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ORIGINAL PAPER

Chimeric $hBPI_{23}$-Fcγ protein shows bactericidal activity against drug-resistant Gram-negative bacteria and protects mice from lethal challenge

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Abstract

The antimicrobial peptides, such as host immune system-derived human bactericidal/permeability-increasing protein (hBPI), are the potential drugs for antibiotic-resistant Gram-negative bacterial infection. However, whether the purified chimeric $hBPI_{23}$-Fcγ protein has bactericidal activity against drug-resistant Gram-negative bacteria (GNB) and the relevant mechanisms have not been fully elucidated. In this study, the chimeric $hBPI_{23}$-Fcγ protein, which consisting of the functional N terminus of BPI and Fcγ1, were expressed and purified in a lab-scale. The chimeric $hBPI_{23}$-Fcγ protein showed longer half-life up to 148.2 min in vivo. The $hBPI_{23}$-Fcγ protein also showed significant bactericidal activity against standard and clinically isolated drug-resistant *Acinetobacter baumannii* (*A. baumannii)* and *Escherichia coli* (*E. coli*). In addition, the $hBPI_{23}$-Fcγ protein markedly decreased biofilm formation, neutralized bacterial lipopolysaccharides (endotoxin) and enhanced the opsonization of phagocytes, as well as significantly improved the survival rate of minimal lethal dose (MLD) of drug-resistant *E. coli* -infected mice. These results indicate that the BPI_{23}-Fcγ protein protected mice from drug-resistant GNB infection not only by direct bactericidal effect, but also by promoting opsonophagocytosis of macrophages. In conclusion, the chimeric BPI_{23}-Fcγ protein may be as a promising candidate of non-antibiotic biological agent for drug-resistant GNB infection.

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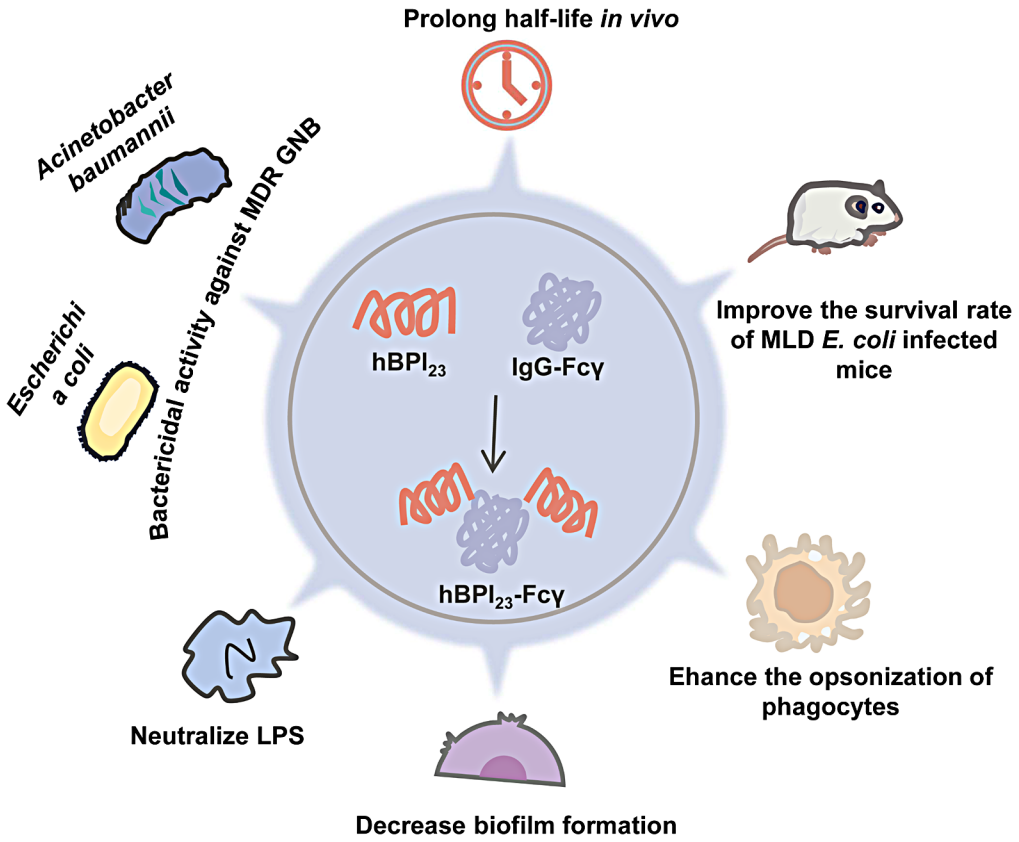
Graphical Abstract



Keywords Bactericidal/permeability-increasing protein (BPI) · Drug-resistant gram-negative bacteria · Opsonization · Antimicrobial peptides

