SI Appendix gram-positive

Two distinct amphipathic peptide antibiotics with systemic efficacy

Jayaram Lakshmaiah Narayana[a], Biswajit Mishra[a], Tamara Lushnikova[a], Qianhui Wu[a], Yashpal S. Chhonker[b], Yingxia Zhang[a], D. Zarena[a], Evgeniy Salnikov[c], Xiangli Dang[a], Fangyu Wang[a], Caitlin Murphy[a], Kirk W. Foster[a], Santhi Gorantla[d], Burkhard Bechinger[c,e], Daryl J. Murry[b], and Guangshun Wang[a,*]

[a]*Department of Pathology and Microbiology, College of Medicine, University of Nebraska Medical Center, 985900 Nebraska Medical Center, Omaha, NE 68198-5900, USA.*

[b]*Clinical Pharmacology Laboratory, Department of Pharmacy Practice and Science, College of Pharmacy, University of Nebraska Medical Center, Omaha, NE 68198, USA.*

[c]*Chemistry Institute UMR7177, University of Strasbourg/CNRS,* [e]*Institut Universitaire de France, F-67008 Strasbourg, France.*

[d]*Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE, USA.*

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Part I: ADDITIONAL METHODS

Peptide and chemicals

All peptides were chemically synthesized and purified to >95% (Genemed Synthesis, TX). The quality of each peptide was determined based on mass spectrometry (MS) and high performance liquid chromatography (HPLC). Peptide stock solutions were freshly made by solubilizing in autoclaved distilled water and their concentrations were quantitated using UV spectroscopy based on the tryptophan residues at 280 nm. Antibiotics used include vancomycin, doripenem monohydrate, colistin sulfate (Sigma, USA), linezolid, nafcillin (Chem Impex Int'l Inc, USA), tigecyclin (Calbiochem, USA), rifamycin (Alfa Aesar, USA), tobramycin (AK Scientific, Inc.), and daptomycin (TSZ CHEM, USA). Other chemicals were purchased from Sigma (MO, USA) or Thermo Fisher Scientific (MA, USA) unless specified.

Bacterial strains and growth media

The bacterial strains used in this study included the gram-positive bacteria methicillin-resistant *Staphylococcus aureus* (MRSA) USA300 LAC, *Enterococcus faecium* V286-17 (VRE), *S. aureus* M838-17 (MRSA) and gram-negative bacteria *Escherichia coli* E423-17, *E. coli* E416-7 (ampC), *Pseudomonas aeruginosa* E411-17, *P. aeruginosa* PA01, *Klebsiella pneumoniae* E406-17, *K. pneumoniae* ATCC13883, and *Acinetobacter baumannii* B28-16, as well as a series of clinical strains for both *S. aureus* and *K. pneumoniae*. *S. aureus* clinical strains include MU50, USA200, USA300, USA400, UAMS-1, Newman, B243-16 MSSA, B254-16 MSSA, B255-16 MSSA, B262-16 MSSA, B269-16 MSSA, M106-16, M108-16, M109-16, M111-16, M112-16, M113-16, M114-16, M116-16, M117-16, M118-16, M119-16, M120-16, M121-16, M122-16,

M123-16, M126-16, M127-16, M128-16, M129-16, M130-16. In addition, the wild type *S. aureus* strain JE2 and its mutant *mprF* from the Nebraska Transposon Mutant Library (*1*) were also used. The *K. pneumoniae* clinical strains are KP-B42-15, KP-B53-15, KP-B73-15, KP-B77-15, KP-231-18, KP-232-18, KP-245-18, KP-249-18, KP-253-18, KP-256-18, KP-257-18, KP-262-18, KP-270-18, KP-B1725-18, KP-8-22-1 to KP-8-22-8. Bacteria were cultivated in tryptic soy broth (TSB) from BD Bioscience MD, USA.

Antimicrobial assays

The antibacterial activity of peptides/antibiotics was evaluated using a standard broth microdilution protocol (*2*) with minor modifications (*3*). In brief, Peptides/antibiotics were diluted in a 96-well polystyrene microplate (10 μL each well). Logarithmic phase bacterial cultures in TSB (i.e., optical density OD at 600 nm ≈ 0.5) were diluted to 0.001 and aliquots of 90 μL were added to each well. Untreated bacterial culture and medium were included as positive and negative control wells. Numerous conventional antibiotics (above) were included for comparison. Plates were incubated at 37^{o}C overnight in a chamber with a water-filled petri dish. Post incubation, the plates were read at 630 nm using a ChroMate Microplate Reader (GMI, Ramsey, MN). The minimal inhibitory concentration (MIC) is the lowest peptide concentration that completely inhibited the bacterial growth. To study the influence of medium conditions on the antimicrobial activity of peptides against *S. aureus* USA300, the pH of the media was adjusted to 6.8, 7.2, and 8.0 followed by autoclave. The volume of media was adjusted to incorporate 150 mM sodium chloride (NaCl), 5-10% of human serum, mouse plasma, or 50% peritoneal fluid to evaluate their influence on peptide activity.

Peptide stability in human Liver microsomes

The *in-vitro* metabolism studies were performed utilizing human liver microsomes (HLM) (XenoTech, LLC, Lenexa, KS, USA) to assess phase I metabolism. Microsomal stability experiments were conducted with tris buffer (50 mM, pH 7.4), microsomal protein (1.0 mg/mL), magnesium chloride (10 mM) and NADPH (2 mM) in a final volume of 1.0 mL. The mixture was pre-incubated at 37°C for 10 min in a water bath maintained at 60 rpm. The reaction was initiated by adding 1 μM final concentration of each peptide. At selected time intervals (0, 5, 15, 20, 30, 45 and 60 min), samples (100 μL) were collected and quenched with acetonitrile (100 μL) containing internal standard (IS, the D-form of WW291) at a concentration of 1.0 μg/mL. The samples were vortexed and centrifuged at 17,950 × g for 15 min and the supernatant collected and 5 μL injected into the LC-MS/MS system. Testosterone and diclofenac were used as positive controls to ensure that HLM and incubation condition was appropriate to conduct metabolism studies. Negative control was the test mixture without NADPH. Samples were analyzed by LC-MS/MS. Experiments were performed with three biological replicates.

The result of metabolic stability was expressed as the percentage of drug remaining. The in vitro metabolic elimination rate constant was determined from the first order plot of a natural logarithm of the area ratio versus time. The slope of the linear regression equation is the elimination rate constant "k" (min^{-1}). The in vitro intrinsic clearance (CL_{int}) was determined by using the following equation (Eq-1). The intrinsic clearance was further extrapolate to *in-vitro* hepatic clearance ($CL_{in\text{-}vitro\ H}$: mL/min/kg of body weight) by using the scaling factors below (*4*,*5*).

In-vitro half-life and intrinsic clearance was calculated using following equation: $T_{1/2} = \frac{0693}{K\ (min^{-1})}$

$$cl_{int} = \frac{0.693}{t_{1/2}} \times \frac{Volume\ of\ reaction\ mixture\ (mL)}{mg\ of\ protein} \text{ ... Eq.1}$$

$$cl_{in\ vitro\ H} = cl_{int} \times \frac{mg\ of\ protein}{gram\ of\ liver} \times \frac{gram\ of\ liver}{kg\ of\ body\ weight} \text{ ... Eq.2}$$

Peptide stability in the presence of murine plasma or biological fluids

Stability of horine and verine was evaluated in the presence of mouse plasma and peritoneal fluid using SDS-PAGE electrophoresis. Briefly, a peptide with 5% mouse plasma or 50% peritoneal fluid in 10 mM PBS buffer (pH 7.4) was incubated at 37°C for 24 h. Aliquots (10 µL) of the reaction solutions were taken and mixed with 20 µL of 2× SDS loading buffer and boiled in a water bath to stop the reaction. For the SDS gel analysis, 10 µL of each sample was loaded to the well of a 5% stacking/ 18% resolving tricine gel and run at a constant current of 35 mA. The gels were stained using the Coomassie Brilliant Blue G-250 dye.

Peptide stability in human serum evaluated by mass spectrometry

In human serum stability studies, horine and verine (2 µg/mL) were incubated in human plasma for 1 h in a shaking water bath (37°C and 90 rpm). The organic content in the reaction mixture was kept within 0.5%. At selected time intervals (0, 5, 15, 30, and 60 min), sample aliquots (100 µL) were obtained and 10 µL of the above internal standard was added. Samples were mixed and extracted via the solid phase extraction method as described and after reconstitution were injected into the LC-MS/MS. Stability was calculated as percent parent remaining at select time

points relative to the parent at 0 minutes (100% parent). The data are represented as mean ± standard deviation (SD).

Hemolytic assay

The hemolytic analysis of peptides was performed using an established protocol (*3*). Briefly, human red blood cells (hRBCs) (UNMC Blood Bank) were washed three times with Blood Bank Saline (isotonic solution 0.90% w/v, Fisher) and diluted to a 2% solution. After peptide treatment, incubation at 37°C for 1 h, and centrifugation at 13,000 rpm, aliquots of the supernatant were transferred to a fresh 96-well microplate. The amount of hemoglobin released was measured at 545 nm. The percent lysis was calculated by assuming 100% release when human blood cells were treated with 1% Triton X-100, and 0% release when incubated with PBS buffer. The peptide concentration that caused 50% lysis of hRBCs is defined as HL_{50} as a measure of peptide cytotoxicity.

Cell viability assay

Peptide toxicity was also assessed using other cells, including human hepatic progenitor cell line (HepaRG), human embryonic kidney cells (HEK-293), human lung fibroblasts (MRC9), and mouse splenocytes. Briefly, cells were seeded at a density of 1×10^4 per well in a tissue culture 96-well plate. For HepaRG cells, the working medium is prepared by adding the HepaRG Supplement to 100 mL/500 mL of Williams' Medium E and 1mL/5mL of GlutaMAX-1. For HEK-293 cells in DMEM medium, MRC in EMEM, and mouse splenocytes in RPMI 1640 were supplemented with 10% fetal bovine serum (FBS), and incubated at 37°C in a 5% CO_2 atmosphere

for 24 h. Culture medium was aspirated and replaced with fresh serum free media. Cells were exposed to different concentrations of peptides for 1 h at 37°C in a 5% CO_2 atmosphere. Post-incubation, the cells were washed and incubated with 100 µL of the corresponding medium for each cell line containing 20 µL of MTS reagent for 2 h at 37°C. Absorbance was read at 492 nm using a ChroMate microplate reader (GMI, Ramsey, MN).

Killing kinetics of exponential bacteria and persisters

Killing kinetics experiments were conducted similar to antibacterial assays described above with the following modifications. The bacterial cells were induced into persisters in the presence of nafcillin as described (*6*). In brief, *S. aureus* USA300 LAC (0.1 OD) was treated with nafcillin at 100 × MIC for 24 h. Cells were washed with PBS. Aliquots of the bacterial culture (~10^5 CFU) treated with different peptides were taken at 15, 30, 60, 90, and 120 min, diluted 100-fold, and plated on Luria-Bertani agar plates and incubated at 37°C overnight. The bacterial CFU was then enumerated.

