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Supplementary Information for

The antimicrobial peptide ZY4 combats multidrug-resistant *P. aeruginosa* and *A. baumannii* infection

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This PDF file includes:

Supplementary Information Text

Materials and Methods

Peptide synthesis

All peptides in Fig. 1A were synthesized by GL Biochem Ltd. (Shanghai, China), and their purity was determined to be higher than 98% using reversed-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry.

Animals

6-8 weeks old female or male C57BL/6 mice (20-22 g) were used for all in vivo experiments. Mice were obtained from Kunming medical university. Mice experiments were approved and handling of animals followed guidelines set by the Animal Care and Use Committee of Kunming Institute of Zoology, Chinese Academy of Sciences (SMKX-2018017).

Bioinformatic analysis and structure modeling

Physical and chemical parameters of the designed peptides were analyzed through ExPASy Bioinformatics Resource Portal (http://www.expasy.org/tools/). The helix-wheel structures of the peptides were constructed by HeliQuest (http://heliquest.ipmc. cnrs.fr/).

Molecular dynamic (MD) simulation of cathelicidin-BF15-a3 and cathelicidin-BF15-a4 (ZY4) was performed to evaluate their stability, folding, and conformational changes. All MD simulations were performed using Amber99SB-ILDN force field(1) using GROMACS 5.1 package(2), running on high-performance Linux system (National Supercomputing Center in Shenzhen). During MD simulations, all the systems were solvated using TIP4P(3) water model in a periodic box, followed by addition 10 Cl^- ions to neutralize the systems. Before MD simulations, energy minimization, NVT, and NPT equilibration were performed. Finally, MD simulations were run for 10 ns time scale under constant temperature (300 K) and pressure (1 atm). Snapshots were collected from the end of MD simulations and PDBs were generated. Discovery Studio 3.1 (Accelrys Software Inc.) and GROMACS tools were used to analyze the stability of each system.

Bacteria strains preparation and growth conditions

Six strains of *P. aeruginosa* (CICC21625, CMCC10104, and 4 clinically isolated strains presented as C1, C2, C3 and C5), six strains of *A. baumannii* (22933, and 5 clinically isolated strains presented as CN40, 18C116, 18C132, 18C135 and 18C136), *S. aureus* (ATCC2592), *E. coli* (25922), *C. albicans* (SC5341) and *B. subtilis* were obtained from the First Affiliated Hospital of Kunming Medical University. The strains of *A. baumannii* were cultured in brain heart infusion (BHI) broth (HKM, Guangzhou, China). YPD (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) was used for routine growth of *C. albicans*. The other bacterial strains were cultured in Luria-Bertani (LB) broth.

Antibacterial properties *in vitro*

Minimal inhibitory concentration (MIC) of ZY4 against all strains of bacteria was determined using the tube microdilution assay as described in our previous studies(4-6). Briefly, bacterial strains described above were grown to mid-logarithmic phase at 37 ℃ in their corresponding broth, harvested by centrifugation at 3500 rpm for 5 min and cells pellets were washed twice with PBS (1.5 mmol/L KH_2PO_4, 2.7 mmol/L Na_2HPO_4, 0.15 mol/L NaCl, pH 7.4) and resuspended in RPMI 1640 media with 10% fetal bovine serum (FBS) and diluted to 2×10^5 CFU/ml in the same medium. 100 ul of this suspension were mixed in 96-well plates with 100 ul of ZY4 in PBS solution at different stock concentrations (1-200 μg/ml). The suspensions were then incubated at 37 ℃ for 18 to 24 h, bacteria growth was determined by measuring the absorbance at 600 nm using a microplate reader (Epoch Etock). For comparison, the MICs of colistin sulfate and other antibiotics including tobramycin, levofloxacin, kanamycin, and carbenicillin disodium were also determined. The MIC was defined as the lowest concentration of test samples that resulted in no detectable bacterial growth.

Hemolysis and cytotoxicity assays

To evaluate possible side effects of ZY4, red blood hemolysis and cytotoxicity assays were performed as described in our previous reports(4, 5) with minimum modifications. For hemolysis assay, 100 ul human red blood cell suspension was incubated with 100 ul of ZY4 at different concentrations ranging from 2.5 to 320 μg/ml. After incubation for 24 h at 37 ℃, the cells were centrifuged and the absorbance of the supernatant was measured at 540 nm. The value for "zero hemolysis" was determined using sterile PBS, while 100% hemolysis was established using 1% (v/v) Triton X-100. Hemolysis of testing sample was calculated as the percentage of Triton X-100-induced hemolysis.

Cytotoxicity was determined using mammalian cells. Briefly, human HEK293 embryonic cells (1×10^5 cells/ml) and human HaCaT keratinocyte cells (1×10^5 cells/ml) from Cell Bank of Kunming Institute of Zoology, Chinese Academy of Sciences were cultured in 96-well plates with Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% FBS and penicillin (100 U/ml)-streptomycin (100 μg/ml) at 37 ℃ in a humidified 5% CO_2 atmosphere. After 24 h incubation, fresh medium with or without testing peptides at different concentration was added to the wells and incubated for another 24 h under same conditions, cell viability was determined by MTS assay kit (ab197010, Abcam) based on the reduction of a tetrazolium compound by viable cells to generate a colored formazan dye soluble in cell culture media. After 24-h incubation with testing samples, fresh medium and 20 ul of MTS (5 mg/ml) was added to each well and incubated for 2 to 5 h at standard cell growth conditions and the absorbance at 490 nm of the resulting solution was measured. The experiments were conducted in triplicate.

Effects of human plasma on ZY4 antibacterial activity

To determine the stability of ZY4 in human plasma, ZY4 (final concentration 10 mg/ml) was mixed with 100 % human plasma for 0, 1, 2, 4, 6, 8 and 10 h at 37 ℃, respectively. Residual antibacterial activity for each incubation time point was evaluated by disk diffusion assay. In brief, bacteria were seeded on nutrient agar plates, 6 mm paper disks were placed on top and 6 μl aliquots of the plasma-peptide mixture was added to the paper disks. After 24 h incubation, the inhibitory zones against *P. aeruginosa* CICC21625 and *A. baumannii* 22933 were measured and recorded.

