in 2 mL sterile and chilled PBS using an IKA T10 basic homogenizer (IKA, Germany) to yield tissue suspensions. Blood at the volume of 50 µL from each septic mouse was also homogenized in cold PBS to disrupt clots. Tissue suspensions, blood and BALF from pneumonia mice were diluted in sterile PBS ten-, hundred- and thousand-fold. The original prepared samples and its dilutions (5 µL each) were then plated on LB plates. The bacterial colony-forming units (CFU) were counted after 16 h incubation at 37 °C. The observed counts were corrected by weight or dilution multiple.

Survival studies
For comparing the rescue effect in vivo, all experimental mice including peritoneal sepsis and bacterial pneumonia models were monitored for physical state and survival after injection. Mice were monitored for activity and food intake without external interference. Given the different severity course induced by different models, mice were under surveillance for 24, 72 and 120 h respectively for E. coli (ATCC 25922)-induced peritoneal sepsis, and pulmonary infections caused by S. aureus (ATCC 25923) or K. pneumoniae (ATCC 13883). For revealing the in vivo safety profile of 10, healthy C57BL/6 mice were intraperitoneally injected with compound 10 in a high dosage (64, 128, or 256 mg/kg), and then the survival rates were observed and recorded.

Histopathological analysis
Organs from the septic mice were fixed in 4% formaldehyde solution overnight. Formalin-fixed paraffin-embedded tissue sections, 4 µm thick, were stained by hematoxylin and eosin (H&E). Images were acquired with a light microscope (Olympus, Tokyo, Japan). Histological scores for the kidney were taken as previously described by evaluation of hemorrhages, the disappearance of brush border, and cast formation (13). The degree of lung damage was calculated by examination of alveolar fluid amass and alveolar hyalinosis (13). The measurement of spleen damage was based on the degree of bleeding and rupture (18). Renal, pulmonary, and splenic morphological alterations were graded on a scale of 0 to 3+: 0, normal; 1+, slight; 2+, moderate; and 3+, severe. Liver injury score was based on the severity of bleeding and infiltration (13). The criteria in detail were depicted as follows. Hemorrhages: normal = 0, mild (< 30% of focal region) = 1, moderate (30%−50% of focal region) = 2, and severe (> 50% of focal areas). Infiltration: normal = 0, mild (2−3-fold inflammatory cells) = 1, moderate (3−10-fold of inflammatory cells) = 2, and severe (> 10-fold inflammatory cells) = 3. Histopathological evaluation was performed by two examiners without prior knowledge of the experimental procedures. They examined 6 randomly selected regions per high-magnification field (HMF) at 200× magnification for each sample involving kidney, lung and spleen, while 3 areas were picked at random for splenic sample per section at 100× magnification to systemically observe the histological structures. The corrected quantitative result of histopathological analysis was the mean value from all selected regions of each section.

Pharmacokinetic analysis
ICR mice were injected intraperitoneally with a single dose of 4 mg/kg peptidomimetic antibiotic 10, in saline containing 5% DMSO. Blood samples were collected from 9 mice for single administration in each group at each time point (0.25, 0.5, 1, 2, 3, 4, 8, 24 h) post-dose, and subsequently centrifuged at 3000 g for 10 min at 4 °C. Blood samples obtained from healthy mice were diluted in saline, and used to generate a standard curve (R² = 0.9960) by adding known concentrations (2, 5, 10, 20, 50, 100, 200, 500 ng/mL) of peptidomimetic antibiotic 10. Resultant supernatant samples were analyzed on LC-MS/MS platform to determine the plasma
concentrations of peptidomimetic antibiotic 10. A Waters XEVO TQ-S micro system (Waters, USA) was employed to quantify tested compound. An ACQUITY UPLC® BEH C18 column (2.1 mm × 50 mm, 1.7 μm) was used for the separation with a flow rate of 350 μL/min with solvent A (0.1% (v/v) formic acid in Milli-Q water) and solvent B (acetonitrile). The initial concentration of 10% solvent B was maintained for 0.5 min, followed by an increase to 90% for 1 min and maintained for 1 min, and finally back to 10% solvent B that maintained for 1 min. MS parameters were as follow: desolvation temperature, 500 °C; desolvation gas flow, 1000 L/h; capillary voltage, 3.5 kV. The average plasma concentration and standard deviation at each time point were calculated, and WinNonLin 8.0 was employed to analyze the pharmacokinetic parameters using a non-compartmental analysis model.