Real time fluorescence based membrane permeation

The experiment was performed as described previously with minor modifications (*3*). Serially diluted 10× peptides (10 µL each well) were created in 96-well microplates. Propidium iodide (2 µL) at a fixed concentration of 20 µM were added to each well followed by 88 µL of the *S. aureus* USA300 culture (a final OD_{600} ~0.1 in TSB media or PBS). The plate was incubated at 37ºC with continuous shaking at 100 rpm in a FLUOstar Omega (BMG LABTECH, NC, USA) microplate reader. The sample fluorescence was read at every 5 min for 24 cycles with an

excitation and emission wavelengths of 584 nm and 620 nm, respectively. Plots were generated based on the average values of duplicated experiments using GraphPad Prism 7.

Membrane depolarization of bacteria

The experiment was conducted as described (*7*). In brief, an overnight culture of *S. aureus* USA300 was re-inoculated in a fresh TSB medium and grown to the exponential phase. Cells were spun using centrifugation and washed 2 × with PBS, and re-suspended in twice the volume of PBS containing 25 mM glucose and incubated at 37°C for 15 minutes. For membrane depolarization measurements, 500 nM (final concentration) of the dye DiBAC4 (3) bis-(1, 3-dibutylbarbituric acid) trimethine oxonol (ANASPEC, CA, USA) was added and vortexed gently. Aliquots of 90 µL of the energized bacteria solution were loaded to the wells and the plate (Corning COSTAR) was placed in a FLUOstar Omega (BMG LABTECH Inc., NC, USA) microplate reader. Fluorescence was read for 20 min at excitation and emission wavelengths of 485 nm and 520 nm, respectively, to get dye normalization. Then 10 µL of peptide solutions were added and gently mixed. Fluorescence readings were recorded for 40 min, where triton X-100 (0.1%) was used as a positive control.

Effects of peptides on 48 h established biofilms

The anti-biofilm potency of the peptide against 48 h established biofilms was evaluated against multi-drug resistant bacteria (MDR) using an established protocol (*3*). Briefly, *S. aureus* USA300 LAC and *K. pneumoniae* E406-17 (10^5 CFU/mL) were prepared from the exponentially

phase. Each well of the microtiter plate (Corning Costar Cat No. 3595) was aliquoted with 180 µL of inoculum and the plates were incubated at 37°C for 48 h to form biofilm. Culture treated with water served as a positive control while media without bacterial inoculation served as the negative control. Media was aspirated post incubation and the attached biofilms were washed with 1× PBS to remove the planktonic bacteria. Each well was aliquoted with 20 µL of various MIC fold of peptides (horine or verine) and antibiotics (doripenem or nafcillin) solution and 180 µL of the fresh TSB medium. Plates were further incubated at 37°C for 24 h. Media were aspirated from the wells and washed with 1× PBS to remove planktonic cells. Live cells in the biofilms were quantitated using the XTT [2, 3-bis (2-methyloxy-4-nitro-5-sulfophenyl)-2H-tertazolium-5-carboxanilide] assay by following the manufacturer's instructions with modifications. 180 µL of fresh TSB and 20 µL of the XTT solution was added to each well and the plates were further incubated at 37°C for 2 h. Absorbance was read at 450 nm (only media with XTT-containing wells served as the blank) using a ChromateTM microtiter plate reader. Percentages of biofilm growth for the peptide was plotted by assuming 100% biofilm growth in bacterial control alone. The data are represented as mean ± SD, the significance at $p<0.05$. Plots were generated using GraphPad prism 7.

Peptide effects on biofilms observed by confocal microscopy

An exponential phase of *S. aureus* USA300 LAC and *K. pneumoniae* E406-17 (10^5 CFU/mL) was prepared as described above for antimicrobial assays. For biofilm formation, two mL of the bacterial culture was aliquoted into the chambers of cuvette (Borosilicate cover glass systems) and incubated at 37°C for 48 h. Later, media was aspirated and chambers were washed with 1× PBS to remove non-adhered cells. To test the peptide effect on the preformed biofilms, 200 µL of 10× stocks of the peptide was added followed by 1800 µL of TSB. Control cuvettes

were treated with water instead of peptide. The cuvettes were further incubated at 37°C for 24 h. Post incubation, the media was aspirated and chambers were washed with 1× PBS. The biofilms were stained with 10 µL of the LIVE/DEAD kit (Invitrogen Molecular Probes, USA) according to the manufacturer's instruction. The samples were examined with a confocal laser scanning microscope (Zeiss 710) and the data were processed using Zen 2010 software.

Scanning electron microscopy (SEM)

Exponential phase bacteria, such as *S. aureus* USA300 LAC and *K. pneumoniae* E406-17, were treated with 2× MIC of horine and verine, respectively, and fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS. Next, the samples were washed with Sorensen's Buffer with three changes, 5 min each change. Samples were post-fixed in 1% osmium tetroxide in water for 30 min. After post-fixation, all samples were washed in Sorensen's buffer with three changes at 5 min each change. All samples were dehydrated through a graded ethanol series (50%, 70%, 90%, 95%, 100% with × 3 changes) for 10 min each dehydration step. Subsequently, samples were placed in HMDS 100% for 10 min for 3 changes and left in HMDS in open dishes in the fume hood overnight to allow the HMDS to evaporate. The following day the samples were mounted on 25 mm aluminum SEM stubs with carbon adhesive tabs. Silver paste was placed around the edges of the samples. Samples were Sputter Coated with 50 nm of gold/palladium in a Hummer VI Sputter Coater (Anatech Ltd.) and examined in a FEI Quanta 200 SEM operated at 25 KV using the Electron Microscopy Core Facility on campus.

In vitro multiple passages to observe bacterial resistance development to the peptides

This experiment was performed similar to the MIC determination as described (*8*). In short, an exponential phase *S. aureus* USA300 culture (i.e., OD at 600 nm ≈ 0.5) was diluted and partitioned into a 96-well polystyrene microplate with ~10^5 colony forming units (CFU) per well (90 μL aliquots). After treatment with 10 μL of peptides: horine and verine or antibiotics; nafcillin and linezolid solutions at various concentrations using autoclaved ddH_2O, microplates were incubated at 37^{o}C overnight and read on a ChroMate Microplate Reader at 600 nm (GMI, Ramsey, MN). The MIC was defined as the lowest peptide concentration that fully inhibited bacterial growth. The wells with sub-MIC levels of the peptides that retained growth approximately half the growth of the control wells were again re-inoculated in fresh TSB with sub-MIC concertation of peptides or antibiotics to attain exponential phase for MIC determination. In total, 16 sequential passages of the bacterial cultures were conducted. The increase in the fold change (MIC on given passage/ MIC recorded in first day of passage) was used to determine the degree of drug resistance.

Ethics and husbandry conditions for animal studies

a) Ethics statement. All animal studies were performed by following the protocols approved by the institutional animal care and use committee (IACUC #16-076-08-FC, 17-104-12-FC, and 19-097-10-FC) at University of Nebraska Medical Center. On the study termination day, animals were euthanized humanely using CO_2 followed by harvesting vital organs for laboratory analysis. All efforts were made to minimize animal pain and suffering.

b) Mice husbandry. Experimental and control mice (5 per cage) were kept in ventilated cages (IVCs) at a room temperature of 20 to 24°C, humidity of 50 to 60%, 60 air exchanges per hour and a 12/12-hour light/dark cycle. Mice were fed with standardized feed (Teklad Laboratory diet for rodents) and water (Hydropac® Alternative Watering System) from an animal's ad libitum (free

feeding). All materials, including IVCs, lids, feeders, bottles, bedding, and water, were autoclaved before use. All animal manipulations were performed in a class II laminar flow biological safety cabinet.

c) Rat husbandry. Rats (3 per cage based on body weight) were kept in ventilated micro isolator cages at a room temperature ranging from 20 to 26°C, 60 air exchanges per hour and a 12/12-hour light/dark cycle. Rats are normally housed on direct contact bedding (corn cob) which allows them to express normal behaviors such as digging and burrowing. Rats were provided Nylabones® for chewing, fed with Envigo #7012 Serializable Diet and autoclaved water from an animal's ad libitum (free feeding). All materials, including micro isolator cages, lids, feeders, bottles, bedding, and water, were autoclaved before use. All animal manipulations were conducted in a class II laminar flow biological safety cabinet.

In vivo toxicity via intraperitoneal and intravenous routes in mice

Female C57BL/6 and BALB/c mice (6 weeks old) were purchased from Charles River. The in vivo toxicity was evaluated by injecting a single dose of horine at 10 mg/kg to C57BL/6 mice intraperitoneally or BALB/c mice intravenously via the tail vein (n=3-5). Animals were injected daily for a week and monitored for adverse effects and mortality at least twice a day. Body weights and animal behaviors were recorded and scored. Prior to euthunization, blood was collected for blood chemistry and cell count evaluation. Organs such as kidney and liver were harvested for histology analysis via the standard H & E staining.

Peptide toxicity evaluation in rats

a) Rat treatment. Female CD Sprague Dawley rats (6 week old) from Charles River were purchased. The peptide doses in rats were projected based on our results in mice by using the dose conversion equation (*9*). We adopted a multiple-ascending dose (MAD) regimen. Animals (n=3) were separated into three groups: (1) vehicle-treated control; (2) Peptide dose-I; and (3) peptide dose-II. Daily peptide injections were made based on animal body weight. For the control, the animals were treated with the vehicle daily for six days. For Dose-I, horine was daily treated intraperitoneally at the following ascending doses: 2.5, 5, 10, 15, 20, and 30 mg/kg for six days. For Dose-II, horine was daily treated intraperitoneally at the following ascending doses: 5, 10, 15, 20, 30, and 40 mg/kg for six days. As indicators for toxicity, animals were observed and recorded for changes in body weight and behavior, including piloerection, subdued normal behavior patterns, interaction with peers, hunched transiently (especially after drug injection), transient vocalization, oculonasal discharge, normal respiration, tremor, convulsion, prostration, and self-mutilation, especially before and after drug treatment. Rat blood was collected prior to euthanization on day 7, and rat organs, such as liver and kidney, were harvested. Part of the tissues was fixed in 4% paraformaldehyde, processed and stained by H&E staining for a standard histology analysis.

b) Evaluation of nephrotoxicity in rodents. Prior to euthanisation on day 7, blood was collected into two tubes: (1) K_3EDTA coated (minicollect, Greiner Bio-one, Austria) and (2) Lithium heparin coated tubes (BD microtainer, USA). The blood samples in EDTA coated tubes were used for cell counting on the Abaxis HM-5 instrument. The heparin coated sample tubes were processed for kidney toxicity (*10*) profile using Abaxis VS-2 at the Center for Drug Delivery and Nano Medicine, University of Nebraska Medical Center. For the kidney toxicity profile, the following were quantitated: albumin (ALB), calcium (CA^{2+}), chloride (CL^-), creatinine (CRE), glucose

(GLU), phosphorous (PHOS), potassium (K^+), sodium (NA^+), total carbon dioxide (tCO2), and blood urea nitrogen (BUN) in heparinized whole blood/plasma. For blood cell analysis, the HM5 analyzer provides various cell parameters: white blood cell (WBC), lymphocyte (LYM), monocyte (MON), neutrophils (NEU), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT = RBC%), the mean corpuscular volume (MCV, average volume of red cells), mean corpuscular hemoglobin (MCH), platelet count (PLT), and mean corpuscular hemoglobin concentration (MCHC, the average amount of hemoglobin inside a single red blood cell). The data were represented as mean± SD.

c) Alanine transaminase (ALT) assay in rats

The ALT assay (*11*) was conducted per the Manufacturer's instruction as described (*3*). In brief, a known weight of liver tissue from each animal was placed into a 96-well plate and washed with cold PBS. Liver tissues were then transferred into a tube containing 1 mL ice cold PBS and were homogenized using an Omni homogenizer (Omni International, USA). Homogenates were spun at 12,000 rpm for 5 min at 4°C and about 20 µl of the stock lysate was diluted 5× using cold PBS and used to quantitate alanine transaminase (ALT) activity as per the manufacture's instruction (enQuireBio, QKIT64-100T). The data was processed and converted with reference to the standard curve. Results are represented as mean ± SD.