Plasma stability assay

To test the stability of ZY4 after in plasma, the peptide was first diluted in mouse plasma (use EDTA-K_2 as anticoagulant from a 8-week old male C57BL/6 mice) to a final concentration of 10 μt/ml. After incubation for 0, 2, 4, 6, 8 and 10 h at 37 ℃, an aliquot of 10 μl sample was taken for protein precipitation with an equal volume of 4% H_3PO_4, the mixture was vortex-mixed for 2 min and then centrifuged at 13000 rpm for 15 min at 4 ℃. Three microliter of supernatant was injected for liquid chromatography-tandem mass spectrometric detection (LC/MS/MS, Exactive Plus, Thermo Scientific).

Pharmacokinetic analysis

C57BL/6 mice (6-8 weeks, male, 20-22 g) were used for pharmacokinetic analysis of ZY4. Briefly, mice (n = 6) were i.v. injected with 8 mg/kg ZY4, blood samples were drawn retroorbitally under short-term anesthesia at different time points (1, 5, 10, 30, 60 min, and 2, 5, 10, 18, 24 h) and placed into EDTA-K_2-treated tubes. Plasma was separated by centrifugation at 2000 rpm for 10 min at 4 ℃ and an aliquot of 10 μl plasma was then taken for protein precipitation with an equal volume of 4% H_3PO_4, the mixture was vortex-mixed for 2 min and then centrifuged at 13000 rpm for 15 min at 4 ℃. Three microliter of supernatant was injected for LC/MS/MS analysis. A series of dilutions of ZY4 in plasma were performed to obtain a standard calibration curve. The pharmacokinetic parameters such as half-life (t1/2) and the area under curve (AUC) were determined using a Microsoft add-in tool, PKSolver(7).

***In vivo* acute toxicity**

To determine *in vivo* acute toxicity of ZY4, female C57 mice (6 to 8 weeks old) were i.v. injected with a single dose of ZY4 or colistin sulfate at 10, 20, and 40 mg/kg. After injection, mice (n = 10) were visually inspected for signs of toxicity three times a day for 96 h. Toxicity was rated based

on the following observations: ruffled fur and poor motility = mild signs; hutching, very ruffled fur and complete immobility even under stimulation = manifest signs.

Evaluation of bacteria membrane permeabilization

Effect of ZY4 on bacteria membrane was evaluated using flow cytometry and scanning electron microscopy (SEM). For flow cytometry, mid-log phase *P. aeruginosa* CICC10104 culture was harvested by centrifugation at 3500 rpm for 10 min. After two washes with PBS, the bacterial pellets were re-suspended in the same buffer at a dilution of 1×10^6 CFU/ml. The suspension was then incubated for 1 h at 37 ℃ with ZY4 (1 × and 2 × MIC), or with PBS as control. 1 h post incubation the suspensions were centrifuged and the pellets re-suspended in 1 ml PBS followed by addition propidium iodide (PI) (Sigma Aldrich) at a final concentration of 5 μg/ml. The suspension was lightly vortexed to mix and incubated at room temperature in the dark for 5 min before being analyzed by flow cytometry (BD LSRFORTESAA^{TM}, USA).

For kinetics of PI staining, a mid-log phase cultures of *P. aeruginosa* CICC10104 and *A. baumannii* 22933 were diluted in PBS to 1×10^6 CFU/ml. The bacteria suspension was then incubated for 15 minutes on ice in the dark with PI dye at a final concentration of 10 μg/ml. After incubation, PI fluorescence was measured over time using the flow cytometer. As controls bacteria was exposed to PBS without ZY4 and for the test, bacteria samples were treated with ZY4 (5 μM) and the samples fluorescence measured immediately during five minutes.

For SEM to determine membrane morphology was performed as described in our previous report(5). *P. aeruginosa* CICC21625 and *A. baumanii* 22933 was cultured in LB broth and BHI broth, respectively, to exponential phase. After two washes with 0.15 M saline, the bacterial pellets were re-suspended and incubated with or without ZY4 (2 × MIC) at 37 ℃ for 30 min. After incubation, the suspension was centrifuged at 1000 rpm for 10 min and the residual pellets fixed with 2.5% buffered glutaraldehyde at 4 ℃ for 2 h then post-fixed in 1% buffered osmium tetroxide for 2 h. Subsequently, the pellets were dehydrated in a graded series of ethanol, frozen in liquid nitrogen cooled tertbutyl alcohol and vacuum dried overnight. Finally, the bacteria pellets were mounted onto aluminum stubs and vacuum sputter-coating with gold and the membrane morphology analyzed with a Hitachi S-3000N (Japan) SEM.

Bacterial killing kinetic assay

The bacterial killing kinetic assay was performed according to a previous method with minor modifications(8). *P. aeruginosa* CICC21625 and *A. baumanii* 22933 were first cultured to exponential phase and diluted to 1×10^6 CFU/ml with fresh LB and BHI broth, respectively. ZY4 (1, 5, 10 × MIC) or colistin (1, 5, 10 × MIC) was added to the bacterial suspension and incubated at 37 ℃ for 0, 1, 10, 30 60 and 180 min, respectively. Fifty microliter aliquots were extracted at each time point and diluted with fresh broth for 1000 times, 50 ul of the dilution was seeded on agar plates. After incubation at 37 ℃ for 24 h, the viable colonies were determined.

Biofilm inhibition assay

To determine ZY4 biofilm inhibition ability, biofilm inhibition assay was done as described elsewhere with little modifications(9). Briefly, 200 ul of 1×10^6 CFU/ml *P. aeruginosa* CICC21625 and *A. baumanii* 22933 was cultured in RPMI 1640 medium with 10% FBS at 37°C for 24 h, in 96-well plates, with or without ZY4 (0.5-8 × MIC). After 24-h incubation, the planktonic bacteria were removed by washing three times with sterile PBS solution. Afterward, 99% of methanol was added and fixed for 15 min. After aspiration, the plates were allowed to dry. Dried wells were stained with 100 ul of 0.1% crystal violet for 5 min and the excess stain was gently rinsed off with tap water. The stain was resolubilized in 95% ethanol and the absorbance measured at 600 nm.

Biofilm eradication assay

Biofilms of *P. aeruginosa* CICC21625 and *A. baumanii* 22933 were grown in 96-well plate by adding 100 ul of bacteria (1×10^5 CFU/ml) in RPMI 1640 medium with 10 % FBS. Plates were incubated at 37 °C for 24 h. The wells containing biofilm were washed three times with PBS. Serial dilutions of ZY4 (0.5-8 × MIC) were prepared in a fresh 96-well plate using the same media and 100 ul of the suspension was transferred to the biofilm containing plate. 100 μl sterile media

was added to the negative control wells. Plates were then incubated in a shaking incubator (100 rpm) for at 37°C for 24 h. After incubation, the wells were emptied and washed three times with 200 ul PBS and air fixed for 1 h under aseptic condition. The percentage of biofilm removal was quantified by measuring the absorbance after applying crystal violet stain as described above.

