Supplementary information for

Molecular mechanism underlying the TLR4 antagonistic and antiseptic activities of papiliocin, an insect innate immune response molecule

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Supplementary Materials and Methods

Bacterial strains. E. coli KCTC 1682, S. aureus KCTC 1621 were obtained from the Korean Collection for Type Cultures (KCTC) (Jeongeup-si, South Korea), A. baumannii KCCM 40203, P. aeruginosa KCCM 11328 from the Korea Culture Center of Microorganisms (KCCM) (Seoul, South Korea). E. coli K1 ATCC 700973 was purchased from American Type Culture Collection (Rockville, MD, USA). K. pneumonia NCCP 16054, and carbapenem-resistant gram-negative bacteria (CREC NCCP 16044, 16046, 16284; CRKP NCCP 15782, 15864, 15866; CRAB CCARM 12004, 12005, 12006) were obtained from the National Culture Collection for Pathogens (NCCP) (Osong, South Korea) and Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women’s University (Seoul, South Korea).

Antimicrobial activity. Antimicrobial activities of papiliocin against all strains were assessed using various known conventional antibiotics (imipenem, meropenem, and PMB) in addition to melittin. To determine MICs, 2 × 10^5 CFU/mL bacterial suspensions were treated with the peptides in Mueller-Hinton (MH) media for 16 h at 37°C. By averaging three measurements, MIC was calculated as the minimum inhibitory concentration of peptide that inhibited over 99% of bacterial growth.

Time-dependent killing assay. The time-dependent antibacterial activity of papiliocin and antibiotics (papiliocin, PMB, melittin, imipenem and meropenem) was monitored as described previously(1). In brief, the 1 μM (MIC of papiliocin) peptides and antibiotics were treated with 2 × 10^6 CFU/mL bacterial suspensions in MH medium at 37°C. We checked the time required to kill standard E. coli KCTC 1682 and CREC NCCP 16046 for up to 480 min. To measure the number of bacteria, the diluted supernatant was plated on a Luria-Bertani (LB) agar plate for 12 h at 37°C. The surviving bacteria were quantified based on the CFU count.

Depolarization assay. The depolarization capability of peptides to E.coli was evaluated using an membrane potential sensitive dye, 3,3'-dipropylthiadicarbocyanine iodide (dSC3-5)(2). Briefly, E.coli cells were washed using wash buffer (5 mM HEPES, 20 mM glucose, pH 7.4). After resuspending the cells in dilution buffer (5 mM HEPES, 20 mM glucose, 0.1 M KCl, pH 7.4), they were treated with dSC3-5 dye (1 h). Simultaneously, spheroplasts of E.coli cells (including plasma membrane and peptidoglycans) were generated by disrupting the outer membrane with osmotic shock, as previously reported (1). Finally, the fluorescence of peptide-treated cells, negative control (dyed cells), and positive control (1% triton X-100) was measured using a fluorescent spectrophotometer and represented as a percentage of depolarization.

Biofilm inhibition assay. The standard E. coli and carbapenem-resistant CREC NCCP 16046 were cultured in LB medium. To quantify the biofilm inhibitory activity, the peptides and antibiotics were treated with 2 × 10^5 CFU/mL bacterial suspensions in MH medium with 0.2% glucose for 16 h at 37°C. As a control, medium without cells was added to the wells. To quantify the biofilm formation, the culture media were discarded and cells were fixed by adding 100% methanol (100 μL) and incubated for 15 min. After the removal of methanol, the biofilms formed in each plate were stained with 0.1% crystal violet and 0.25% acetic acid for 1 h. The plate was washed with distilled water, dried, and 100% ethanol (200 μL) was added to each well. After drying the plates, biofilm formation activity was measured at 595 nm using an absorbance microplate reader SpectraMAX (Molecular Devices, San Jose, California, U.S.A).