Statistics
EC50 values in in vitro bactericidal assays were calculated by GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA) using nonlinear regression [agonist] versus response, constraining the bottom to 0, fitting by a four-parameter dose-response model. Similar method was also applied in calculation of CC50 values in in vitro cytotoxic assays using nonlinear regression [inhibitor] versus response, constraining the bottom to 0, fitting by a four-parameter dose-response model. A two-tailed Student’s t-test, or one/two-way analysis of variance with Bonferroni corrections was performed to determine the statistical significance of experimental data. Hierarchical clustering analysis and volcano plots were performed using R program and Heatmap package. P value < 0.05 were considered statistically significant.

Data and Materials Availability
All data are available in the main text and/or supporting information.

Supporting Text
We initially established three-dimension coordinate of A. baumannii OmpA using homologous modelling based on the NMR structure of K. pneumoniae OmpA (SI Appendix, Fig. S2 A and B). The top 10 ranked complexes for HD5/OmpA predicted by molecular docking showed high conformational similarity (SI Appendix, Fig. S2 C and D). Structurally, HD5 fits well with OmpA through embedding into the β-barrel channel opening that possibly influences the substance exchange between gram-negative bacteria and external environment, and thus disturbs bacterial membrane physiology (SI Appendix, Fig. S2 E and F). To illuminate critical residues responsible for interaction between HD5 and OmpA, the molecular mechanics/generalized Born surface area (MM/GBSA) binding free energy of top 10 ranked complexes was decomposed into the contributions of each residue. The results demonstrated that the T2-C3-Y4-C5-R6-T7-G8-R9-C10-A11-T12-R13 and R25-L26-Y27-R28-L29-C30-C31-R32 motifs contribute to the most favorable interactions with OmpA (SI Appendix, Fig. S2G). To identify important residues required against Gram-positive pathogens, we also studied structural interactions between HD5 dimer and peptidoglycan precursor lipid II using molecular docking simulation. The top 10 ranked complexes clearly showed strong and similar interaction between lipid II and either monomer of HD5 (SI Appendix, Fig. S3 A). Fig. S3B illustrated the conformation and detail interactions of the best-rank pose of HD5/lipid II complex generated from DockThor, in which A1, R6, T7, G8, and R25 in HD5 exhibit favorable contacts with lipid II through forming nonbonding interactions. To further investigate the interactive details and strength between HD5 and lipid II, the best-rank structure of HD5/lipid II complex was submitted to molecular dynamics (MD) simulation. Results clearly
presented the dynamic trajectories of HD5 and lipid II in aqueous microenvironment, which showed that lipid II always preserved steady and tight contact with HD5 within the total 30-ns simulation (SI Appendix, Fig. S3 C and D, and Supporting Movie). Afterward, the MM/GBSA binding free energy between HD5 and lipid II was calculated and decomposed into the contributions of each residue in HD5 based on last 20-ns trajectories. Results demonstrated that T²-C³-Y⁴-C⁵-R⁶-T⁷-G⁸ and E²¹-I²²-S²³-G²⁴-R²⁵-L²⁶-Y²⁷-R²⁸ motifs were critical for lipid II binding (SI Appendix, Fig. S3E).