Antibacterial properties against persister cells

Antibacterial activity of ZY4 against persister cells was carried out according to the method described previously with minor modifications(10). *P. aeruginosa* CICC21625 and *A. baumanii* 22933 was first cultured to exponential phase and diluted to 1×10^8 CFU/ml with fresh culture broth. 100 µl of this suspension was transferred to 96-well plate and incubated at 37°C for 24 h. The planktonic bacteria were removed by washing three times with sterile PBS, and 100 µl of fresh medium with colistin (50 × MIC) was then added to the wells and incubated for another 24 h under same conditions. After incubation, planktonic bacteria were first removed by washing three times with PBS, and the adherent persisters were dislodged in 100 µl of fresh PBS by 5 min sonication. Serial dilutions of ZY4 (0.5-8 × MIC) were added to the persisters suspension and incubated at 37°C for 12 h, the number of viable bacteria was determined microbiologically. PBS was used as a control, and the experiments were conducted in triplicate.

Cytokine release from LPS-induced RAW264.7 cells

Mouse RAW264.7 cells were seeded in 12-well plates (1×10^6 cells/well) and incubated at 37 °C overnight. Cells were then incubated for another 1 h in the presence or absence of ZY4 (5-20 µg/ml) and after 1 h incubation, 100 ng/ml LPS was added and the cells were incubated for another 5 to 6 h at 37 °C. TNF-α and IL-6 levels in the samples were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's guidelines (R&D Systems). Colistin (10 µg/ml) was used as a control.

Induction of resistance and determination of cross-resistance

24-h serial passages were used to evaluate the development of selective resistance following long term exposure to ZY4 or colistin. *P. aeruginosa* (CICC21625, C1 and C2) and *A. baumanii* (22933, CN40 and 18C116) were cultured in RPMI 1640 medium with 10 % FBS with constant shaking at 160 rpm at 37 °C. 20 ul of bacteria was transferred daily into 2 ml of fresh medium in the presence or absence of test compounds at sub-MIC concentrations, every passage was incubated for 24 h with constant shaking at 160 rpm. The incubation sub-MIC concentration of the test substances was doubled after every 10 passages. After every 5 passages, the bacteria suspension was mixed with 20% sterile glycerol and stored at -20 °C. The MIC values for resistant induced, control and original strains were assessed after every 5 passages as described above. In another experiment cross-resistance to other AMPs (ZY13 and LZ1) and antibiotics (levofloxacin and tobramycin) was determined using the disk diffusion assay as described above.

Induction of *P. aeruginosa* lung disease

P. aeruginosa C1 was cultured and prepared as described above and diluted to 1×10^6 CFU/ml in NaCl 0.9%. Female C57BL/6 mice (n = 8) were lightly anesthetized with inhaled isoflurane (RWD Life science Co, Ltd, China) and an inoculum of 1×10^6 CFU in 40 µl of the suspension administered intranasally (this infection dose was able to produce a clinical infection in C57 mice without mortality). Control mice were inoculated with 40 ul of sterile saline alone. 1 h post bacteria inoculation, the mice were intravenously administered with increasing concentration of ZY4 (2, 4 and 8 mg/kg, respectively) to test for dose dependence and colistin (2 and 4 mg/kg) was used as a control. Subsequently, mice were intravenously administered with the test drugs 2 times per day for 3 days. The vehicle group was administered with 0.9% NaCl.

Preparation of lung homogenates

At 72 h post inoculation, mice were anesthetized via cervical dislocation, blood was collected via retro-orbital bleeding for cytokine evaluation and lungs were harvested and homogenized in 1 ml of PBS with a hand tissue grinder. 10-fold serial dilutions of the homogenates were made in pyrogen-free NaCl and equal volumes were plated on LB agar plates and incubated at 37 °C. CFU were counted after 24 h. For assessment of cytokine levels, lung homogenates were

centrifuged at 10,000 rpm for 20 minutes at 4 °C and the supernatants were collected and stored at -20 °C.

Histopathological examination

For histopathological analysis, lungs sections were fixed in 4% paraformaldehyde, embedded in paraffin and stained with hematoxylin & eosin.

***P. aeruginosa*-induced bacteremia model**

Dissemination of *P. aeruginosa* to target organs was evaluated in *P. aeruginosa*-induced bacteremia model. *P. aeruginosa* C1 was grown to mid-log phase, harvested by centrifugation at 3500 rpm for 5 min, washed twice in PBS to remove cell detritus and diluted in the same buffer to 2×10^8 CFU/ml. A hundred microliters of this bacterial suspension were injected i.p. into female C57BL/6 mice (n = 10). One h after inoculation, ZY4 (10 mg/kg), colistin (10 mg/kg), levofloxacin (100 mg/kg) or vehicle (PBS) were i.p. administrated into the mice to assess their therapeutic effects. To evaluate bacteria dissemination to target organs, mice were sacrificed 12 h post bacteria inoculation and the spleens, livers, and kidneys were harvested, weighed and placed in 1 ml PBS. The excised organs were homogenized on ice with a hand tissue grinder. Following a 10-fold serial dilution in PBS, the homogenates were plated on LB agar plates and CFU were determined.

Evaluation of cytokine levels in endotoxin shock model

Female C57BL/6 mice (n = 8) were i.p. injected with sub-lethal concentration (15 mg/kg) of *E. coli* LPS (Sigma Aldrich). Thirty minutes post LPS injection, mice were i.p. injected with ZY4 (10 mg/kg) or PBS (control). 6 and 18 h post LPS injection, mice were sacrificed by an overdose of isoflurane and blood was collected by retro-orbital bleeding. The blood was centrifuged at 5000 x g for 10 min to collect plasma. Cytokine levels of TNF-α, MCP-1, IL-6, IL-10, and IFN-γ in plasma were evaluated by standard ELISA kits (R&D systems) following the manufactures guidelines.