Electron microscopy analyses. For SEM and TEM observation, E. coli at mid-log phase was aliquoted in MH media followed by 2 μM of papiliocin for 1, 2, and 4 h. To prepare samples for SEM, the cells were washed and fixed in 2.5% glutaraldehyde overnight at 4°C. The cells were then washed using 10 mM PBS, and fixed in 1% osmium tetroxide for 1 h, and dehydrated using a graded ethanol series. After dehydration, the cells were first incubated with a mixture of ethanol
and isoamyl acetate, followed by incubation with pure isoamyl acetate for 10 min each. Finally, the cells were dehydrated using hexamethyldisilazane reagent for 40 min, coated with platinum, and visualized by SEM (SU8020; Hitachi, Tokyo, Japan) at Tech laboratories in Konkuk university (Seoul, Korea).

TEM sample was prepared similarly to that used for SEM until after the dehydration step (The difference is that fixed in 2% glutaraldehyde and 2% paraformaldehyde). After dehydration, the cells were treated with a mixture of ethanol and propylene oxide, followed by treatment with pure propylene oxide for 20 min each. The cells were then transferred to a mixture of propylene and eponate 812 resin for 20 min and then to pure eponate 812 resin for incubation overnight. Samples were finally baked at 60°C in a constant-temperature incubator overnight, sectioned using an ultramicrotome, stained with uranyl acetate and lead citrate, and visualized by TEM (JEM-2100F; JEOL, Japan) at Korea Basic Research Institute (KBSI) (Chuncheon, Korea).

**LPS-neutralization assay.** The LPS-neutralizing activity of peptides was determined using the Pierce LAL chromogenic endotoxin quantitation kit (ThermoFisher Scientific, MA, USA) as described previously(3). Briefly, 10 μL of peptides (3.13, 6.25, 12.5, 25, and 50 μM) were allowed to interact with LPS (2ng/mL) for 10 min at 37°C. Then 20 μL of LAL enzyme was added to peptide-LPS complex (10 min, 37°C). 40μL of chromogenic substrate was added followed by 5 min incubation (37°C), after the reaction was stopped using 100 μL of acetic acid (25% v/v). The absorbance was measured at 405 nm against endotoxin standard and the values are expressed as endotoxin units (EU) per milliliter.

**BODIPY-TR cadaverine (BC) displacement assay.** The BC displacement assay was performed as described previously (4, 5). Initially, the probe complex was prepared by incubating LPS (50 mg/mL, *E. coli* O55:B5, Sigma-Aldrich, St. Louis, MO, USA) with 5 mg/mL of BC (ThermoFisher Scientific, Waltham, MA, USA) in 50 mM Tris buffer (pH 7.4) for 6 h at room temperature. Increasing concentrations of peptides were aliquoted to a 96-well dark fluorescence plate followed by 30 min incubation with LPS-BC solution. The fluorescence intensity was recorded at an excitation wavelength of 580 nm and an emission wavelength of 620 nm using a fluorescence microplate reader (Molecular Device, USA). The values were converted to %ΔF(A.U.) using the following equation: %ΔF(A.U.) = 100 × (F – F0)/(F1 – F0), where F is the fluorescence intensity shown by the peptides, F0 is the fluorescence intensity without the peptides, and F1 is the fluorescence intensity with 64 μM PMB (a potent LPS-binding peptide).

**Isothermal titration calorimetry (ITC).** All measurements were carried out with a MicroCal Auto-iTC200 (Malvern Panalytical, Malvern, UK) at KBSI (Ochang, Korea). Binding affinity was measured using 0.2 mM of papilocin and PMB to 370 μl of LPS from *E. coli* O111:B4 (0.025 mM, M.W 20,000) in Dulbecco's phosphate-buffered saline (DPBS, pH7.0) with an injection duration of 4 s intervals for 80 s at 37°C while 0.1 mM of PapN and PapC were injected to 370 μl of 0.025 mM LPS. Overall, 38 injections were performed for each experiment and the data were analyzed by MicroCal Origin software (MicroCal origin, USA).

**Secreted alkaline phosphatase (SEAP) assays.** HEK-Blue™ hTLR4 cells were purchased from Invivogen (San Diego, CA, USA). Cells were seeded in 96-well plates (2.5 × 10^4 cells/well) in HEK-Blue detection medium (Invivogen, San Diego, CA, USA) and peptides (0–100 μM) were treated for 1h followed by LPS (20 ng/mL) stimulation for 16 h. The SEAP production was then determined as described previously(6).