In vivo pharmacokinetics study

a) Mice and peptide administration. Female BALB/c mice (6 weeks old) were purchased from Charles River. The in vivo pharmacokinetics were evaluated by intravenous injection of horine and D-horine at a dose of 10 mg/kg along with control (vehicle only) to mice. Animals were injected with peptides intravenously via the tail vein. Blood was collected at selected intervals (5,

15, 30, 45 min, 1, 2, 3, 4, 5, 6, and 24 h). Blood samples were spun to collect plasma and transferred into fresh tubes and stored at -80^{o}C until further analysis by LC-MS/MS.

b) Quantitative estimation of horine and D-horine peptides in mouse plasma using LC-MS/MS

LC-MS/MS condition for peptides:

a) Mass spectrometric detection was performed on a Shimadzu 8060 mass spectrometer (LC-MS/MS 8060, Shimadzu Scientific, Japan) equipped with a DUIS source operated in the positive ESI mode. The LC system equipped with two pumps (LC-30 AD), column oven (CTO-30AS) and an auto-sampler (SIL-30AC). The MS/MS system was operated at unit resolution in the multiple reaction monitoring (MRM) mode, using precursor ion > product ion combinations of 448.75>159.15 *m/z* for horine or D-horine and 425.15>132.20*m/z* for the internal standard (IS) (WW337). The compound dependent mass spectrometer parameters, such as temperature, voltage, gas pressure, etc., were optimized by auto method optimization via precursor ion search for each analyte and the internal standard (IS) using a 0.5 µg/mL solution in methanol. Lab Solutions LCMS Ver.5.6 controlled both the UPLC and MS systems (Shimadzu Scientific, Inc.).

The peptide D-horine and IS resolution and acceptable peak shape were achieved on a Metacil AQ C18, 120A (5µm, 2.1 × 50 mm, Agilent Technologies, Wilmington DE) column protected with a C_{18} guard column (Phenomenex, Torrance CA). The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and methanol: acetonitrile (1:1) (mobile phase B), at a total flow rate of 0.5 mL/min. The chromatographic separation was achieved using 5.3 min gradient elution. The initial mobile phase composition was 10% B, increased to 65% B over 4

min, and finally brought back to initial conditions of 10% B in 0.1 min followed by 1.2-min re-equilibration (injection volume of all samples is 5 µL).

b). Peptide stock, standards and quality control samples preparation

Stock solutions (1 mg/mL) of peptides horine, D-horine and the IS, were dissolved in water then further diluted with methanol. The calibration standard stocks of analytes were prepared by stepwise dilution of the stock solution in methanol (MeOH) over the concentration range (CCs) of 1–1000 ng/mL. Quality control samples (QCs) at four different concentrations were used i.e., lower limit of quantification (LLOQ - 1 ng/mL), low quality control (LQC - 3 ng/mL), middle quality control (MQC – 200 ng/mL) and high quality control (HQC- 750 ng/mL). QCs were prepared separately in three replicates, independent of the calibration standards. IS was diluted to 1000 ng/mL in MeOH for spiking to CC, QCs and samples.

c) Plasma sample preparation

Solid phase extraction (SPE) was utilized for all samples, standards and controls. Oasis HLB cartridges, (30mg 1mL cartridge, Waters) were used for the plasma sample preparation of mouse samples. All CCs and QCs were prepared by the addition of working standard solution into 100 µL of blank mouse plasma. For in vivo mouse study samples, 100 µL plasma was added into a 2 mL polypropylene (PP) tube. The IS (20 µL) and 400 µL of 0.1% trifluoroacetic acid (TFA) were added to all CCs, QCs and study samples prior to SPE. The entire sample was loaded onto Oasis HLB SPE cartridges that had been activated with1 mL of acetonitrile followed by 1 mL of 0.1% TFA. Loaded cartridges were washed with 1 mL of 0.1% TFA and eluted with 1 mL of 70% acetonitrile (ACN). All eluates were evaporated under a gentle stream of nitrogen at 50 ºC temperature and reconstituted in 100 µL of methanol (MeOH)/ACN: 0.1% FA (10:90). Five microliters of the reconstituted sample was injected into LC-MS/MS for analysis.

d) Method Validation

The developed LC-MS/MS method was validated as per US-FDA guidance with respect to selectivity, specificity, lower limit of quantification (LLOQ), accuracy, precision, and matrix effect (*12*).

Mouse survival study

Female C57BL/6 mice (8 per group) were made neutropenic by administering two doses of cyclophosphamide on day 1 (150 mg/kg) and Day 4 (100 mg/kg) (*13*). Mice were then infected on day 5 with bacterial suspension via intraperitoneal injection. This CFU was able to cause at least 90% mortality within 48 h after infection. At 2 h post-infection, the *S. aureus* infected treatment groups (~2×10^7 CFU per mouse) were treated with horine or vancomycin (positive control) at a single dose of horine at 10 mg/kg per mouse, while the *K. pneumoniae* infected treatment groups (~1×10^7 CFU per mouse) were treated with a single dose of verine or doripenem (positive control) at 15 mg/kg per mouse. Mouse survival was observed at least twice a day and continued for 5 days. The mortality of mice for both the peptide treated and untreated control groups was reported using the Kaplan–Meier plot.

Neutropenic murine infection model

Female C57BL/6 and BALB/c mice (6 weeks old) were purchased from Charles River. The mice were induced neutropenic by administering two doses of cyclophosphamide on day 1 (150 mg/kg) and Day 4 (100 mg/kg) (*13*). On day 5, mice were infected with *S. aureus* USA300 (~2×10^6 CFU per mouse) or K. *pneumoniae* E406-17 (~5×10^5 CFU per mouse) via intraperitoneal injection (i.p.). For the *S. aureus* infected treatment groups, mice were treated 2 h

post infection with peptide horine or vancomycin (positive control) at a single dose of 10 mg/kg per mouse; for K. *pneumoniae* infected treatment groups, verine or doripenem (positive control) were treated at a single dose of 15 mg/kg per mouse. For drug treatment, two routes were used: i.p. administration for C57BL/6 mice and intravenous (i.v.) treatment for BALB/c mice. At the end of the experiments, all animals were euthanized by exposing to CO_2 by following the institutional guidelines. Organs such as spleen, liver, lung, and kidney were harvested, weighed and placed in 1 mL sterile PBS and stored on ice. Harvested organs were subsequently homogenized using an Omni Homogenizer. Homogenates were serially diluted to achieve countable colonies. The homogenates were plated onto blood agar plates and incubated at 37ºC overnight. The CFU of each mouse was plotted as an individual dot and error bars represent the deviation within the experimental group. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, and NS represents no significance (determined by *t* test).

Non-neutropenic (normal) murine infection model

Female C57BL/6 and BALB/c mice (6 weeks old) were purchased from Charles River. On the day of infection, mice were infected with *S. aureus* USA300 LAC (~2×10^8 CFU per mouse) via intraperitoneal injection. Two drug treatment routes were used: intraperitoneally for C57BL/6 mice and intravenously for BALB/c mice. Two hours post infection, a single peptide dose was injected: 10 mg/kg for horine and 15 mg/kg for verine. At the termination day of the experiments, all animals were euthanized according to institutional guidelines. Organs, including spleen, liver, lung, and kidney, were harvested, weighed, placed in sterile PBS and stored on ice. Harvested organs were subsequently homogenized using an Omni Homogenizer. Proper dilutions were made to get countable colonies. The homogenates were plated onto blood agar plates followed by

incubation at 37ºC overnight. The CFU for each mouse was plotted as an individual dot and error bars represent the deviation within the experimental group. * p<0.05, ** p <0.01, ***p <0.001, and NS represents no significance (determined by *t* test).

Humanized mice model

a) Humanized mice generation. To generate human CD34+ human hematopoietic stem cell (HSC) reconstituted humanized mice, new born NOD.Cg-Prkdc^{scid} Il2rgt^{m1Wjl}/SzJ (NSG) mice were irradiated with a sub-lethal dose of radiation (1Gy) using a RS-2000 X-Ray Irradiator (Rad Source Technologies) and reconstituted with human cells as described previously (*14-19*). CD34+ Hu-NSG were enriched from human cord blood using immune-magnetic beads (CD34+ selection kit; Miltenyi Biotec Inc., Auburn, CA, USA). CD34+ cell purity was >90% as confirmed by flow cytometry and then injected intra-hepatically (IH) at 50,000 cells/mouse. Each donor derived cells were used to reconstitute from 4 to 10 mice depending on the yield of CD34+HSC per sample. Mice were bled at subsequent time points from the submandibular vein into ethylenediaminetetraacetic acid (EDTA)-coated tubes and screened for human immune cells using flow cytometry. For flow cytometric analysis we used a multichromatic panel of antibodies comprised of FITC-conjugated mouse anti-human CD45, Alexa Fluor 700-conjugated mouse anti-human CD3, APC-conjugated mouse anti-human CD4, and BV421-conjugated mouse anti-human CD8, PE-conjugated mouse anti-human CD14 and PE-Cy5-conjugated mouse antihuman CD19 antibodies as a six-color combination to measure human pan-CD45, CD3, CD4, CD8, CD14 and CD19 positive cells to confirm successful humanization with human immune system. Before and after infection to assess the immune activation antibody to human BV510 conjugated CD45RO was added to the panel. A second panel to assess myeloid cell activation was also included with

antibodies to mouse CD45, mouse Ly6c, human CD45, CD14, CD16 and HLA-DR. Flow cytometric analysis was performed using Attune NxT flow cytometer from Thermo Fisher Scientific, Waltham, MA. Antibodies and isotype controls were obtained from BD Pharmingen (San Diego, CA, USA), and staining was analyzed with a FlowJo (BD Immunocytometry Systems (Mountain View, CA, USA). Results were expressed as percentages of total number of gated lymphocytes and the gating strategy is shown in the figure legends.

b) Infection and treatment studies. Humanized mice were transferred from the Humanized Mice Core Facility (Dr. Gorantla) to the animal facility granted to Dr. Wang (both at the University of Nebraska Medical Center) for infection and treatment studies. A mixture of male/female mice were separated into two groups: infected and infected/treated. Mice were infected with *S. aureus* USA300 LAC (~2×10^8 CFU per mouse) via intraperitoneal injection. Mice in the treatment group (n=3-5) were intraperitoneally administered (a) with a single peptide dose at 10 mg/kg per mouse 2 h post infection or (b) two peptide doses at 2 and 3 h post infection at 10 mg/kg or (c) two peptide doses at 2 and 3 h post infection at 15 mg/kg per mouse. At the termination day of experiments, animals were euthanized using CO_2. Spleen, liver, lung, and kidney were harvested, weighed, placed in sterile PBS and stored on ice. Harvested organs were subsequently homogenized using an Omni Homogenizer. Additional dilutions were made to get countable colonies. The homogenates were plated onto blood agar plates followed by incubation at 37ºC overnight. The CFU for each mouse was plotted as an individual dot and error bars represent the deviation within the experimental group. * $p<0.05$; ** $p<0.01$; ***$p<0.001$; and NS represents no significance (determined by *t* test).