***A. baumannii*-induced bacteremia model**

A. baumannii-induced bacteremia model was also used to evaluate the dissemination of bacteria to target organs. MDR *A. baumannii* CN40 was first cultured in BHI broth to mid-log phase, after centrifugation at 3500 rpm for 5 min, the bacteria were harvested and washed twice in PBS. 1×10^8 CFU of the bacteria in 200 µl PBS solution was i.p. injected into female C57BL/6 mice (n = 8). 1 h after the injection, ZY4 (1, 2, 4 mg/kg), colistin (1 mg/kg) and vehicle (PBS) were i.p. applied. Five h after injection of the bacteria, the mice were sacrificed and the blood, lung, liver and spleen were collected and homogenized as described above to assess the bacterial burden. Partial blood was used to isolate plasma as described above to determine the levels of IL-1β, IL-6, IL-10 and TNF-α.

Statistical analysis

The differences in mean values among different groups were assessed and data are expressed as mean ± SD or SEM. Data were assessed for statistical significance using Student's (unpaired) *t*-test with a significance accepted at $p < 0.05$. All data were analyzed using GraphPad Prism 6.0 software.

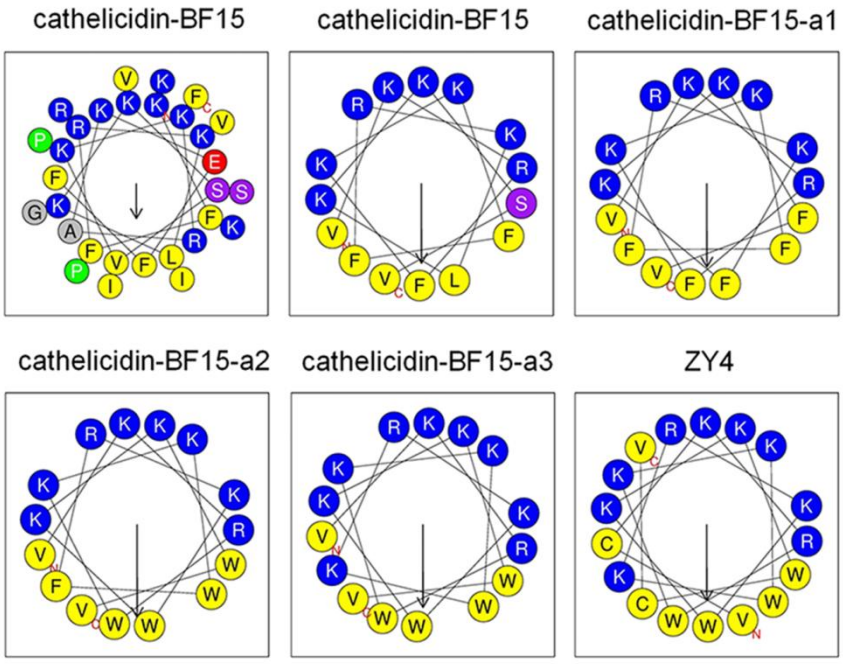


Fig. S1. Helical wheel projection diagrams of cathelicidin-BF and its analogs.

The hydrophobic residues are presented in yellow color, positively charged hydrophilic residues blue, the noncharged polar residue purple, and negatively charged hydrophilic residue red.

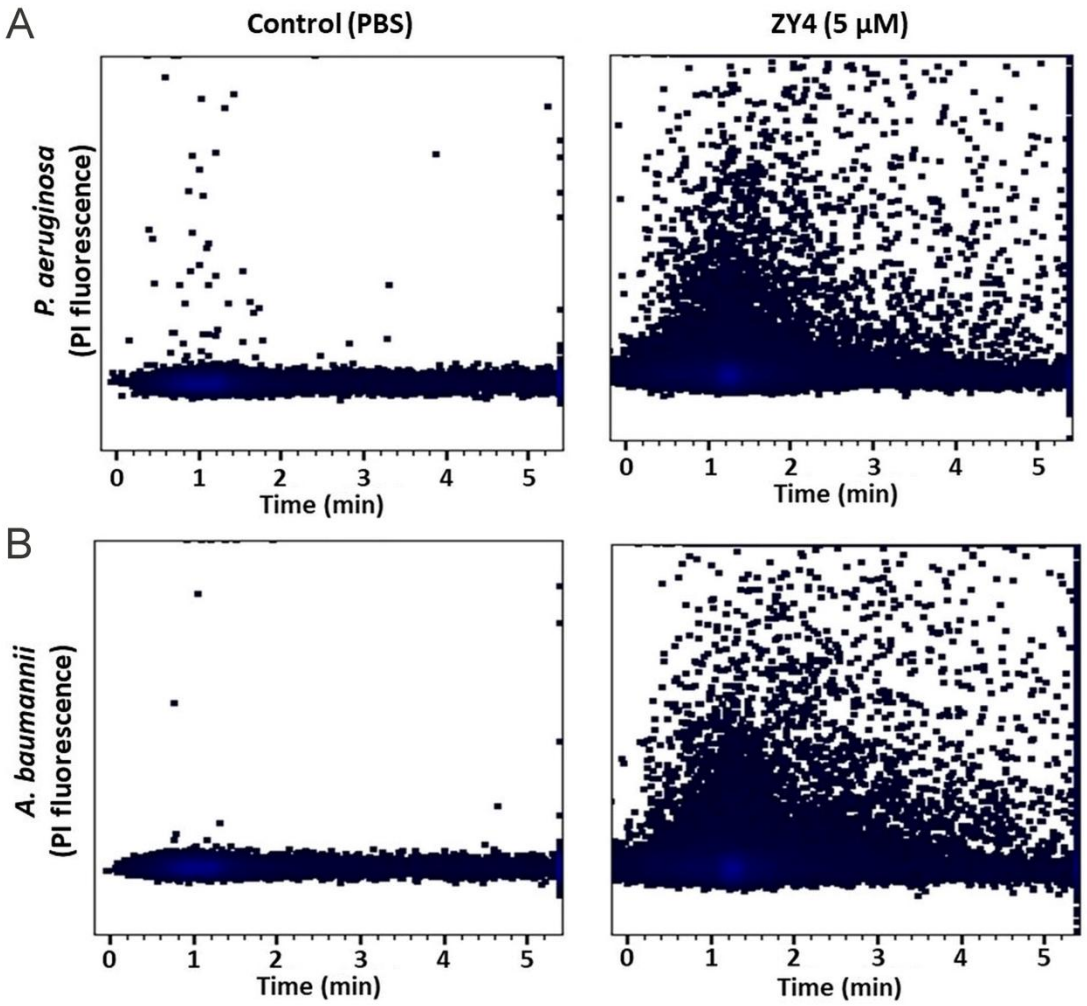


Fig. S2. Kinetics of PI influx in *P. aeruginosa* and *A. baumannii*.