**ELISA analysis.** RAW 264.7 cells were obtained from Korean cell line bank (Seoul, Korea) and the cells were maintained in DMEM culture media (Thermo Fischer Scientific Inc., MA, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin at 37°C in a humidified 5% CO₂ incubator. RAW 264.7 cells (1x10^4) were treated with varying concentrations of peptides (0 – 10 μM, 1 h) and then stimulated with LPS (20 ng/mL) for 16 h. After incubation, the levels of
inflammatory cytokines in cell supernatant using corresponding ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

**Saturation Transfer Difference (STD)-NMR.** STD-NMR experiments were recorded at 298 K on a Bruker 900 MHz spectrometer at KBSI (Ochang, Korea). The STD-NMR spectra were obtained with selective saturation of 1.3 μM TLR4/MD-2, 1.7 μM TLR4, and 4 μM MD-2 (R&D Systems, Minneapolis, MN, USA) resonances at -3.0 ppm (40 ppm for reference spectra) with a protein to peptide ratio of 1:100. A cascade of 40 selective Gaussian-shaped pulses of 50 ms duration were used in all STD-NMR experiments with a total saturation time of 2 s with scan numbers of 8K, 7K, and 1K for TLR4/MD-2, TLR4 and MD-2, respectively. Subtraction of the two spectra (on resonance-off resonance) leads to the difference spectrum, which contains signals arising from the saturation transfer. Peptide samples were freeze-dried and dissolved in 10 mM deuterated sodium phosphate buffer at pH 7.4 for TLR4/MD-2, pH 7.2 for TLR4 and pH 6.8 for MD-2 respectively.

**Binding assay.** Binding affinity measurements for peptides with TLR4/MD-2, TLR4 and MD-2 protein (R&D Systems, Minneapolis, MN, USA) were performed using SPR on a Biacore T200 Instrument (GE Healthcare, Sweden). The receptors were covalently immobilized onto two flow cells of a CM5 chip using a standard EDS/NHS amine coupling method with sodium acetate buffer (pH 4.0 for TLR4/MD-2 and TLR4, pH 5.0 for MD-2 protein, respectively). One flow cell was used as a reference and was immediately blocked with 1 M ethanolamine. In the other flow cells, protein was injected 30 μg/mL to a CM5 chip surface, such that the resonance values were 2500 for TLR4/MD-2, 4000 for TLR4 and 2400 for MD-2. The binding affinities of the peptides to TLR4/MD-2, TLR4, and MD-2 protein were measured using a 1:1 binding assay using Biacore T200 Evaluation Software 3.0 (GE Healthcare, CA, USA).

**Prediction of binding model.** AutoDock Vina implemented in YASARA software version 20.7.4 (YASARA Bioscience,Wien, Austria)(7) was used for molecular docking simulation of TLR4/MD-2/papiliocin. The crystal structure of the human TLR4/MD-2/LPS complex (PDB ID: 3FXI)(8) was used to prepare a receptor coordinate by removing all molecules except for one TLR4/MD-2 complex. As the sidechain of R90 in MD-2 is located to interfere the entrance of the ligand into the pocket of MD-2, we kept the atoms in the sidechain of R90 flexible during the simulation. Papiliocin (PDB ID: 2LA2) was used as a potential antagonist, with all the backbone atoms fixed except for R1 and W2 at the N-terminus, K37 at the C-terminus and hinge region while side chains of all other residues were kept flexible in the structure. An entire TLR4/MD-2 complex was set as the binding site, and molecular docking calculation with 200 docking runs was performed. The docked conformations in all the clusters were analyzed based on the binding score. We selected the best binding model with lowest binding energy and highest population, and the best binding model satisfied the intermolecular interactions which agree with the experimental data.

**In vitro and in vivo toxicity measurements.** RAW 264.7 cells were obtained from Korean cell line bank (Seoul, Korea) and the cells were maintained in DMEM culture media (Thermo Fischer Scientific Inc., MA, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin at 37°C in a humidified 5% CO2 incubator. The cytotoxic capacity of peptides were analyzed using WST-8 Cell Proliferation Assay Kit (Biomax Co, Ltd, Seoul, South Korea)(2). Briefly, cells (1x10⁶) were seeded in 96-well plate and then treated with papiliocin and PMB (0–100 μM) for 24 h and then incubated with WST-8 reagent for another 2 h. The change in absorbance was read at 450nm against reagent blank.