Nuclear magnetic resonance (NMR) structural studies

For NMR measurements, the peptide was solubilized in 0.3 mL of aqueous solution of 90% H_2O and 10% D_2O containing deuterated dodecylphosphocholine (DPC) at 25°C. The peptide/DPC molar ratio was ~1:60. The pH of each sample was adjusted and measured directly in the 5-mm NMR tube with a calibrated micro-pH electrode (Wilmad-Labglass). All proton NMR data (NOESY, TOCSY and DQF-COSY) (*20*) were collected using a spectral width of 8,000 Hz in both dimensions at 25°C on a Bruker 600 MHz NMR spectrometer equipped with a triple-resonance cryoprobe. Water was suppressed by low power presaturation during the relaxation delay in NOESY, TOCSY and DQF-COSY experiments. To obtain backbone ^{15}N, $^{13}C\alpha$, and $^{13}C\beta$ chemical shifts, natural abundance HSQC spectra were collected. All NMR data were processed on an Octane workstation using the NMRPipe software (*21*). NMR data were apodized by a 63° shifted squared sine-bell window function in both dimensions, zero-filled prior to Fourier transformation to yield a data matrix of 2,048 × 1,024. NMR data were analyzed with PIPP (*22*). The peptide proton signals were assigned using the standard procedure (*20*) and validated using ^{15}N and ^{13}C chemical shifts of the peptides.

Three-dimensional structure of the peptide bound to membrane-mimetic micelles

The 3D structure of the peptide was determined based on both distance and angle restraints by using the NIH-Xplor program (*23*). The distance restraints were obtained by classifying the NOE cross peak volumes into strong (1.8–2.8 Å), medium (1.8–3.8 Å), weak (1.8–5.0 Å), and very weak (1.8–6.0 Å) ranges. Peptide backbone restraints were predicted based on backbone $^{1}H\alpha$, $^{13}C\alpha$, $^{13}C\beta$, and ^{15}N chemical shifts (*24*). In total, 100 structures were calculated. An ensemble of 20 structures with the lowest total energy was chosen. This final ensemble of the accepted structures satisfies the following criteria: no distance violations greater than 0.30 Å, rmsd for bond

deviations from the ideal less than 0.01 Å, and rmsd for angle deviations from the ideal less than 5°.

Solid-state NMR spectroscopy

a) Materials.

Natural abundance and palmitoyl chain-deuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) (POPG) and 1-palmitoyl-2-oleoyl-s*n*-glycero-3- phosphoethanolamine (POPE) were from Sigma-Aldrich (Sigma, St Quentin Fallavier, France).

b) Preparation of non-oriented samples.

The preparation of non-oriented samples started by mixing POPE/POPG-*d31* (5.35 mg/2 mg) or POPE-*d31*/POPG (2 mg/0.7 mg), and the appropriate amount of peptide to reach 2 mole% (0.27 mg or 0.1 mg, respectively) in methanol/chloroform 1/1 (v/v). The sample solution was vortexed and sonicated in a bath sonicator for 10 minutes and the solvent was evaporated by exposure to a stream of nitrogen and to high vacuum overnight in such a manner to form a film along the walls of a glass tube. The sample was resuspended in 37.5 µl or 13.5 µl of 10 mM phosphate buffer (pH 7.1) and involved vortexing and bath sonication, as well as 5 chill/heat cycles at 0ºC and 40ºC. The glass tube (6 mm outer diameter) with the sample was inserted into the solenoidal coil of the solid-state NMR probe.

c) Preparation of samples for oriented solid-state NMR spectroscopy.

Uniaxially oriented samples were prepared as described in detail and illustrated in reference (*25*). Lipid mixture [POPE (27 mg) / POPG (9.1 mg), 3/1 mole/mole] was co-dissolved with the appropriate amount of peptide to obtain a 2 mole% concentration (1.35 mg) in

methanol/chloroform 1/1 (v/v). The sample solution was vortexed and sonicated in a bath sonicator for 10 minutes and the solvent partially evaporated. The viscous sample was spread onto ultrathin cover glasses (8 × 12 mm, thickness 00; Marienfeld, Lauda-Königshofen, Germany), dried by exposure to air and thereafter in high vacuum overnight. The lipid bilayers were equilibrated in an atmosphere of 93% relative humidity at 37°C in order to be in the membrane fluid-phase.

d) Solid-state NMR spectroscopy

Proton-decoupled ^{31}P solid-state NMR spectra were acquired at 121.577 MHz using a Bruker Avance wide-bore 300 solid-state NMR spectrometer equipped with a commercial double-resonance flat-coil probe (Bruker, Rheinstetten, Germany) (*26*). A Hahn-echo pulse sequence (*27*) was used with a $\pi/2$ pulse of 5 μs, a spectral width of 100 kHz, an echo delay of 40 μs, an acquisition time of 10.2 ms, and a recycle delay of 3 s. External 85% H_3PO_4 at 0 ppm was used as a reference.

^{2}H solid-state NMR spectra of deuterated lipid samples were acquired using a quadrupole pulse-echo sequence (*27*) with a recycle delay of 0.3 s, an echo time of 100 μs, a dwell time of 0.5 μs and a $\pi/2$ pulse of 6.1 μs. Before Fourier transformation of the free induction decay an exponential apodization with line broadening of 100 Hz was applied.

Proton-decoupled ^{15}N cross-polarization (CP) spectra were recorded at 76.016 MHz on a Bruker Avance wide bore NMR spectrometer using a cross polarization pulse sequence and an e-free double-resonance flat-coil probe (*28*). The spectral width, acquisition time, CP contact time, and recycle delay time were 100 kHz, 3.9 ms, 0.6 ms, and 2 s, respectively. A field strength of 35 kHz was used during CP, for the ^{1}H $\pi/2$ pulse and the SPINAL-64 heteronuclear decoupling (*29*). An exponential apodization with 300 Hz line broadening was applied before Fourier transform for

the spectrum in Fig. 2K and with 100 Hz line broadening for the one in Fig. 2L. An external reference of $^{15}NH_4Cl$ was used for calibration of the ^{15}N chemical shift scale (39.3 ppm) (*30*). All NMR measurements were performed at 37°C.

e) Deuterium order parameters.

The deuterium order parameters (S_{CD}) of each CD_2 and CD_3 group was determined according to: $S_{CD}^{i} = \frac{4}{3}\frac{h}{e^2qQ}\Delta^{i}\upsilon$, where $\Delta^{i}\upsilon$ is the quadrupolar splitting of segment i and (e^2qQ/h) is the static quadrupole coupling constant (167 kHz) observed for deuterons within C–D bonds (*31*).

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For Additional Methods

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For Fig. S1

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For Fig. S4

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PART II. SUPPORTING TABLES S1-S6

Table S1. Antibacterial activity of the designed peptides

Peptide	Amino acid Sequence	Minimal inhibitory concentration (MIC, μM)		
		S. aureus USA300 LAC	***E. coli*** ATCC 25922	***K. pneumoniae*** ATCC 13883
WW291[a]	WWWLRKIW	3.1	6.2-12.5	12.5
WW292	WWLRKIWW	6.2	25	>50
WW293	WLRKIWWW	3.1-6.2	12.5-25	25
WW294	LRKIWWWW	3.1-6.2	12.5	6.2-12.5
WW295	RKIWWWWL	3.1-6.2	6.2	3.1-6.2
WW296	KIWWWWLR	3.1	6.2-12.5	12.5
WW297	IWWWWLRK	3.1	12.5	12.5-25
WW298	WWWWLRKI	1.5-3.1	12.5	6.2-12.5

[a] All the peptides are C-terminally amidated. To facilitate peptide numbering, we have shortened the name of TetraF2W-KR (Appendix ref. *32*) to WW291 (WW means W-rich).

Table S2. **Structural statistics of two designer antimicrobial peptides WW291 and WW295 in complex with membrane-mimetic micelles** [a]

	WW291	**WW295**
NMR Restraints		
Total NOE-derived distances	229	133
Intra	76	58
Sequential	81	37
Short range	62	36
Long range	0	2
Chemical shift-derived angles	10	10
Structural quality		
Backbone rmsd (Å)	0.064	0.23
All atoms rmsd (Å)	0.80	0.69
Ramachandran plot		
Most favored region	83.3%	10%
Additional allowed region	0%	80%
Generally allowed	16.7%	10%
Total in the allowed regions	100%	100%

[a] Structures were determined by the improved 2D NMR method for high quality. Sample conditions for WW291 are 4 mM peptide in complex with perdeuterated dodecylphosphocholine (DPC) (peptide:DPC molar ratio 1:57) at pH 6.2 and 25^{o}C (Appendix ref. *33*). Sample conditions for WW295 are 3 mM peptide in complex with perdeuterated dodecylphosphocholine (DPC) (peptide:DPC molar ratio 1:60) at pH 5.8 and 25^{o}C (this study).

Table S3. Structure-based improvement of WW299, an arginine analog of WW291 [a]

Peptide	**Amino acid sequence**	**MIC (μM)**			**HL_{50} (μM)**
		S. aureus USA300 LAC	***P. aeruginosa*** PAO1	***K. pneumonia*** ATCC 13883	
WW299	WWWLRRIW	3.1	>50	25	35
WW300	**R**WWLRRIW	12.5	12.5	>50	>200
WW301	W**R**WLRRIW	6.2	6.2	25	100
WW302	WW**R**LRRIW	12.5	12.5	>25	>200
WW303	WWW**R**RRIW	12.5	25-50	>25	>200
WW304 (Horine)	WWWLRR**R**W	3.1	12.5	>25	150
WW305	WWWLRRI**R**	12.5	12.5	>25	>200

[a] MIC, Minimum inhibitory concentration; HL_{50} is the peptide concentration that causes 50% hemolysis; MIC data for *S. aureus* were taken from Appendix ref (*33*).