P. aeruginosa CICC10104 (A) and *A. baumannii* 22933 (B) were incubated with PI for 15 minutes on ice in the dark, the samples were then measured with a flow cytometer with or without ZY4 for 5 min. Images are representative of at least three independent experiments.

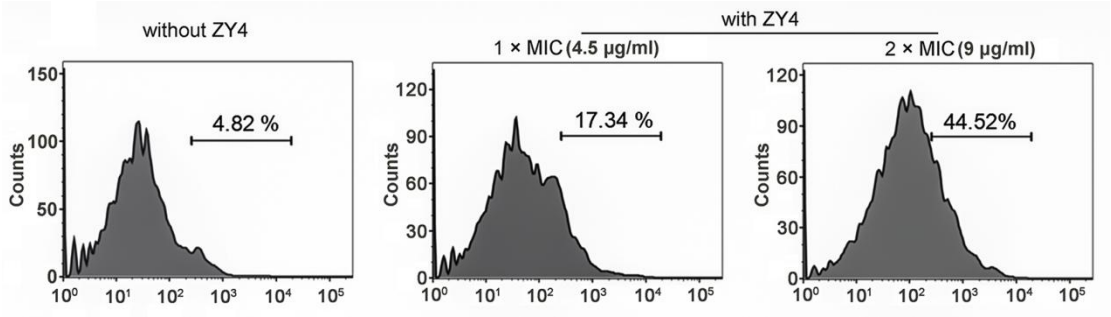


Fig. S3. Bacterial membrane permeabilization of ZY4.

Bacterial membrane permeabilization was determined by flow cytometry by measuring the intensity of PI dye following incubation of *P. aeruginosa* CICC21625 in the presence or absence of ZY4 (1 and 2 × MIC).

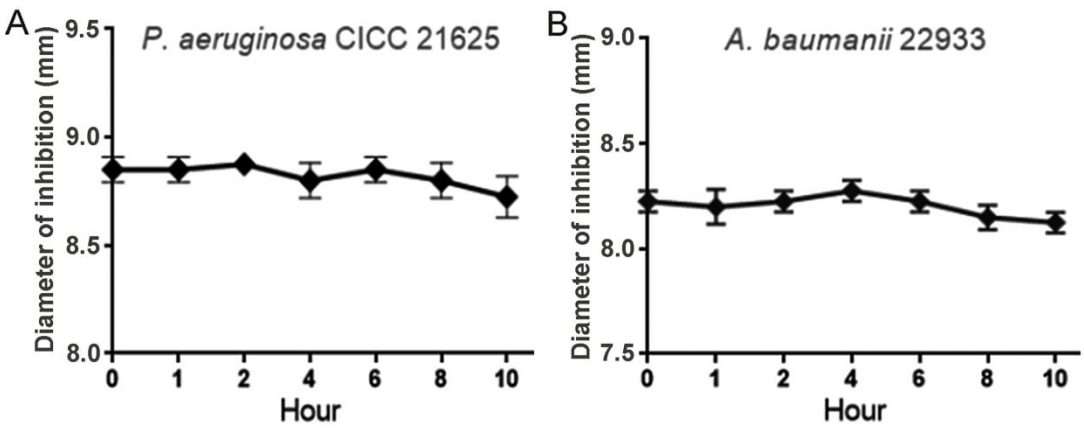
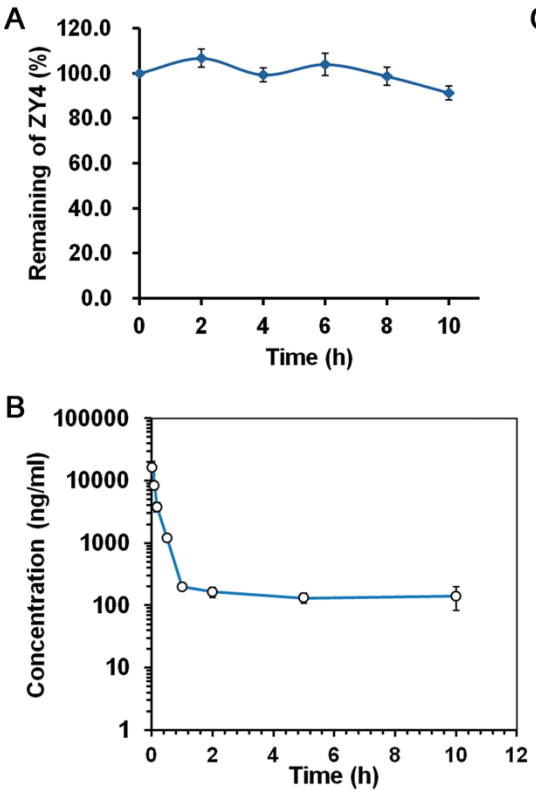


Fig. S4. ZY4 maintains its antibacterial activity in plasma.

ZY4 was incubated with human plasma (final concentration 10 mg/ml) and the stability of ZY4 determined by evaluating the antibacterial activity of ZY4 against *P. aeruginosa* CICC21625 (A) and *A. baumannii* 22933 (B) using the disk diffusion assay by measuring the diameter of zone of inhibition after incubation for 0-10 h. Data represent mean ± SD of three independent experiments.



C

Parameter	Unit	Value
Lambda_z	1/h	0.37271189
t1/2	h	1.8597399
Tmax	h	0.0167
Cmax	ng/ml	16222.9391
C0	ng/ml	19199.9155
Clast_obs/Cmax		0.00862976
AUC 0-t	ng/ml*h	4104.42943
AUC 0-inf_obs	ng/ml*h	4480.05468
AUC 0-t/0-inf_obs		0.9161561
AUMC 0-inf_obs	ng/ml*h^2	12148.2023
MRT 0-inf_obs	h	2.7116192
Vz_obs	(μg)/(ng/ml)	0.0958216
Cl_obs	(μg)/(ng/ml)/h	0.03571385
Vss_obs	(μg)/(ng/ml)	0.09684236

Fig. S5. *In vitro* plasma stability and *in vivo* pharmacokinetic studies of ZY4.