Supporting Information Figures

Figure S1. Schematic illustrating multifaced antimicrobial mechanisms of HD5 based on previous investigations.
Figure S2. Molecular modelling of interaction between HD5 and OmpA. (A) Superimposition of NMR structure of *K. pneumoniae* OmpA (PDB code: 2K0L) and homologous structure of *A. baumannii* OmpA. (B) Ramachandran plot of the homology models of *A. baumannii* OmpA. Residues located in Ramachandran favorable regions are 86.44%. (C and D) Side (C) and top (D) view of the superimposition of the top 10 ranked docking conformations of HD5 in complex into *A. baumannii* OmpA. (E) Top view of best-ranked pose of HD5/OmpA complex, which is visualized as cartoon model. HD5 and OmpA are colored cyan and white, respectively. (F) Close-up view of molecular interactions between HD5 and OmpA. Residues constructed from the binding pocket are labelled, and contact residues located in HD5 or OmpA are marked red or black texts, respectively. Nonbond interactions are indicated as dashed lines. (G) Contributions of individual residues in the full-length structure of HD5 to MM/GBSA binding free energy. The sequences of motifs that form favorable interactions are pointed out. Red text indicates basic residues; sky blue text indicates hydrophilic residues; gray text indicates hydrophobic residues; and yellow text indicates cysteines. Data are collected from the top 10 ranked interactive conformations predicted by ZDOCK.
Figure S3. Molecular modelling of the interaction between HD5 dimer and lipid II. (A) Superimposition of the top 10 ranked binding poses of lipid II in complex into HD5 dimer. HD5 dimer is visualized using cartoon model at color of white, lipid II is presented as stick model. (B) Representational images of the best-ranked pose of the HD5 dimer in complex with lipid II. The HD5 dimer is visualized as a cartoon or space-filling model with white color. The lipid II molecule is visualized as a stick model with a cyan color. Residues that formed nonbond interactions with lipid II are labeled. (C) Superimposition and structural analysis of the original structure, 10-ns snapshot, 20-ns snapshot and 30-ns snapshot of HD5/lipid II complex output from MD simulation. (D) RMSDs of Cα atoms are shown as a function of time for HD5/lipid II. (E) Contributions of individual residues in the full-length structure of HD5 to MM/GBSA binding free energy. The sequences of motifs that form strong interactions are pointed out. Red text indicates basic residues; sky blue text indicates hydrophilic residues; gray text indicates hydrophobic residues; and yellow text indicates cysteines.
Figure S4. *In vitro* antimicrobial activity of HD5. Dose-dependent bactericidal effects of HD5 against ESKAPE pathogens including *S. aureus*, MRSA, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. Bacteria (5×10^5 CFU/ml) were incubated with HD5 at concentrations ranging 2-fold from 0.195 to 100 μM. The bactericidal rates were calculated as the colonies from HD5-treated wells relative to those from vehicle-treated control wells. All experiments were repeated independently three times, data are shown as the mean ± SD. EC50 values for HD5 against each pathogen are calculated using GraphPad prism 8 nonlinear regression [agonist] versus response, constraining the bottom to 0, fitting by using a four-parameter dose-response model.
**Figure S5. In vitro antimicrobial activity of peptidomimetic antibiotic 3 (A), 4 (B) and 5 (C).**