The ICR mice (n = 5 per group) were intraperitoneally (i.p.) injected with papiliocin and PMB (5 mg/kg/daily for 5 days and 20 mg/kg twice a day for 3 days, each). After experiment, serum creatinine was analyzed using DeteclX® Serum Creatinine Kit (Arbor Assays, Ann Arbor, MI, USA) and AST, ALT, and BUN levels were determined using a standard kit from Asan Pharmaceutical as described previously (6). For histopathology, paraffin-blocked kidney tissue sections were
processed for hematoxylin and eosin (H&E) staining and were examined for morbid changes under light microscopy (Eclipse Ni; Nikon, Tokyo, Japan).
Supplementary Figures

Fig. S1. Saturation transfer difference (STD) NMR analysis showing the binding interaction of papiliocin to TLR4 and MD-2. (A) 1D $^1$H NMR spectra of 0.17 mM papiliocin plus 1.7 μM TLR4. (B) STD NMR spectrum obtained on sample A (Papiliocin plus TLR4). (C) 1D $^1$H NMR spectra of 0.2 mM papiliocin plus 2 μM MD-2. (D) STD NMR spectrum obtained on sample C (Papiliocin plus MD-2). Aromatic protons of W2 and F5 are marked in green box.
Fig. S2. Surface plasmon resonance (SPR) sensorgrams of (A) TLR4, and (B) MD-2 protein interaction with varying concentrations of papiliocin.
Fig. S3. (A) Sequence alignment of papiliocin with cecropins from various insects, including moths (Bombyx mori, Antheraea pernyi, Heliothis virescens, Hyalophora cecropia, Trichoplusia ni and Danaus plexippus). Yellow box indicates the highly conserved hinge sequence while blue and red boxes represent the conserved positively charged residues and hydrophobic residues interacting with TLR4/MD-2, respectively. (B) Sequence alignment of papiliocin with cecropins from Aedes aegypti, Drosophila melanogaster and Sarcophaga peregrina. Completely conserved residues (100%) are highlighted in black-filled boxes, sequences showing more than 80% conservation are boxed in black lines in the alignment.
Fig. S4. Surface plasmon resonance (SPR) sensorgrams of TLR4/MD-2 complex, TLR4, and MD-2 protein interaction with varying concentrations of PapN (A-C) and PapC (D-F) peptides.
Fig. S5. Surface plasmon resonance (SPR) sensorgrams of TLR4/MD-2 complex interaction with varying concentrations of (A) papiliocin, (B) P24G and (C) G23P peptides.
Fig. S6. (A) Effects of papiliocin, PapN, PapC, P24G, and G23P on the inhibition of (A) Nitrite, (B) TNF-α, (C) IL-6 levels in LPS-stimulated RAW 264.7 cells (LPS, 20 ng/mL; peptides, 1.25, 2.5, 5, and 10 μM). Each bar represents mean ± SEM of three independent experiments. ***P < 0.001, and ns, non-significant compared with control by two-way analysis of variance with Dunnett's comparison test.
Fig. S7. Papiliocin and its analogs’ effects on (A) Concentration-dependent (0–8 μM) depolarization of intact E. coli. (B) LAL assay demonstrating the ability of peptides to neutralize LPS (LPS, 2 ng/mL; peptides, 3.1, 6.3, 12.5, 25, and 50 μM). (C) Isothermal titration calorimetry (ITC) measurement showing the binding interaction of K3E, K6E, R13E and R16E (0.1 mM, each) to 0.025 mM LPS. Upper panels represent the heat (μcal/s) of the injectant and lower panels show the enthalpy (kcal/mol) of the injectant. The supplements show the binding affinity (KD) for each interaction. Each bar represents mean ± SEM of three independent experiments. **P < 0.01, ***P < 0.001, and ns, non-significant compared with control in two-way analysis of variance with Dunnett’s comparison test.
Fig. S8. Anti-inflammatory activities of LPS-interacting papiliocin analogs (K3E and K6E) (A) Nitrite, (B) TNF-α, (C) IL-6 levels in LPS-stimulated RAW 264.7 cells (LPS, 20 ng/mL; peptides, 1.25, 2.5, 5, and 10 μM). (D) Effects of peptides (0–100 μM) on TLR4 inactivation measured in SEAP assays in LPS (20 ng/mL)-stimulated HEK-Blue™ hTLR4 cells. Error bar represents the mean ± SEM of three independent experiments. ***P < 0.001, and ns, non-significant compared with control in one-way ANOVA with Dunnett’s comparison test (A–C) and IC₅₀ using non-linear regression analysis (D).