(A) ZY4 was incubated with human plasma (10 μg/ml) and the remaining of ZY4 was determined by LC/MS/MS. (B). ZY4 plasma levels at different time points *in vivo* by pharmacokinetic analysis. (C) The pharmacokinetic parameters analyzed by PKSolver were also shown. Data represent mean ± SD of six independent experiments.

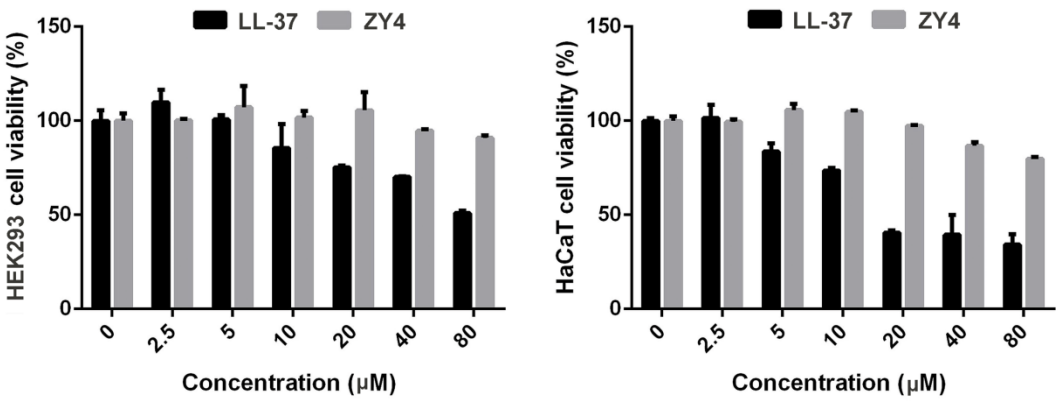


Fig. S6. ZY4 causes a negligible toxicity to mammalian cells.

Potential toxicity of ZY4 (2.5-80 μM) on human HEK293 embryonic kidney cells (A) and HaCaT keratinocytes cells (B). LL-37 (2.5-80 μM) was used as a positive control. Data represent mean ± SD of five individual experiments.

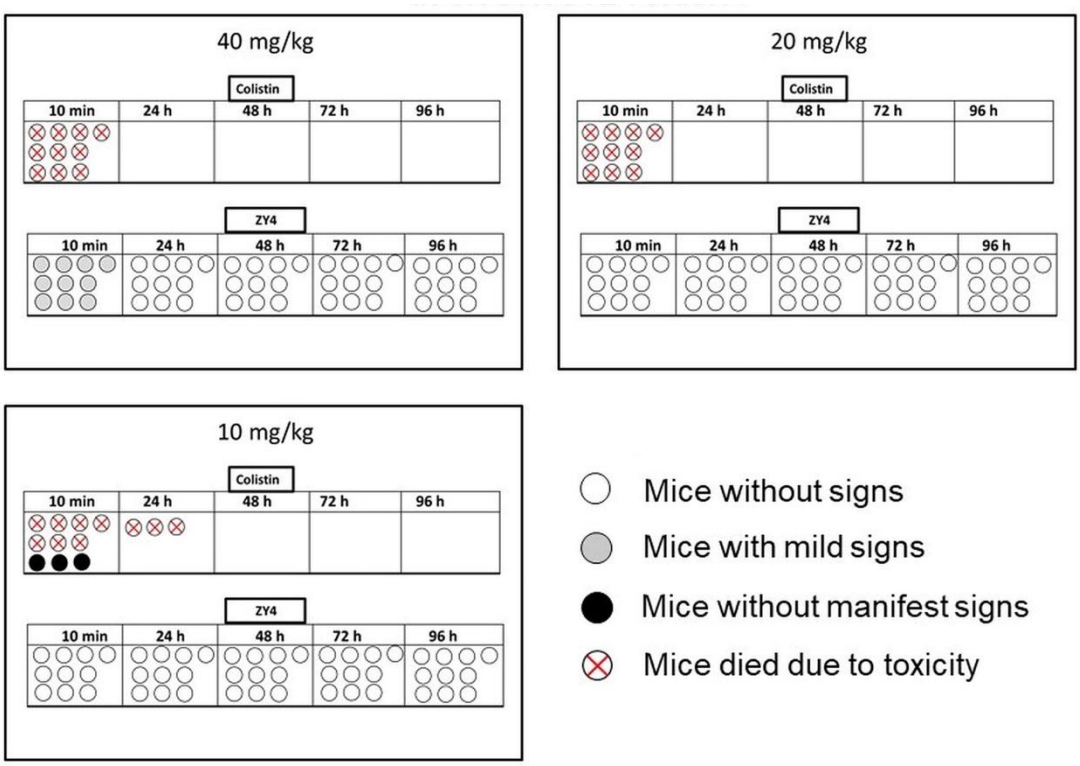


Fig. S7. *In vivo* acute toxicity of ZY4 and colistin sulfate.

Female C57 mice (n = 10 mice/group) were i.v. injected with a single dose of ZY4 or colistin sulfate at 10, 20 and 40 mg/kg and monitored for 96 h. Circles each represent one mouse and toxicity score is indicated by different circle shading as outlined in the legend.