Dose-dependent bactericidal effects of 3, 4 and 5 against ESKAPE pathogens including *S. aureus*, MRSA, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. Bacteria (5×10^5 CFU/ml) were incubated with peptidomimetics at concentrations ranging 2-fold from 0.195 to 100 μM. The bactericidal rates were calculated as the colonies from compound-treated wells relative to those from vehicle-treated control wells. All experiments were repeated independently three times, data are shown as the mean ± SD. EC50 values for peptidomimetics against each pathogen are calculated using GraphPad prism 8 nonlinear regression [agonist] versus response, constraining the bottom to 0, fitting by using a four-parameter dose-response model.
Figure S6. *In vitro* cytotoxicity of peptidomimetic antibiotic 3 (A), 4 (B) and 5 (C) to mouse peritoneal macrophages, hepatocyte BNL CL.2 and fibroblast 3T3. Mouse peritoneal macrophages (5×10$^3$ cell/well), hepatocyte BNL CL.2 (3.5×10$^3$ cell/well) and fibroblast 3T3 (3.5×10$^3$ cell/well) were respectively incubated with peptidomimetic antibiotics at concentrations ranging 2-fold from 1.56 to 50 μM. Survival rates of cells were calculated after 24-h incubation, using MTT assay. All experiments were repeated independently three times, data are shown as the mean ± SD. CC50 values for peptidomimetic antibiotics against each pathogen are calculated using GraphPad prism 8 nonlinear regression [inhibitor] versus response, constraining the bottom to 0, fitting by using a four-parameter dose-response model.
Figure S7. *In vitro* antimicrobial activity of peptidomimetic antibiotic 6 (A) and 7 (B). Dose-dependent bactericidal effects of 6 and 7 against ESKAPE pathogens including *S. aureus*, MRSA, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. Bacteria (5×10⁵ CFU/ml) were incubated with peptidomimetics at concentrations ranging 2-fold from 0.195 to 100 μM. The bactericidal rates were calculated as the colonies from compound-treated wells relative to those from vehicle-treated control wells. All experiments were repeated independently three times, data are shown as the mean ± SD. EC50 values for peptidomimetics against each pathogen are calculated using GraphPad prism 8 nonlinear regression [agonist] versus response, constraining the bottom to 0, fitting by using a four-parameter dose-response model.
Figure S8. *In vitro* cytotoxicity of peptidomimetic antibiotic 6 (A) and 7 (B) to mouse peritoneal macrophages, hepatocyte BNL CL.2 and fibroblast 3T3. Mouse peritoneal macrophages (5×10^3 cell/well), hepatocyte BNL CL.2 (3.5×10^3 cell/well) and fibroblast 3T3 (3.5×10^3 cell/well) were respectively incubated with peptidomimetic antibiotics at concentrations ranging 2-fold from 1.56 to 50 μM. Survival rates of cells were calculated after 24-h incubation, using MTT assay. All experiments were repeated independently three times, data are shown as the mean ± SD. CC50 values for peptidomimetic antibiotics against each pathogen are calculated using GraphPad prism 8 nonlinear regression [inhibitor] versus response, constraining the bottom to 0, fitting by using a four-parameter dose-response model.
Figure S9. *In vitro* cytotoxicity of peptidomimetic antibiotic 8 (A), 9 (B) and 10 (C) to mouse peritoneal macrophages, hepatocyte BNL CL.2 and fibroblast 3T3. Mouse peritoneal macrophages (5×10^3 cell/well), hepatocyte BNL CL.2 (3.5×10^3 cell/well) and fibroblast 3T3 (3.5×10^3 cell/well) were respectively incubated with peptidomimetic antibiotics at concentrations ranging 2-fold from 1.56 to 50 μM. Survival rates of cells were calculated after 24-h incubation, using MTT assay. All experiments were repeated independently three times, data are shown as the mean ± SD. CC50 values for peptidomimetic antibiotics against each pathogen are calculated using GraphPad prism 8 nonlinear regression [inhibitor] versus response, constraining the bottom to 0, fitting by using a four-parameter dose-response model.
Figure S10. *In vitro* antimicrobial activity of peptidomimetic antibiotic 8 (A), 9 (B) and 10 (C). Dose-dependent bactericidal effects of 8, 9 and 10 against ESKAPE pathogens including *S. aureus*, MRSA, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. Bacteria (5×10⁵ CFU/ml) were incubated with peptidomimetics at concentrations ranging 2-fold from 0.195 to 100 μM. The bactericidal rates were calculated as the colonies from compound-treated wells relative to those from vehicle-treated control wells. All experiments were repeated independently three times, data are shown as the mean ± SD. EC50 values for peptidomimetics against each pathogen are calculated using GraphPad prism 8 nonlinear regression [agonist] versus response, constraining the bottom to 0, fitting by using a four-parameter dose-response model.
Figure S11. Summary of LC99 values of HD5 and peptidomimetic antibiotic 3~10 against S. aureus, MRSA, E. coli, K. pneumoniae, P. aeruginosa, and A. baumannii.