Introduction

Antibiotic resistance has become as a major health concern globally. And the death toll will increase to 10 million by 2050, if effective strategies are not developed (Li et al. 2019; Ruckert et al. 2020). The World Health Organization (WHO) has warned that humans will enter the "post-antibiotic era" in which no sensitive antibiotics is available, if bacterial resistance can't be solved (Hasan et al. 2021). In 2017, nine of twelve kinds of drug-resistant bacteria were Gram-negative bacteria (GNB), supporting that GNB is major threat to human health (Hasan et al. 2021). In addition, these multi-drug-resistant (MDR) GNB infections occur in immunosuppressed patients, and cause death (Shrivastava et al. 2018; Almudena et al. 2019). Unfortunately, the development of new antibiotics lags behind the emergence of drug resistance of bacteria (Fernandes and Martens 2017; Hoffman 2020). Thus, it is important to develop alternative biological agents or drugs.

Antimicrobial peptides are crucial components of innate immunity against bacterial infections and an emerging alternative strategy for overcoming MDR bacterial infection (Hou et al. 2020; Lazzaro et al. 2020; Mba and Nweze 2022).Bactericidal/permeability-increasing protein (BPI) derived from neutrophil and epithelial cells is a cationic antimicrobial protein and plays crucial roles in innate immunity against GNB infection through lipopolysaccharide (LPS)-binding/bactericidal activity (I et al. 1978; J et al. 1992). The N-terminal functional domain of BPI (molecular weight of approximately 23 kDa) can neutralize LPS with high affinity, block LPS-induced inflammation, and has antimicrobial potential and cytotoxicity activity (J et al. 1992; P and J 1993; Iovine et al. 1997; Jomkuan et al. 2021). Recombinant full-length human BPI protein can effectively neutralize LPS and improve the survival of mice suffering from lethal septic shock elicited by LPS in vitro and *in vivo (*Alexander et al. 2004). In phase III clinical trial, recombinant N-terminal bioactive fragment of BPI is safe and effectively neutralizes LPS and sterilize GNB, as well as significantly reduces morbidities and improves the serious complications of children with severe meningococcemia (Levin et al. 2000; Giroir et al. 2001). Our previous results

also demonstrated knockout of BPI increased serum levels of LPS and aggravated acute colitis (Qingli et al. 2021). These make BPI as an attractive agent to prevent and treat GNB infection.

However, the short half-life (<45 min) of recombinant full-length or N-terminal fragment of BPI limit their clinical application and result in disappointing outcomes in relevant clinical trials (Wiezer et al. 1998; Levin et al. 2000). We previously constructed recombinant Ad5-BPI_{23}-Fcγ1 or AAV2-BPI_{23}-Fcγ1 virus, which expressed the chimeric hBPI_{23}-Fcγ protein containing human functional N-terminal of human BPI and the Fc segment of human immunoglobulin G1 (Li et al. 2006, 2008; Chen et al. 2007; Kong et al. 2012; Lv et al. 2016). Furthermore, transfection with Ad5-BPI_{23}-Fcγ1 or AAV2-BPI_{23}-Fcγ1 virus had the bactericidal activity and opsonophagocytosis, and protected mice against lethal endotoxemia and systemic/lethal *E. coli*/*Klebsiella pneumonia* challenge (Li et al. 2006, 2008; Chen et al. 2007; Kong et al. 2012; Lv et al. 2016). Supportively, transfection with Ad5-BPI_{23}-Fcγ1 virus also protected mice against MDR *A. baumannii* infection through direct bactericidal effects and promotion of neutrophil recruitment, phagocytosis and maturation (Wang et al. 2024). However, whether purified recombinant chimeric hBPI_{23}-Fcγ protein has bactericidal activity against MDR GNB and the relevant mechanisms remain largely unexplored.

The purified recombined chimeric hBPI_{23}-Fcγ1 protein was assessed the potential bactericidal activity, and explored the potential mechanisms against drug-resistant *A. baumannii* and *E. coli* infection. These laid the foundation for the further exploring the roles, mechanisms, pathological toxicology and drugability of chimeric BPI23-Fcγ protein in vivo.

Materials and methods

Drug-resistant GNB strains

Bacterial strain *A. baumannii* (ATCC BAA-1605) is resistant to ceftazidine, gentamicin, ticarcillin, aztreonam, cefepime, ciprofloxacin, imipenem, and meropemem. *E. coli* (ATCC BAA-2469) produces New Delhi metallo-beta-lactamase (NDM-1), which can be resistant to carbapenem (imipenem and ertapenem). Clinical strain of *A. baumannii* (No. 31781) obtained from the China-Japan Friendship Hospital is resistant to amikacin, ticarcillin/clavulanic acid, cefoperazone/sulbactam, piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, tobramycin, cotrimoxazole, minocycline, and doxycycline. Clinical strain of *E. coli* (No. 41598) obtained from the China-Japan Friendship Hospital is resistant to cefoperazone/sulbactam, ticarcillin/clavulanic acid, cefepime, imipenem, ciprofloxacin, cotrimoxazole, minocycline, piperacillin/tazobactam, ceftazidime, aztreonam, meropenem, levofloxacin, and doxycycline. Strains *E. coli* pBR322/BL21 (DE3) and *E. coli* pET28a-EGFP/BL21 (DE3) are respectively resistant to ampicillin/tetracycline and kanamycin, and preserved in our laboratory.