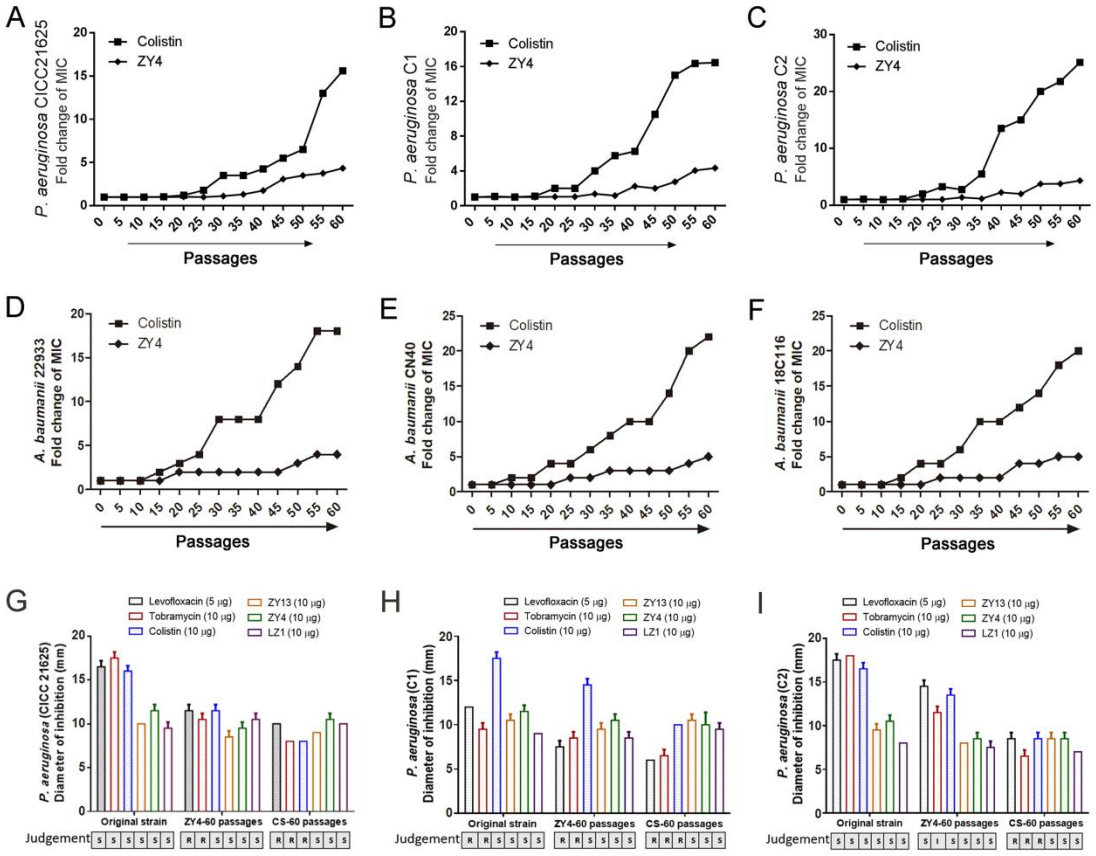


Fig. S8. ZY4 shows low propensity to induce resistance and cross-resisitance.

Resistance development of *P. aeruginosa* CICC21625 (A), C1 (B) and C2 (C) and *A. baumanii* 22933 (D), CN40 (E) and 18C116 (F) to ZY4 and colistin after 60 passages. (G-I) Evaluation of the development of cross-resistance to AMPs (ZY13 and LZ1) and antibiotics (levofloxacin and tobramycin). Cross-resistance was determined by comparing the zone of inhibition values of original bacteria strains and 60-passages resistance-induced strains using disk-diffusion assay. S: sensitive, I: intermittent, and R: resistant. Data represent mean ± SD values of three independent experiments performed in duplicate.

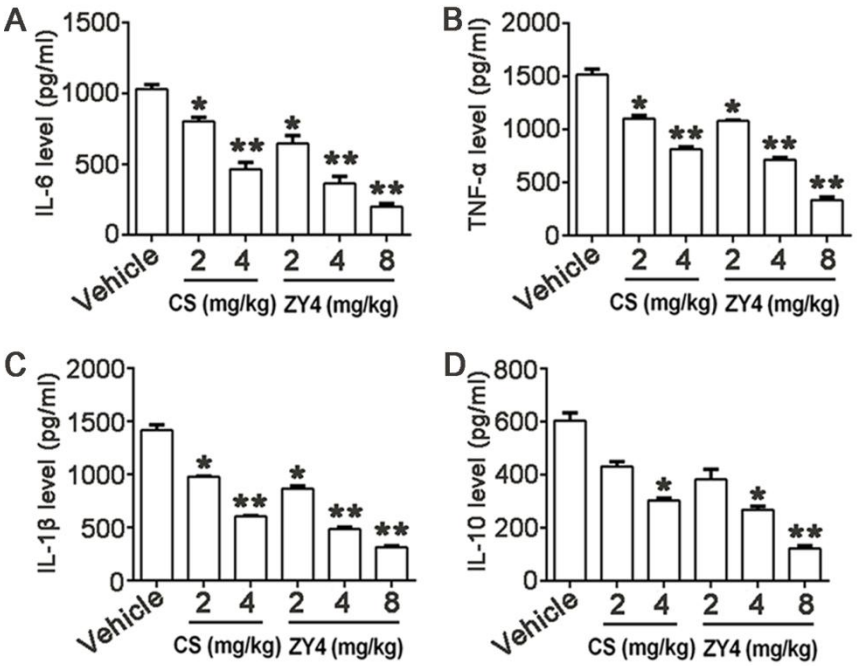


Fig. S9. ZY4 inhibits *P. aeruginosa* lung inflammation *in vivo*.

Following intranasal inoculation of mice (n = 8) with *P. aeruginosa* C1, subsequent intravenous administration of ZY4 (2, 4 and 8 mg/kg) or colistin (2 and 4 mg/kg) twice per day for 72 h to examine their therapeutic effects. Amounts of IL-6 (A) TNF-α (B) IL-1β (C) and IL-10 (D) were determined by ELISA assay on supernatants of lung homogenates. Data represent the mean ± SD of 8 individual experiments. *$p < 0.05$, **$p < 0.01$.

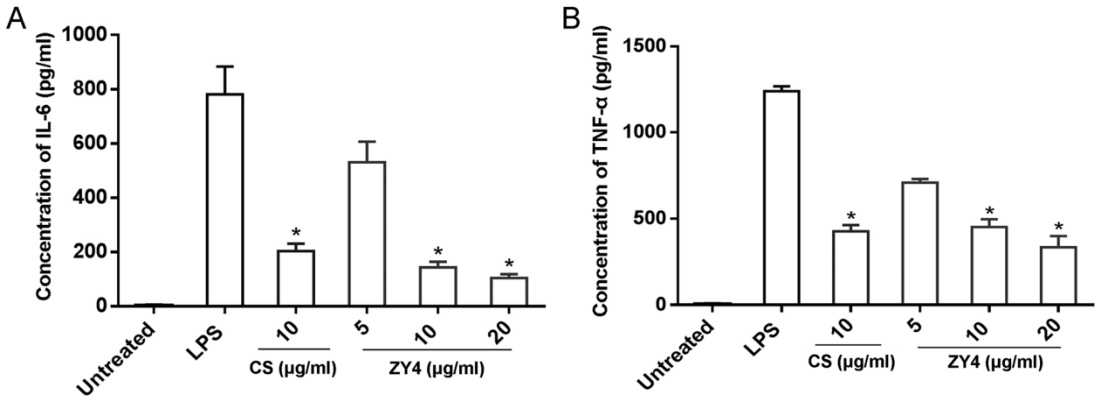


Fig. S10. ZY4 inhibits of cytokine release by LPS-stimulated RAW264.7 cells.