Fig. S9. Binding interaction between papiliocin and TLR4/MD-2. (A) Binding model of papiliocin to TLR4/MD-2 complex showing hydrophobic C-terminal helix of papiliocin inserted into the hydrophobic pockets of MD-2. Papiliocin is in red ribbon while TLR4 in green and MD-2 in gray are in surface representation. (B) Binding interactions between papiliocin and TLR4/MD-2 complex. Hydrogen bonds and electrostatic interactions are shown on the left side and hydrophobic interactions are shown on the right side. The N- and C-terminal helices of papiliocin are represented as separated boxes connected by a solid line indicating the hinge region. Residues in TLR4 and MD-2 that are also involved in LPS binding are shown in red. Hydrogen bonds (blue lines) and electrostatic interactions (purple lines) are shown on the left side and hydrophobic interactions (brown lines) are shown on the right side. The N- and C-terminal helices of papiliocin are represented as separated boxes connected by a solid line indicating the hinge region. Residues in TLR4 and MD-2 that are also involved in LPS binding are shown in red.
**Fig. S10.** Effect of the alanine substituted papiliocin analogs (R13A and R16A) on biological activity in comparison to those of papiliocin, R13E, R16E. (A) TNF-α, (B) IL-6 levels in LPS-stimulated RAW 264.7 cells (LPS, 20 ng/mL; peptides, 1.25, 2.5, 5, and 10 μM). (C) Effects of peptides (0–100 μM) on TLR4 inactivation measured by SEAP assays in LPS (20 ng/mL)-stimulated HEK-Blue™ hTLR4 cells. Error bar represents mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, and ns, non-significant compared with control by one-way ANOVA with Dunnett's comparison test (A, B) and IC₅₀ by non-linear regression analysis (C).
Fig. S11. Antibacterial activities of papiliocin in comparison to conventional antibiotics. Time-killing kinetics of papiliocin to (A) E. coli 1682, and (B) CREC 16046 strains. The bacterial strains were exposed to the peptides and control antibiotics at 1 μM and were monitored for different time intervals (0–4 h) to assess the killing ability. (C) Papiliocin affects biofilm formation in a dose-dependent manner against E. coli 1682, and CREC 16046. All peptides were treated with 0–2 μM, for 16 h, and the percentage of biofilm growth was quantified after staining with crystal violet. CREC, carbapenem-resistant E. coli; PMB, polymyxin B. (D) Ultrastructural images of E. coli 1682 cell morphology following treatment with papiliocin at 2 μM for 1, 2, and 4 h and visualized by SEM and TEM (scale bar = 1 μm, scale bar = 500 nm, respectively). Error bar are shown as the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ns, non-significant compared with the control by two-way ANOVA with Dunnett’s comparison test (B).
Fig. S12. (A) Dose-dependent cytotoxic effect of papiliocin and PMB on RAW 264.7 cells. After 24 h treatment with peptides (0–100 μM), cell survival was quantified using WST-8 assay. (B) Effect of papiliocin and PMB on toxic markers (AST, ALT, and BUN) using ICR mice. Mice (n=5 per group) were intraperitoneally (i.p.) injected with papiliocin or PMB (5mg/kg/day for 5 days and 20mg/kg twice day for 3 days), and toxic parameters were quantified after respective durations in the serum. (C) Hematoxylin and eosin-stained kidney sections show the effect of papiliocin and PMB on morbid changes such as tubular necrosis (asterisk) and vascular congestion (black arrow). Magnification, 10x and scale bar, 200 μm. Each bar represents mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, and ns, non-significant compared with control by one-way ANOVA with Dunnett’s comparison test.
**Supplementary Table S1.** Antibacterial activities of papiliocin and its analogs against gram-negative bacteria.