Table S4. Structure-based improvement of WW295 [a]

Peptide	Amino acid sequence	MIC (μM) [a]			HL_{50} (μM)
		S. aureus M838-17	***P. aeruginosa*** E411-17	***K. pneumoniae*** E406-17	
WW295	RKIWWWWL	6.2-12.5	>50	12.5	57
WW306 (Horine-L)	RRRWWWWL	3.1	6.25	6.25	60
WW307 (Verine)	RRRWWWWV	3.1	12.5	6.25	130 [b]
WW308	RRRWWWWA	12.5	25	>50	>200
WW309	RRRRWWWL	6.25	12.5	>50	>200

[a] MIC, Minimum inhibitory concentration; HL_{50} is the peptide concentration that causes 50% hemolysis

Table S5. Antimicrobial activity of horine and verine against 31 clinical strains of *S. aureus* (MIC, μM) [a]

***S. aureus* strain**	**Horine**	**Verine**	**Nafcillin**	**Vancomycin**
mu50	4	8	>8	2
USA200	4	4	>8	0.5
USA300	4	4	0.5	0.5
USA400	4	4	1	1
UAMS-1	8	4	4	2
Newman	4	4	0.25	1
B243-16 MSSA	4	4	<0.25	0.5
B254-16 MSSA	4	4	<0.25	0.5
B255-16 MSSA	4	4	<0.25	1
B262-16 MSSA	2	8	0.5	0.5
B269-16 MSSA	4	4	0.5	1
M106-16	4	4	2	1
M108-16	4	4	1	1
M109-16	4	4	1	0.5
M111-16	4	4	8	0.5
M112-16	4	8	0.5	0.5
M113-16	4	4	0.5	1
M114-16	4	4	0.5	1
M116-16	2	2	<0.25	0.5
M117-16	4	4	0.5	0.5
M118-16	4	4	0.5	1
M119-16	4	4	1	0.5
M120-16	4	4	2	0.5
M121-16	4	4	1	1
M122-16	4	4	1	1
M123-16	4	4	4	0.5
M126-16	4	4	1	0.5
M127-16	4	4	2	0.5
M128-16	4	4	8	1
M129-16	4	4	1	0.5
M130-16	4	4	1	0.5

[a] Bacteria were provided by the clinical laboratory of the University of Nebraska Medical Center.

Table S6. **Minimal inhibitory concentrations (μM) of horine, verine and antibiotics against 22 *Klebsiella pneumoniae* (KP) clinical strains** [a]

Klebsiella strain	Horine	Verine	Tigecycline	Tobramycin
KP-B42-15	>64	8	<2	8
KP-B53-15	>64	16	2	4
KP-B73-15	>64	8	2	4
KP-B77-15	>64	8	<2	4
KP-231-18	>64	16	4	64
KP-232-18	>64	16	8	>64
KP-245-18	>64	32	4	~4-8
KP-249-18	>64	16	2	8
KP-253-18	>64	8	2	8
KP-256-18	>64	8	4	>64
KP-257-18	>64	8	8	>64
KP-262-18	>64	16	4	4
KP-270-18	>64	16	4	16
KP-B1725-18	>64	4	2	8
KP-8-22-1	>64	8-16	2	2
KP-8-22-2	>64	16	4	>32
KP-8-22-3	>64	16	4	4
KP-8-22-4	>64	16	4	>32
KP-8-22-5	>64	8	2	>32
KP-8-22-6	>64	16	4	8
KP-8-22-7	>64	8	2	4
KP-8-22-8	>64	8	4	>32

[a] Bacteria were provided by the clinical laboratory of the University of Nebraska Medical Center.

SUPPORTING FIGURES S1-S11

Figure S1. Structures of the two designed peptides determined by the improved 2D NMR method (Appendix ref. *34*). The superimposed backbone (***A,C***) and side chains (***B,D***) of 20 NMR structures of WW291 (***A,B***) and WW295 (***C,D***). The red bar shows the axis for the folding of polypeptide chains. In the case of WW291, charged amino acids (R5 and K6) are located above the bar, while the hydrophobic side chains (W1, W3, L4, and W8) are below (***B***). In the case of WW295, the charged (R1 and K2) and hydrophobic side chains (W4-L8) are clustered into two sectors, top for the charged and bottom for hydrophobic ones (***D***).

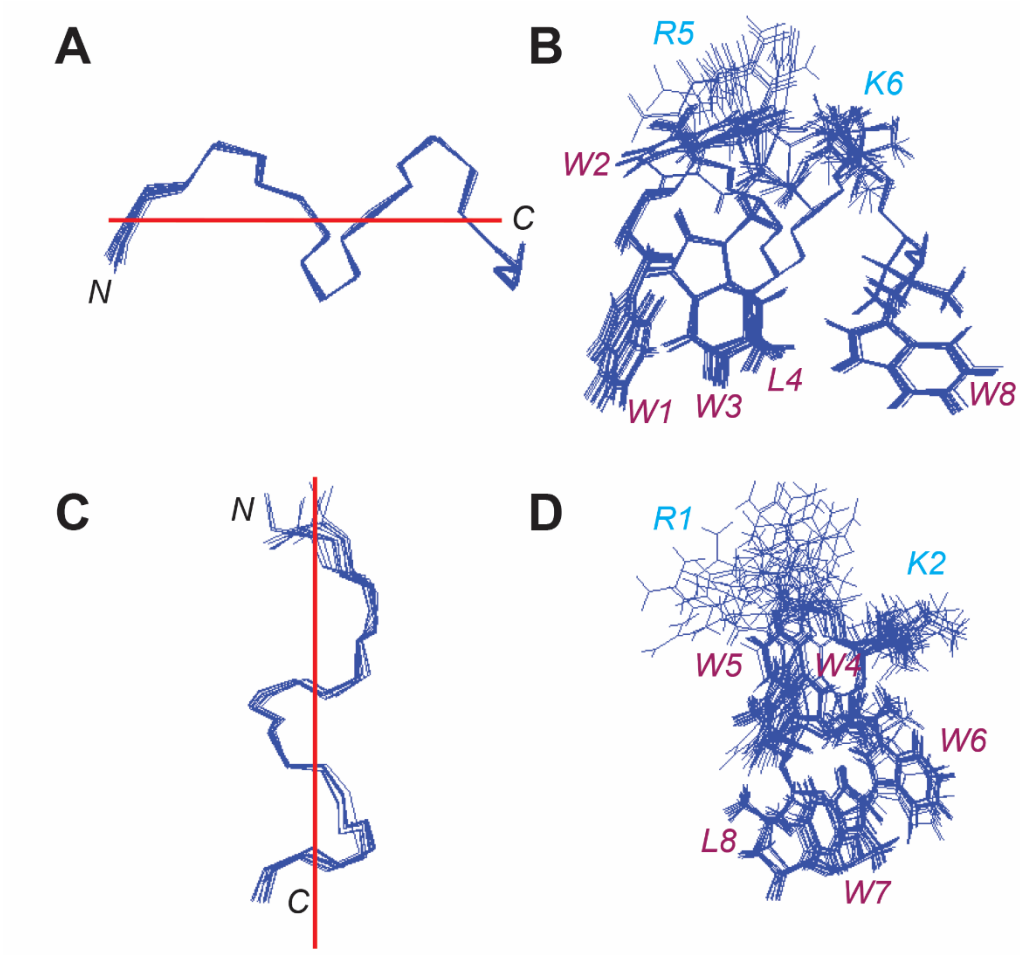


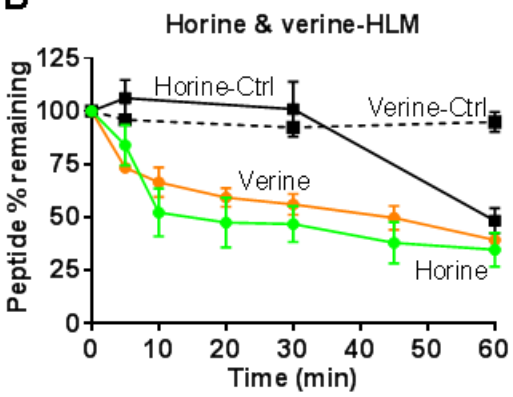
Figure S2. Peptide stability (*A*-*D*), bacteria mutant susceptibility (*E*), and resistance development (*F*). (***A***) Anti-staphylococcal activity of horine and verine in the presence of the homogenates of mouse spleen, lung, kidney tissues (5%) and peritoneal cavity fluid (50%), (***B***) Stability of horine and verine in liver microsomes, (***C***) Both peptides are stable to the proteolytic action of mouse plasma and peritoneal fluids after 24 h incubation at 37^{o}C, and (***D***) Peptides showed only slight degradation after incubation with 5% human serum at 37^{o}C for 24 h based on mass spectrometry quantification. (***E***) Disruption of the *mprF* gene in *S. aureus* made it more susceptible to the killing of horine and verine, leading to a lower MIC of 2 μM. (***F***) Resistance development of *S. aureus* USA300 LAC in a multiple passage experiment in the presence of sub-MIC levels of horine (green circle), verine (gold square), nafcillin (black triangle), and linezolid (purple inverted triangle). The *y* axis is the fold of MIC changes during multiple passaging. The figure presents the mean ± SD of duplicate experiments. The graph is generated using GraphPad Prism 7. *This figure indicates that S. aureus developed resistance to nafcillin but not peptides*.