RAW264.7 cells were incubated with ZY4 at indicated concentration and colistin (Control) following stimulation with LPS (100 μg/ml). The levels of IL-6 (A) and TNF-α (B) in the supernatant were determined. Data represent the mean ± SD of three individual experiments. *p significance compared to LPS values *$p < 0.05$.

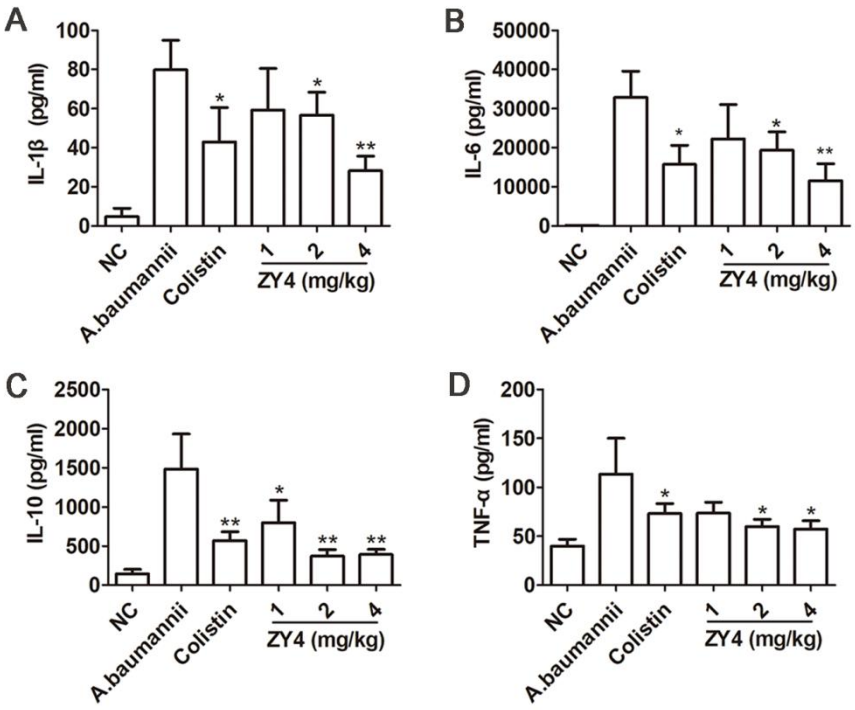


Fig. S11. ZY4 inhibits *A. baumannii*-induced cytokine release *in vivo*.

1×10^8 CFU of *A. baumannii* in 200 µl PBS solution was i.p. injected into female C57BL/6 mice (n = 8). 1 h after the injection, ZY4 (1, 2, 4 mg/kg), colistin (1 mg/kg) and vehicle (PBS) were i.p. applied. Five hours after injection of the bacteria, the levels of IL-1β (N), IL-6 (O), IL-10 (P) and TNF-α (Q) in plasma were determined by ELISA. Data represent the mean ± SD of 8 individual experiments. $*p < 0.05$, $**p < 0.01$.

Table S1. Comparison of the antimicrobial activities of ZY4 and its analogs.

Bacteria strain	MIC (μM)				
	cathelicidin-BF15	**cathelicidin-BF15-a1**	**cathelicidin-BF15-a2**	**cathelicidin-BF15-a3**	**ZY4**
E. coli	9.6	4.6	2.1	1.6	1.5
B. subtilis	38.7	9.2	4.3	2.8	2.0
S. aureus	38.7	9.2	8.5	2.8	2.0
C. albicans	9.6	4.6	4.3	2.8	2.0

MIC values of ZY4 against *E. coli*, *B. subtilis*, *S. aureus* and *C. albicans* in comparison with its analogs.

Table S2. Hemolytic activity of designed peptides on human erythrocytes.

Peptides	HC_{10} (μg/ml) (mean ± SD)	HC_{50} (μg/ml) (mean ± SD)	H_{max} (%) (mean ± SD)
cathelicidin-BF15	>320	>320	9.6 ± 4.1
cathelicidin-BF15-a1	32.1 ± 5.9	>320	31.7 ± 4.2
cathelicidin-BF15-a2	>285 ± 16	>320	12.3 ± 2.8
cathelicidin-BF15-a3	>320	>320	4.5 ± 1.4
ZY4	>320	>320	3.1 ± 1.2

HC_{10} and HC_{50} are the concentrations of peptide causing 10% and 50% hemolysis on human erythrocytes, respectively. H_{max} is the percentage (%) hemolysis at the highest peptide concentration tested (320 μg/ml).

Table S3. Comparison of the antimicrobial activities of ZY4 and antibiotics.

Bacteria strain	MIC (μM)					
	ZY4	Colistin sulfate	Tobramycin	Levofloxacin	Kanamycin	Carbenicillin disodium
P. aeruginosa CICC21625	1.9	6.8	13.0	138.4	85.8	>200
P. aeruginosa CMCC10104	1.9	17.8	26.7	138.4	64.3	>200
P. aeruginosa C1	1.0	0.8	13.0	66.4	85.8	>200
P. aeruginosa C2	1.3	1.1	26.7	66.4	85.8	>200
P. aeruginosa C3	0.8	2.2	26.7	128.2	120.2	>200
P. aeruginosa C5	1.3	6.8	9.6	150.2	32.0	>200
A. baumanii 22933	1.9	0.4	2.6	3.3	4.0	44.3
A. baumanii CN40	4.0	0.8	>200	>200	>200	>200
A. baumanii 18C116	1.9	0.8	＞200	138.4	＞200	＞200
A. baumanii 18C132	1.9	0.8	＞200	138.4	＞200	＞200
A. baumanii 18C135	1.9	0.4	＞200	>200	＞200	＞200
A. baumanii 18C136	1.9	0.4	＞200	103.8	＞200	＞200

MIC values of ZY4 against *P. aeruginosa* and *A. baumannii* in comparison with other antibiotics. These concentrations represent mean values of three independent experiments performed in duplicates. Strains of *P. aeruginosa* C1, C2, C3 C5 and *A. baumanii* CN40, 18C116, 18C132, 18C135, 18C136 are clinically isolated bacteria strain.

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