| Peptides     | Sequence*                                 | MIC (μM) |  |  |  |
|--------------|------------------------------------------|----------|---|---|---|
|              |                                          | E. coli | E. coli K1 | A. baumannii | P. aeruginosa |
| Papiliocin   | RWKIFKKIEKVRGRNVRDGIIKAGPAVAVGQAATVVK   | 1        | 1           | 2            | 4            |
| PapN         | RWKIFKKIEKVRGRNVRDGIIKA                 | 16       | 16          | 32           | 32           |
| PapC         | AVAVGQAATVVK                             | >64      | >64         | >64          | >64          |
| Papiliocin G23P | RWKIFKKIEKVRGRNVRDGIIKAPPAVAVGQAATVVK | 2        | 2           | 4            | 64           |
| Papiliocin P24G | RWKIFKKIEKVRGRNVRDGIIKAGGAVAVGQAATVVK | 1        | 1           | 2            | 4            |
| Papiliocin R1E   | EWKIFKKIEKVRGRNVRDGIIKAGPAVAVGQAATVVK | 8        | 8           | 16           | >64          |
| Papiliocin W2F5A   | RAKIAKKIEKVRGRNVRDGIIKAGPAVAVGQAATVVK | 4        | 8           | 16           | 16           |
| Papiliocin K3E   | RWEIFKKIEKVRGRNVRDGIIKAGPAVAVGQAATVVK | 32       | 16          | >64          | >64          |
| Papiliocin K6E   | RWKIFKIEKVRGRNVRDGIIKAGPAVAVGQAATVVK   | 16       | 32          | >64          | >64          |
| Papiliocin K7E   | RWKIFKERIEKVRGRNVRDGIIKAGPAVAVGQAATVVK | 4        | 4           | 8            | 32           |
| Papiliocin E9R   | RWKIFKIKERKVRGRNVRDGIIKAGPAVAVGQAATVVK | 2        | 2           | 2            | 2            |
| Papiliocin K10E  | RWKIFKKIEEVRGRNVRDGIIKAGPAVAVGQAATVVK  | 2        | 2           | 4            | 32           |
| Papiliocin R13E  | RWKIFKKIEKREVRNVRDGIIKAGPAVAVGQAATVVK  | 2        | 2           | 8            | 32           |
| Papiliocin R13A  | RWKIFKIEKVKGAEVRNVRDGIIKAGPAVAVGQAATVVK| 2        | 2           | 4            | 16           |
| Papiliocin R16E  | RWKIFKIEKVRGNVEDGIIKAGPAVAVGQAATVVK    | 8        | 8           | 32           | 32           |
| Papiliocin R16A  | RWKIFKKIEKVRGNVEDGIIKAGPAVAVGQAATVVK   | 2        | 2           | 4            | 16           |
| Papiliocin V28,29A | RWKIFKKIEKVRGRNVRDGIIKAGPAVAAAGQAATVVK| 2        | 2           | 2            | 4            |
| Papiliocin V35,36A | RWKIFKKIEKVRGRNVRDGIIKAGPAVAVGQAATAAK  | 2        | 2           | 2            | 4            |
| Papiliocin K37E  | RWKIFKKIEKVRGRNVRDGIIKAGPAVAVGQAATVVE  | 2        | 2           | 2            | 16           |

*All peptides have C-terminal amidation.

Abbreviations: MIC, minimum inhibition concentration at which the peptide or antibiotic inhibits over 99% growth of bacteria (MIC values are representative of three biological replicates).

Bold letters: substituted residues.
**Supplementary Table S2.** Antibacterial activities of papiliocin and other antimicrobials against standard and carbapenem-resistant bacteria.