A

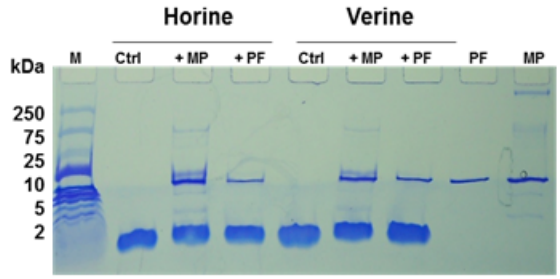
MIC (μM)

Assay condition	Horine	Verine
Free peptide	4	4
+spleen	4	4
+Lung	4	4
+Kidney	4-8	4-8
+Peritoneal fluid	4	4

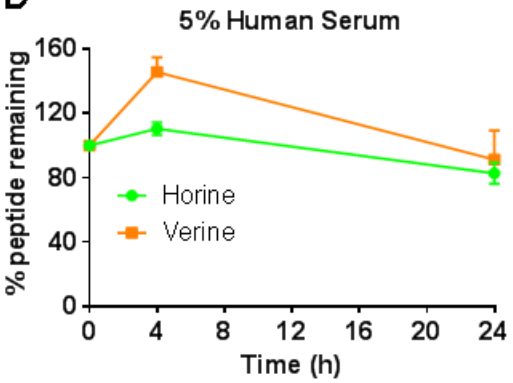
B



C



D



E

MIC (μM)

S. aureus strain	Horine	Verine
USA300	4	2-4
JE2	4	4
mprF	2	2

F

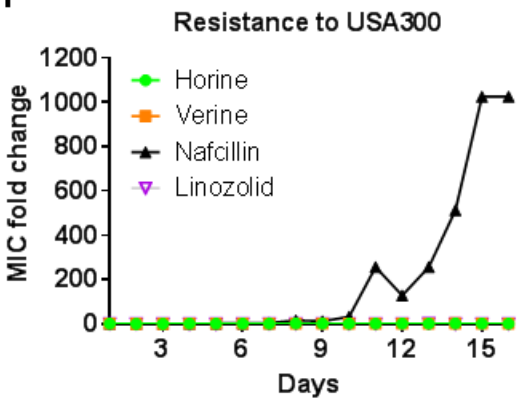


Figure S3. Mechanisms of action of horine and verine. (*A*) Membrane permeation and (***B***) depolarization of *S. aureus* USA300. **(*C*)** Membrane permeation of *E. coli* ATCC 25922. The scanning electron microscopy (SEM) images of (***D***) untreated *S. aureus* (SA), (***E***) horine-treated *S. aureus,* (***F***) untreated *K. pneumonia* (KP), and (***G***) verine-treated *K. pneumoniae* (2 × MIC). **(*H*-*M*)** ^{2}H NMR probes the interaction of the peptide with gram-negative *E. coli* lipids, consisting of palmitoyloleoylphophatidylglycerol (POPG) and palmitoyloleoylphophatidylethanolamine (POPE). In order to further investigate the effect of horine and verine-L on the lipid packing the peptides were reconstituted at 2 mole% into bacterial membrane mimetics where either the palmitoyl chain of POPG (***H***-***J***) or of POPE (***K***-***M***) was deuterated throughout the chain. The resulting deuterium NMR spectra are composed of the spectral contributions from individual CD_2 and the terminal CD_3 segments each giving a quadrupolar powder pattern with characteristic deuterium quadrupolar splitting (***H*** and ***K***). Deconvolution of the NMR spectra (***H*** and ***K***) provides the order parameters of each segment S_{CD} (***I*** and ***L***). To better illustrate the changes by the peptides the order parameters are scaled relative to the values of pure lipid (***J*** and ***M***).

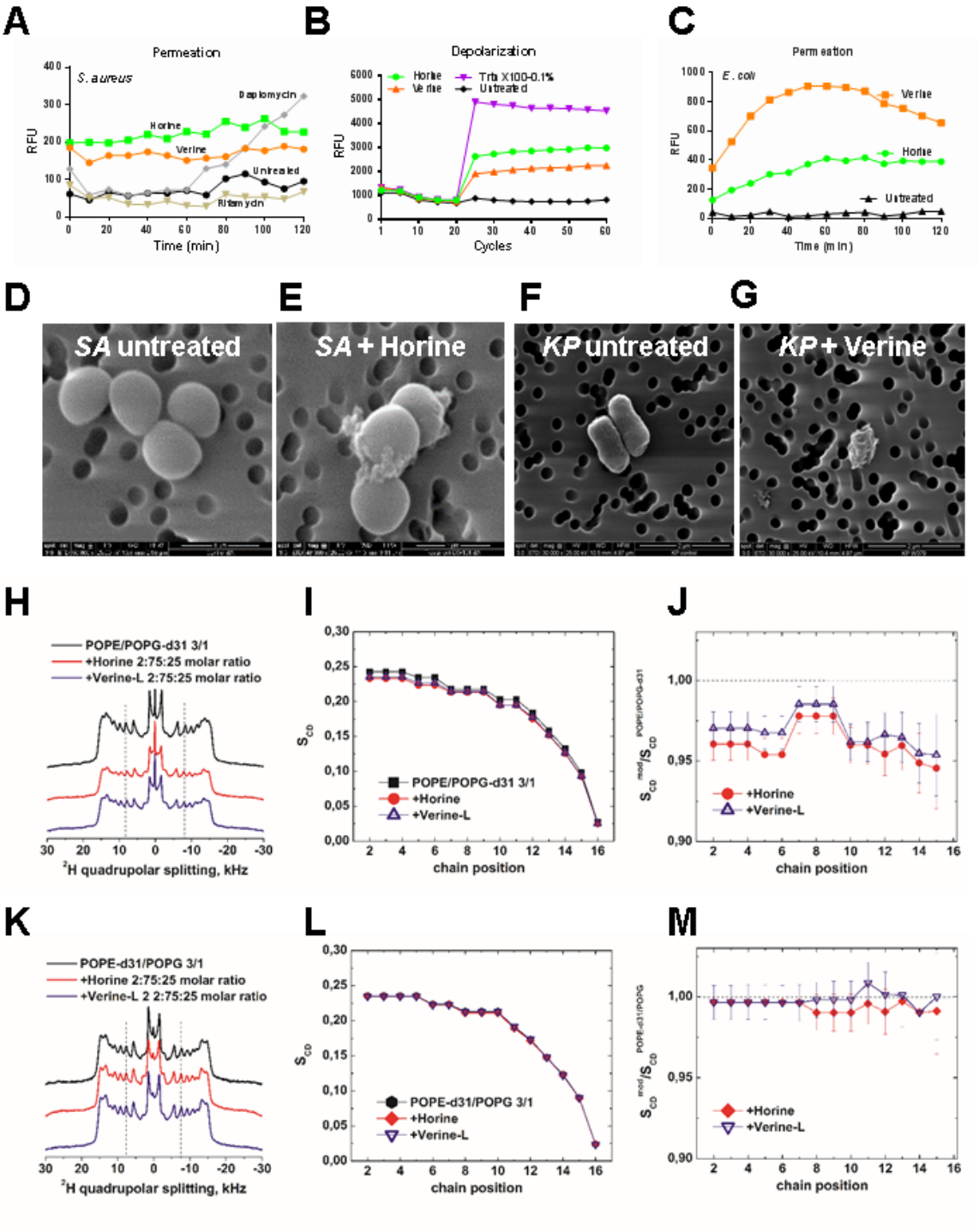

A
Permeation
S. aureus
Daptomycin
Horine
Verine
Untreated
Rifamycin
RFU
Time (min)
B
Depolarization
Horine
Verine
Triton X100-0.1%
Untreated
RFU
Cycles
C
Permeation
E. coli
Verine
Horine
Untreated
RFU
Time (min)
D
SA untreated
E
SA + Horine
F
KP untreated
G
KP + Verine
H
POPE/POPG-d31 3/1
+Horine 2:75:25 molar ratio
+Verine-L 2:75:25 molar ratio
²H quadrupolar splitting, kHz
I
POPE/POPG-d31 3/1
+Horine
+Verine-L
chain position
J
+Horine
+Verine-L
chain position
K
POPE-d31/POPG 3/1
+Horine 2:75:25 molar ratio
+Verine-L 2 2:75:25 molar ratio
²H quadrupolar splitting, kHz
L
POPE-d31/POPG 3/1
+Horine
+Verine-L
chain position
M
+Horine
+Verine-L
chain position

Figure S4. ^{31}P solid-state NMR spectra of oriented lipid bilayers treated with horine (*A*) or verine-L (*B*). The peptides were reconstituted into uniaxially oriented POPE/POPG 3/1 mole/mole membranes (See Materials and Methods), where POPG is palmitoyloleoylphophatidylglycerol and POPE is palmitoyloleoylphophatidylethanolamine. The resulting ^{31}P solid-state NMR spectra are characterized by a predominant intensity at about 25 ppm, indicative of well-oriented supported bilayers in their liquid crystalline state. Some intensities extend into the -15 ppm region as often observed for cationic amphipathic peptides that partition into the membrane interface, indicative of membrane deformations and/or conformational changes of the lipid head groups (Appendix ref. *35*). Thus, ^{31}P solid-state NMR spectra indicate that horine and verine-L exert a considerable curvature strain on the bacterial membrane.

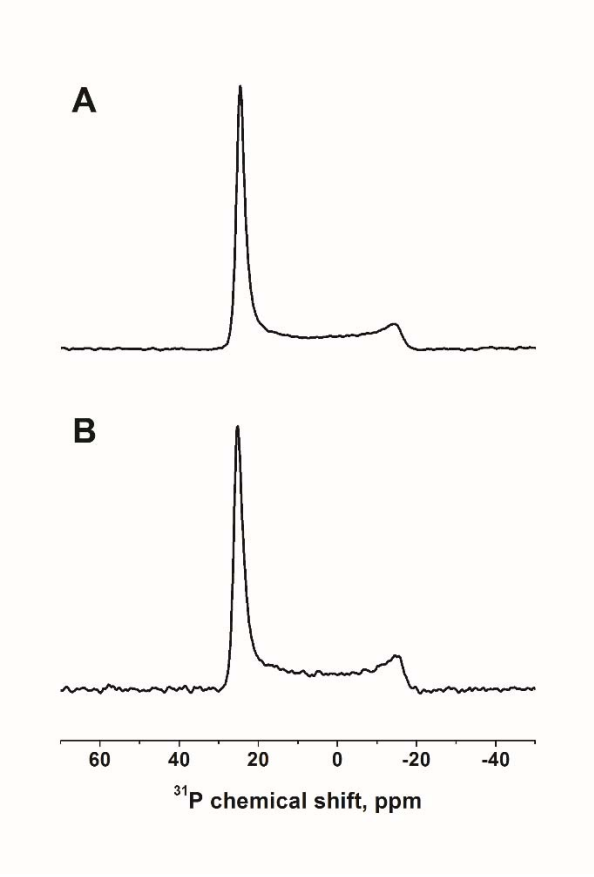
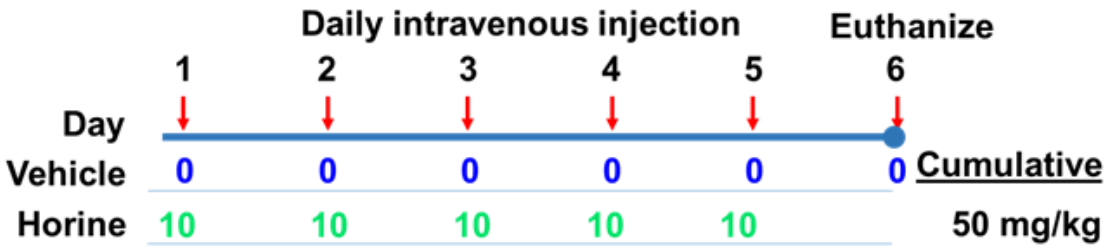
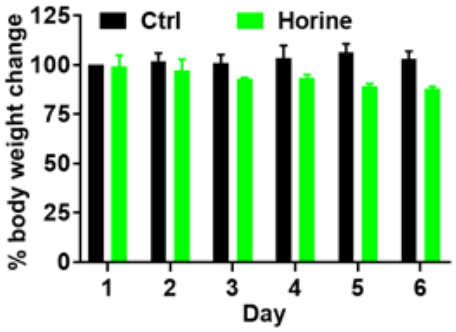


Figure S5. Intravenous toxicity evaluation of horine in a mouse model. For the treatment group, BALB/c mice (n = 3) were intravenously injected with the peptide daily at 10 mg/kg for one week, while the control group (n =4) was untouched to exclude the potential effects of physical stress and chemical treatment on mouse kidney. Shown are (***A***) the scheme of toxicity study, (***B***) body weight change percentage, kidney histology images (H&E stained) for (***C***) vehicle control, (***D***) horine treated tissue (amplification 400), (***E***) nephrotoxicity blood chemistry profile, (***F***) whole blood analysis. The abbreviations for blood cells can be found in Supporting Materials and Methods. The data represent mean ± SD. This study indicates no significant histologic differences between the control and treatment groups, although there is a slight weight loss in 6 days (12.1%). There is no significant acute tubular injury or interstitial inflammation.