Figure S12. *In vitro* antimicrobial activity of PMB against Gram-negative clinical isolates. Dose-dependent bactericidal effects of PMB against Gram-negative clinical isolates including S. typhimurium, CR-K. pneumoniae, CR-P. aeruginosa, PDR-P. retgeri, PDR-A. baumannii. Bacteria (5×10⁵ CFU/ml) were incubated with peptidomimetics at concentrations ranging 2-fold from 0.195 to 100 μM. The bactericidal rates were calculated as the colonies from compound-treated wells relative to those from vehicle-treated control wells. All experiments were repeated independently three times, data are shown as the mean ± SD. EC50 values for peptidomimetics against each pathogen are calculated using GraphPad prism 8 nonlinear regression [agonist] versus response, constraining the bottom to 0, fitting by using a four-parameter dose-response model.
Figure S13. (A) EC50 values of peptidomimetic antibiotic 10 against E. coli in presence of different concentration of OmpA β-barrel domain. (B) Fold change in MBC of peptidomimetic antibiotic 10 against E. coli in presence of different concentration of OmpA β-barrel domain. (C) EC50 values of peptidomimetic antibiotic 10 against E. coli in presence of different concentration of OmpA periplasmic domain. (D) Fold change in MBC of peptidomimetic antibiotic 10 against E. coli in presence of different concentration of OmpA periplasmic domain. Experiments were repeated in triplicate. Statistical significance was calculated by one-way analysis of variance with the Bonferroni correction for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns, not significant.

Figure S14. (a) SPR analysis of interaction between 10/HD5 and OmpA periplasmic domain. (b) Table recording the interactive parameters calculated from SPR assay, including the equilibrium dissociation constant (Kₐ), binding constant (Kₐ) and dissociation constant (Kₐ).
Figure S15. (A) EC50 values and (B) fold change in MBC of peptidomimetic antibiotic 10 against wild-type E. coli (E. coli WT) and OmpA-deficient E. coli (E. coli ΔOmpA). The experiments were repeated nine times. Statistical significance was calculated by one-way analysis of variance with the Bonferroni correction for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns, not significant.

Figure S16. In vitro virtual-colony count assay determining the bactericidal activity of 10 (A), PMB (B) and Kanamycin (C) in presence of different concentrations of LPS. Log survival rates of E. coli in low dose of drugs were also presented using histograms in which highlight significant differences. Data are mean ± SD with triplicate experiments. Statistical significance was calculated by two-way analysis of variance with the Bonferroni correction for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns, not significant.
Figure S17. Transcriptome analysis of DEGs between peptidomimetic antibiotic 10/HD5/PMB- and control E. coli. E. coli ATCC 25922 were treated with 12.5 μM antibiotic 10, HD5 or PMB, and the RNA isolated and sequenced. (A) Hierarchical clustering of DEGs between antibiotic 10 and control E. coli samples. (B) Volcano plot illustrating the DEGs between antibiotic 10 and control E. coli samples. (C) Hierarchical clustering of DEGs between HD5 and control E. coli samples. (D) Volcano plot illustrating the DEGs between HD5 and control E. coli samples. (E) Hierarchical clustering of DEGs between PMB and control E. coli samples. (F) Volcano plot illustrating the DEGs between PMB and control E. coli samples. Genes with |log2(FC)| ≥ 1 and FDR-corrected P < 0.05 were identified as DEGs. Sample for each group were collected from three independent biological replicates.
Figure S18. KEGG enrichment analysis of DEGs of RNA-seq profile in *E. coli* treated with HD5 (A) and PMB (B).

Figure S19. *In vitro* virtual-colony count assay determining the bactericidal activity of 10 (A), PMB (B) and Kanamycin (C) in presence of *E. coli* 70S ribosome. Log survival rates of *E. coli* in low dose of drugs were also presented using histograms in which highlight significant differences. Data are mean ± SD with triplicate experiments. Statistical significance was calculated by two-way analysis of variance with the Bonferroni correction for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns, not significant.
Figure S20. Attenuated organ injury in gram-negative septic mice after treatments using peptidomimetic antibiotic 10 and PMB. (A) Representative images of histopathological H&E sections of liver, spleen, lung and kidney collected from septic mice with or without pharmacological interventions. Scale bar, 200 μm. Representative images of histopathological H&E sections of spleen. Scale bar, 400 μm. (B) Histopathological score quantifying the severity of injury in liver, lung, kidney, and spleen. n = 6 per group. Data are presented as mean ± SD. Statistical significance was calculated by two-way analysis of variance with the Bonferroni correction for multiple comparisons. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure S21. In vivo efficacy of compound 10, PMB, and vancomycin in treating murine models with bacterial pneumonia. (A and B) Kaplan-Meier survival analysis of mice with K. pneumoniae- (A) or S. aureus- (B) induced pneumonia after different interventions. n = 10 per group. (C and D) Determination of bacterial burden in BALF samples collected from K. pneumoniae- (C) or S. aureus- (D) induced pneumonia mice. n = 6 per group. Dashed lines indicate the limit of detection. (E) Total cells (left) and proteins (right) in BALF samples from mice with K. pneumoniae-induced pneumonia. n = 6 per group. (F) Total cells (left) and proteins (right) in BALF samples from mice with S. aureus-induced pneumonia. n = 6 per group. Statistical significance was calculated by one-way analysis of variance with the Bonferroni correction for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure S22. (A) Kaplan-Meier survival analysis of healthy C57BL/6 mice with intraperitoneal administration of high dosage of compound 10. n = 10 per group. (B) In vivo pharmacokinetics of
Samples were analyzed for antibiotic content by LC-MS/MS. Pharmacokinetic parameters such as $C_{\text{max}}$, $T_{\text{max}}$, $T_{1/2}$, and $\text{AUC}_{0-24h}$ are provided. $n = 3$ per time points.