| Microorganism           | Papiliocin | PMB | Imipenem | Meropenem | Melittin |
|-------------------------|------------|-----|----------|-----------|----------|
| *E. coli* (KCTC 1682)   | 1          | 2   | 0.5      | 0.5       | 8        |
| *E. coli K1* (ATCC 700973) | 1          | 1   | 0.5      | 0.5       | 8        |
| *A. baumannii* (KCCM 40203) | 1          | 2   | 1        | 2         | 4        |
| *P. aeruginosa* (KCCM 11328) | 4          | 1   | 1        | 1         | 32       |
| *K. pneumonia* (NCCP 16054) | 2          | 2   | 0.5      | 0.5       | 16       |
| *S. aureus* KCTC 1621† | >128       | >128| 8        | 8         | 4        |
| CREC (NCCP 16044)       | 1          | 2   | 8        | 32        | 8        |
| CREC (NCCP 16046)       | 1          | 2   | 8        | 128       | 8        |
| CREC (NCCP 16284)       | 2          | 4   | >128     | >128      | 16       |
| CRKP (NCCP 15782)       | 2          | 2   | >128     | >128      | 16       |
| CRKP (NCCP 15864)       | 2          | 2   | 8        | >128      | 16       |
| CRKP (NCCP 15866)       | 2          | 2   | 64       | >128      | 16       |
| CRAB (CCARM 12004)      | 1          | 4   | 128      | >128      | 8        |
| CRAB (CCARM 12005)      | 1          | 2   | 128      | >128      | 8        |
| CRAB (CCARM 12006)      | 1          | 8   | 128      | >128      | 16       |

†gram-positive bacteria.
Abbreviations: MIC, minimum inhibition concentration at which the peptide or antibiotic inhibits over 99% growth of bacteria (MIC values are representative of three biological replicates); PMB, polymyxin B; CREC, carbapenem-resistant *E. coli*; CRKP, carbapenem-resistant *K. pneumoniae*; CRAB, carbapenem resistant *A. baumannii*. 
SI Dataset S1 (dataset_one in excel file format)
Papiliocin interacts with LPS and specifically targets TLR4. (Fig.1)

SI Dataset S2 (dataset_two in excel file format)
Binding interaction between papiliocin and TLR4/MD-2. (Fig.2)

SI Dataset S3 (dataset_three in excel file format)
Effect of important structural elements on LPS–interactions of papiliocin. (Fig.3)

SI Dataset S4 (dataset_four in excel file format)
R13 and R16 are the key residues contributing to the TLR4 antagonistic activity of papiliocin. (Fig.4)

SI Dataset S5 (dataset_five in excel file format)
Antiseptic effects of papiliocin and PMB in E. coli K1-induced sepsis mouse model (Fig.5)

SI Dataset S6 (dataset_six in excel file format)
Surface plasmon resonance (SPR) sensograms of TLR4 and MD-2 protein interaction with varying concentrations of papiliocin. (Fig.S2)

SI Dataset S7 (dataset_seven in excel file format)
Surface plasmon resonance (SPR) sensograms of TLR4/MD-2 complex, TLR4, and MD-2 protein interaction with varying concentrations of PapN and PapC peptides. (Fig.S4)

SI Dataset S8 (dataset_eight in excel file format)
Surface plasmon resonance (SPR) sensograms of TLR4/MD-2 complex interaction with varying concentrations of papiliocin, P24G and G23P peptides (Fig.S5).

SI Dataset S9 (dataset_nine in excel file format)
Anti-inflammatory activities of papiliocin, PapN, PapC, P24G, and G23P in LPS-stimulated RAW 264.7 cells (Fig.S6).

SI Dataset S10 (dataset_ten in excel file format)
Membrane depolarization and LPS–interactions of papiliocin, PapN, PapC, P24G, and G23P (Fig.S7).

SI Dataset S11 (dataset_eleven in excel file format)
Anti-inflammatory activities of LPS-interacting papiliocin analogs (K3E and K6E) (Fig. S8)

SI Dataset S12 (dataset_twelve in excel file format)
Effect of the alanine substituted papiliocin analogs (R13A and R16A) on biological activities in comparison to those of papiliocin, R13E, R16E (Fig. S10)

SI Dataset S13 (dataset_thirteen in excel file format)
Antibacterial activities of papiliocin in comparison to conventional antibiotics (Fig. S11)

SI Dataset S14 (dataset_fourteen in excel file format)
Cytotoxic effects of papiliocin and PMB on RAW 264.7 cells, kidney and liver (Fig. S12)

SI Dataset S15 (dataset_fifteen in excel file format)
Antibacterial activities of papiliocin and its analogs (Table S1)

SI Dataset S16 (dataset_sixteen in excel file format)
Antibacterial activities of papiliocin and other antimicrobials against standard bacterial strains and MDR bacteria (Table S2)
Supplementary References

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