A

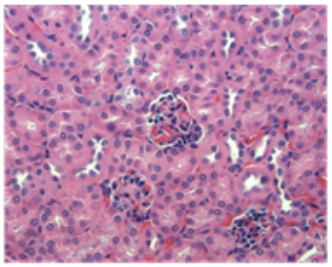


B



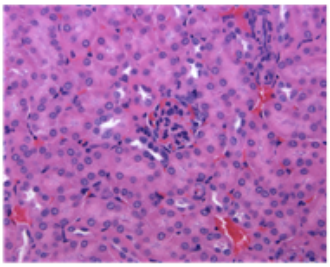
C

Control



D

Horine



E

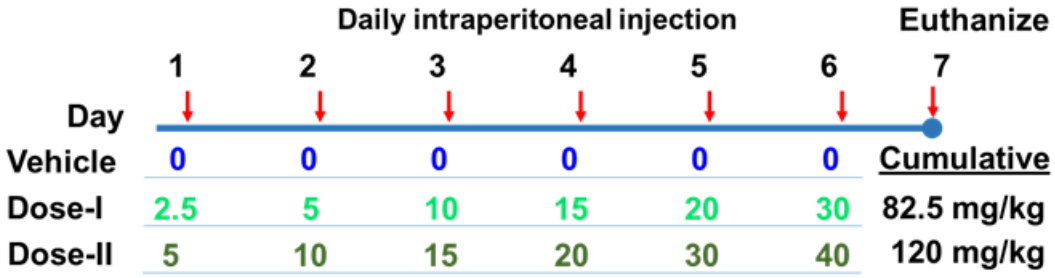
Parameters	Ctrl	Horine
Glucose	7.83±0.9	8.10±0.9
Urea Nitrogen	6.9±1.5	7.17±0.6
Ca^{2+}	2.6±0.05	2.70±0.04
Creatinine	18	18.00
Albumin	45.3±1.15	45.33±3.1
Phosphorous	2.3±0.14	2.05
Na^{+}	153.2±3.1	155±1
K^{+}	8.0±0.64	8.5
Cl^{-}	111.5±3.1	113±1.7
Total CO_2	16.5±3.1	15.33±2.1

F

Parameters	Unit	Ctrl	Horine
WBC	10^9 cells/l	4.7±2.3	5.8±0.5
LYM	10^9 cells/l	4.6±2.3	5.5±0.5
MON	10^9 cells/l	0.06±0.1	0.19±0.2
NEU	10^9 cells/l	0.08 ±0.1	0.1±0.05
LYM	%	96.8±0.3	94.8±3.8
MON	%	1.8±0.1	3.4±3.6
NEU	%	2.05±0.7	1.8±0.7
RBC	10^{12} cells/l	9.1±3.3	10±1.2
HGB	g/l	15.6±5.7	17.3±2.1
HCT	%	43.5±15.6	47.3±4.9
MCV	fl	48	47±1
MCH	pg	17.2±0.3	17.3±0.06
MCHC	g/l	35.8±0.8	36.6±0.7
PLT	10^9 cells/l	355.5±211.7	385±166
MPV	fl	6.2±0.14	6.2±0.3

Figure S6. Peptide toxicity to rats. The CD Sprague Dawley rats (6 weeks old, n = 3) were intraperitoneally injected daily with vehicle control, horine dose I (2.5 to 30 mg/kg), and horine dose II (5 to 40 mg/kg) at an ascending dose daily (***A***) for each group for 6 days. Shown are (***A***) toxicity study scheme in rats, (***B***) percent body weight change, (***C***) alanine amino-transferase activity in liver, (***D***) kidney blood chemistry profile, and (***E***) whole blood cell analysis of control and horine-injected rats at dose-I and dose-II as detailed in panel A. The abbreviations for blood cells can be found in **Additional Methods**. The data represents average mean ± SD. Rat kidney histological images (H&E stained) of (***F***) vehicle-treated, horine treated (***G***) dose I, and (***H***) dose II (amplification 400). *These images indicate no significant histologic differences between the control group and either treatment group, consistent with blood chemistry, indicating no nephrotoxicity*.

A



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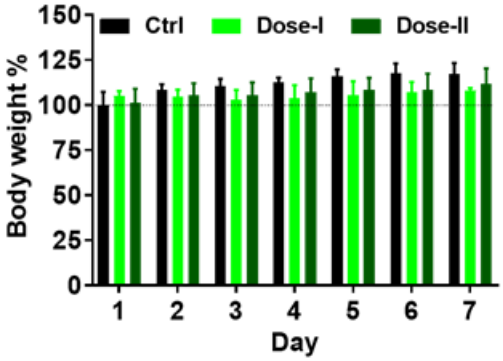

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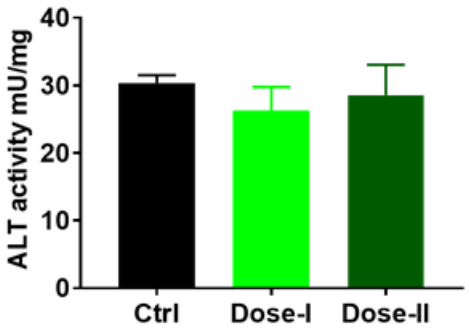

D

Kidney profile chemistry

Parameters	Control	Dose-I	Dose-II
Glucose	10.03±6.7	9.27±0.57	10.77±0.76
Urea Nitrogen	6.03±.4	6.50±0.32	6.73±1.1
Ca++	2.87±0.12	2.89±0.11	2.82±0.1
Creatinine	28.67±10.5	18.67±11.59	30.67±7.57
Albumin	56.33±3.06	48.33±6	47.00±3.61
Phosphorous	3.77±0.28	3.68±0.28	4.06±0.38
Na+	143.67±2.08	148.67±2.65	144.33±1.58
K+	6.67±1.59	6.93±0.21	7.80±0.7
Cl-	102.33±0.58	100.33±3.46	102.67±2.52
Total CO_2	28.67±1.53	28.33±3.51	26.00

E

Whole blood analysis

Parameters	Unit	Control	Dose-I	Dose-II
WBC	10^9 cells/l	13.0±6.6	18.3±5 *	24.7±3.6 *
LYM	10^9 cells/l	12.4±6.5	17.4±4.6 *	23.8±3.4 *
MON	10^9 cells/l	0.4±0.3	0.7±.4	0.7±0.3
NEU	10^9 cells/l	0.1±4.3	4.5±7.4	0.2
LYM	%	96.2±1.5	95.1±1.9	96.7±1.7
MON	%	2.9±1.12	3.9±1.9	2.9±0.8
NEU	%	1.0	1.0	1.0
RBC	10^{12} cells/l	6.6±1.1	7.7±0.3	7.9±0.7
HGB	g/l	14.6±2.8	16.5±1.15	16.3±0.6
HCT	%	42.8±7.2	50.3±1.8	50.7±3.5
MCV	fl	65±1.8	65.3±2.1	64.0±2.
MCH	pg	20.9±1	20.5±0.2	20.7±1.
MCHC	g/l	32.2±1.8	31.4±1	32.2±1

F G H

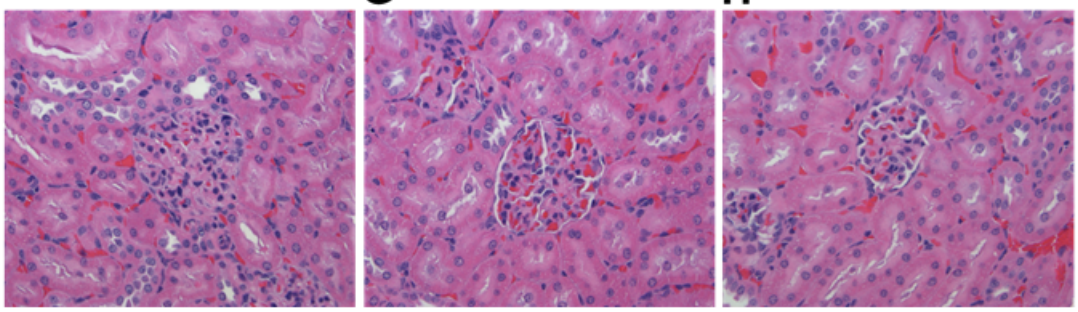

Figure S7. Evidence for the establishment of systemic infection of *K. pneumoniae* in mice. Shown is bacterial distribution in different organs of neutropenic mice 2 h post infection of *K. pneumoniae* E406-17 at 5×10^5 CFU intraperitoneally. As anticipated, no bacteria were detected in the uninfected animals (control group). The experiment was conducted and processed in the exactly the same manner as the peptide treated groups (e.g. Fig. 5). This experiment established a systemic infection model 2 h post infection prior to our peptide treatment.