**Fig. S23.** ESI-MS and HPLC validation of lead peptide 1
Fig. S24. ESI-MS and HPLC validation of lead peptide 2
Fig. S25. ESI-MS and HPLC validation of peptidomimetic antibiotic 3
Fig. S26. ESI-MS and HPLC validation of peptidomimetic antibiotic 4
Fig. S27. ESI-MS and HPLC validation of peptidomimetic antibiotic 5
Fig. S28. ESI-MS and HPLC validation of peptidomimetic antibiotic 6
Fig. S29. ESI-MS and HPLC validation of peptidomimetic antibiotic 7
Fig. S30. ESI-MS and HPLC validation of peptidomimetic antibiotic 8
Fig. S31. ESI-MS and HPLC validation of peptidomimetic antibiotic 9
Fig. S32. ESI-MS and HPLC validation of peptidomimetic antibiotic 10

Table S1. Case report of ICU’s patient with CR-K. pneumoniae infection

| Indexes         | Parameters                                                                 |
|-----------------|----------------------------------------------------------------------------|
| Age (year)      | 75                                                                         |
| Sex             | Male                                                                       |
| Height (cm)     | 167                                                                        |
| Weight (kg)     | 73                                                                         |
| Diagnosis       | 1. Choledocholithiasis with acute suppurative cholangitis;                   |
|                 | 2. Stenosis of bile duct;                                                  |
|                 | 3. Biliary fistula;                                                        |
|                 | 4. Abdominal bleeding;                                                     |
|                 | 5. Multi-organ dysfunction syndrome (MODS).                                 |
| Main pathogens  | CR-K. pneumoniae                                                           |
| Antibiotic usage| Tienam; Sulperazon; Tazocin; Targocid; Tygacil; Polymyxin B; Zavicefta;    |
|                 | Amikacin; Linezolid; Aztreonam                                              |
| APACHE-II scoring| 14                                                                        |
Table S2. Case report of ICU’s patient with PDR-P. rettgeri infection

| Indexes        | Parameters                                                                 |
|----------------|-----------------------------------------------------------------------------|
| Age (year)     | 75                                                                          |
| Sex            | Male                                                                        |
| Height (cm)    | 172                                                                         |
| Weight (kg)    | 67                                                                          |
| Diagnosis      | 1. Acute suppurative obstructive cholangitis; 2. Septic shock; 3. Acute pulmonary edema; 4. Liver failure 5. Acute renal insufficiency (ARRT status); 6. Coagulation dysfunction. |
| Main pathogens | PDR-P. rettgeri                                                             |
| Antibiotic usage | Tienam; Meropenem; Sulperazon; Tazocin; Teicoplanin for Injection; Tygacil; Polymyxin B; Zavicefta; Vancocin; Vancomycin Hydrochloride for Injection; Linezolid; Fosfomycin; Aztreonam |
| APACHE-II scoring | 20                                                                      |
| SOFA scoring   | 14                                                                          |
| Current outcome | Death                                                                      |

Supporting Movie
Movie S1. Molecular dynamic trajectories of HD5 and lipid II in aqueous microenvironment.

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