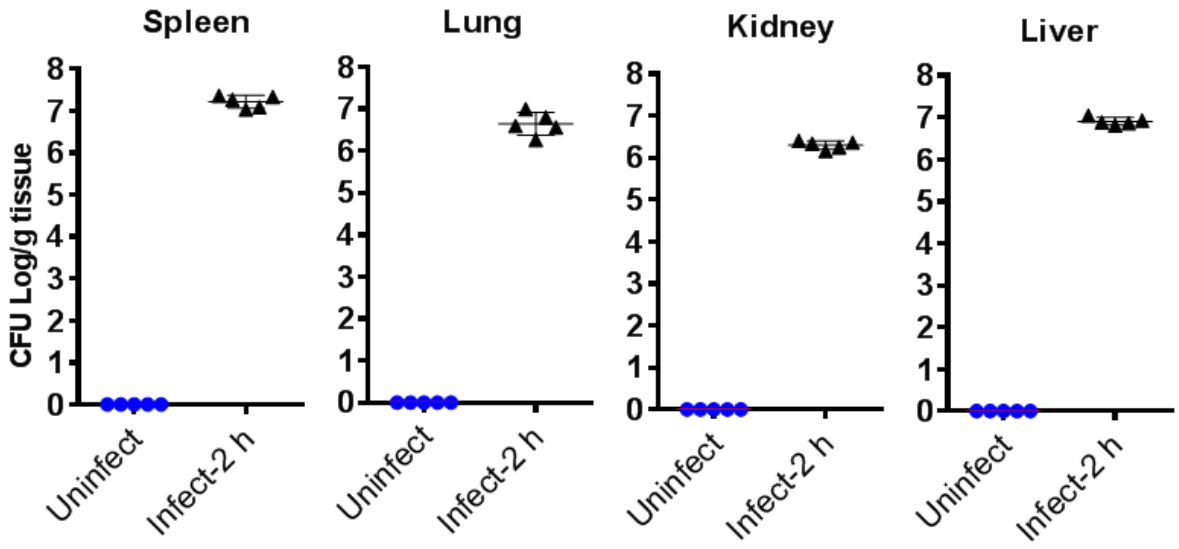

Figure S8. Efficacy of horine (green) and verine (gold) in normal mice (i.e., non-neutropenic). Mice were infected with *S. aureus* USA300 at 2×10^8 CFU per mouse. Two hours post infection, C57BL/6 mice were treated intraperitoneally (***A***) with a single dose of peptide (n = 5 mice per group at 10 mg/kg per mouse), while BALB/c mice were treated intravenously (***B***) with a single dose of peptide (n = 5 mice per group at 10 mg/kg per mouse). Animals were sacrificed 24 h post treatment. Vital organs were harvested and bacterial loads enumerated in spleen, lung, kidney, and liver by plating on blood agars and colony counting. The bacterial loads in tissues were plotted as individual points and error bars represent the deviation from the average. * $P < 0.05$; ** $P < 0.01$; and ***$P < 0.001$ (determined by *t* test).

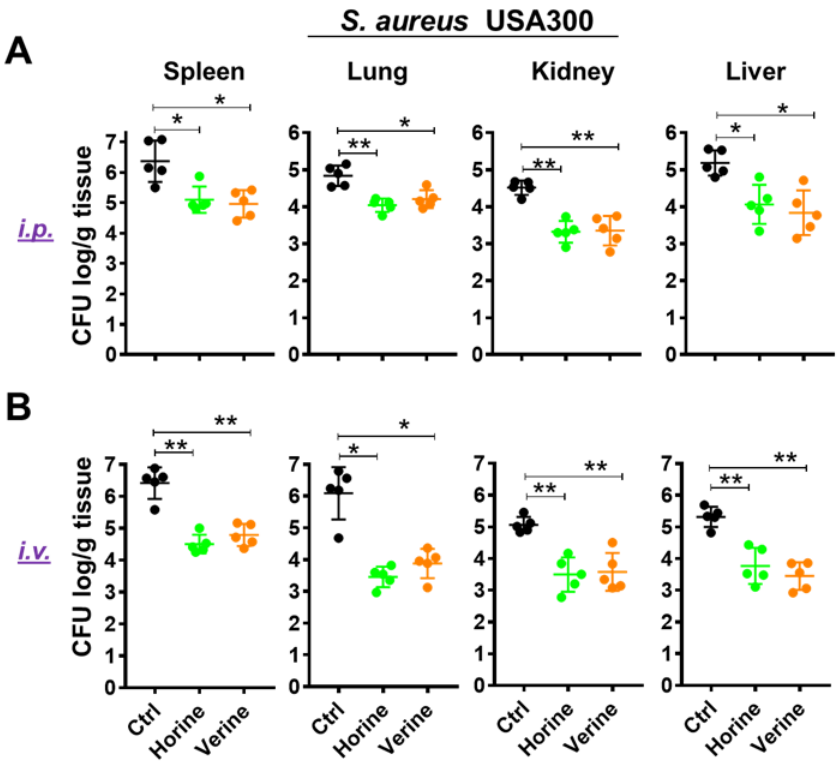

Figure S9. Dose-dependent efficacy of horine (green) in a humanized mouse model. Mice, infected with *S. aureus* USA300 LAC at 2×10^8 CFU, were treated with (***A***) a single dose of horine at 10 mg/kg per mouse (n=3, two male and one female mice in both groups) 2 h post infection, (***B***) two shots of horine at 2 and 3 h post-infection at 10 mg/kg (n = 3, two male and one female mice in both groups), and (***C***) two peptide doses at 2 and 3 h post-infection at 15 mg/kg (n = 5, five male mice in the control group and four male and one female mice in the treated group). Vital organs were harvested and bacterial loads enumerated in spleen, lung, kidney, and liver. The bacterial loads in tissues were plotted as individual points and error bars represent the deviation from the average. Statistical analysis was conducted as described in Fig. S8.

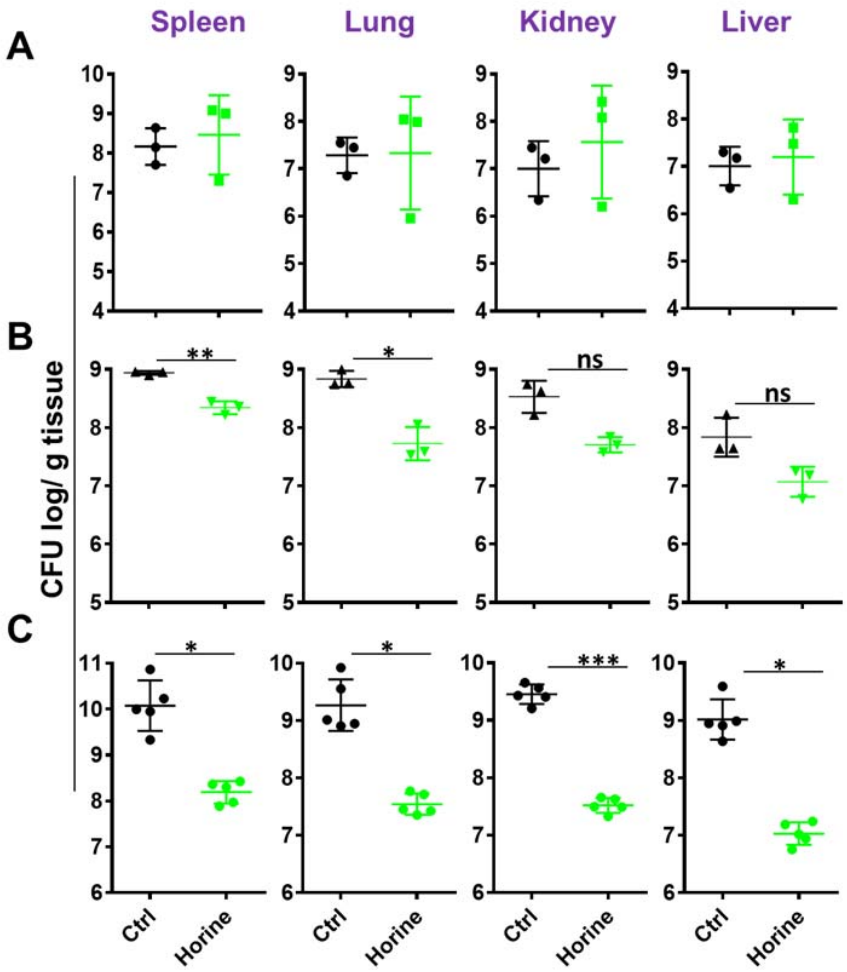

Figure S10. Plasma kinetics and tissue distribution of horine and D-horine. Peptides were intravenously administered at 10 mg/kg to BALB/c mice (mean ± SD, n = 4). Blood samples were collected at various time points from 5 min to 24 h for horine (***A***) and D-horine (***B***). (***C***) The distribution of D-horine in spleen, lung, kidney, and liver of BALB/c mice at 0.5, 2, 4, 6, and 24 h post administration of D-horine. The graph is generated using GraphPad prism 7.

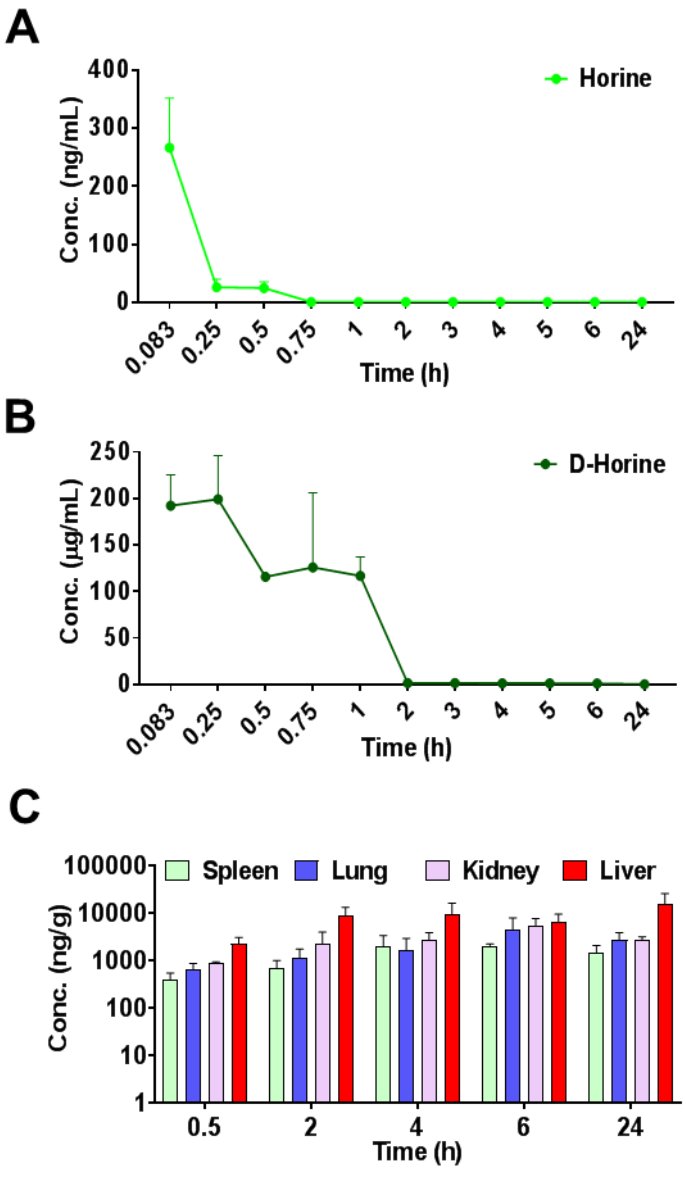

Figure S11. Time-dependent efficacy of horine and D-horine in *S. aureus* USA300 LAC infected neutropenic C57BL/6 mice treated intraperitoneally with a single dose at 10 mg/kg. Infected mice were euthanized after 24, 48, or 72 h treatment. The bacterial loads from each mouse were plotted as individual points and error bars represent the deviation within the experimental group. This study indicates that the peptide efficacy was observed after 24, 48, or 72 h treatment. Statistical analysis was conducted as described in Fig. S8.