Mice

Female BALB/c mice (6–8 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The animal experiments were approved by the Animal Experiment and Experimental Animal Welfare Committee of Capital Medical University (approval No.: AEEI-2018-167).

Expression, purification, and identification of chimeric hBPI_{23}-Fcγ protein

CHO-DG44 cells were cultured in CD DG44 medium (Gibco, A1100001) at 37 °C with 5% CO_2 for 3 d, inoculated into a 24-well plate (4×10^5 cells/well) and cultured for additional 24 h. Following the instructions of the PEI (Polysciences, 23966-1) transfection reagent, the cells in each well were transfected with 30 µL of DNA-PEI complex containing 7.5 µL of pSCm-BPI_{23}-Fcγ plasmid DNA (CGMCC, 16676) (1 µg/µL) and 22.5 µL of PEI (1 µg/µL), and incubated at 37 °C with 5% CO_2 for 6 h. After culturing with new medium for 3 d, the transfected cells were centrifuged (200 g, 5 min), and the supernatant was discarded. Cell pellets were resuspended with CD optiCHO medium (Gibco, 31985-070) containing 25 nM MTX and 8 mM glutamine and transferred to a 96-well plate (5000 cells/400 µL/well) until colony formation. The concentration of MTX was gradually increased to 500 nM to select hBPI_{23}-Fcγ-transfected cells. The hBPI_{23}-Fcγ expression level was measured by an IgG ELISA kit (Alpha Diagnostic International, 1750). Then, 500 mL of the stably transfected cells (4×10^6 cells/mL) with 3.5 L of the optimized serum-free medium were placed in a 5 L cell reactor (BioFlo, 320). The transfected cells were cultured for 14 days. Afterward, the unpurified hBPI_{23}-Fcγ protein was obtained. To obtain the purified hBPI_{23}-Fcγ protein, primary and secondary purification were carried out by using an SP Sepharose Fast Flow cation exchange column (GE Healthcare Lifesciences, 17-0729-10) and protein A affinity chromatography column (GE Healthcare Lifesciences, 17-5280-01), respectively. After purification, the hBPI_{23}-Fcγ protein from the culture supernatant was assessed by Coomassie brilliant blue staining to observe the purification efficiency. Then the purified hBPI_{23}-Fcγ protein was further analyzed by Western

blotting with the primary antibody (mouse anti-human IgG Fc antibody, ab31925, abcam) and secondary antibody (IRDye 800CW goat anti-mouse, 926-32210, LI-COR).

To measure the pharmacokinetics of chimeric $hBPI_{23}$-Fcγ protein in vivo, the blood samples of BALB/c mice were obtained at 5, 45, 90, 180, 270 and 360 min after intravenous injection of $hBPI_{23}$-Fcγ protein (10 mg/kg). The concentrations of $hBPI_{23}$-Fcγ protein in blood samples were determined by the IgG ELISA kit.

Bactericidal assay of chimeric $hBPI_{23}$-Fcγ protein against drug-resistant GNB

The bactericidal activity was evaluated using the pouring plate assay. Briefly, 100 μL of drug-resistant *A. baumannii* or *E.coli* (1×10^4 CFU/mL) was mixed with 100 μL of different concentrations of $hBPI_{23}$-Fcγ protein, bacteria-resistant antibiotic (imipenem) (Sigma-Aldrich, 64221-86-9), bacteria-sensitive antibiotic (polymyxin E) (Sigma-Aldrich, 1264-72-8) or phosphate buffered saline (PBS) in microcentrifuge tubes, respectively. The microcentrifuge tubes with *A. baumannii* were incubated for 1 h, and microcentrifuge tubes with *E.coli* were incubated for 2 h at 37 °C. After vortex mixing, 50 μL of solution from each microcentrifuge tube was taken out and mixed with 45 °C Luria–Bertani (LB) agar in a plate and incubated at 37 °C for 24 h. The colony-forming units (CFU) in each plate were recorded. The bactericidal rate was calculated by the formula:

bactericidal rate = (number of CFU in normal saline plate - number of CFU in experimental plate) / (number of CFU in normal saline plate) × 100%.

Detection of biofilm formation of the drug-resistant GNB

The bacteria of drug-resistant *A. baumannii* (ATCC BAA 1605) were diluted to 5×10^5 CFU/mL with an LB broth containing 0.2% glucose, then added into a 96-well polystyrene plate (200 μL /well) and incubated at 37 °C for 24 h until the bacteria biofilm formed on the plate walls. After the supernatant was discarded, the biofilms were gently rinsed three times with PBS. 200 μL of hIgG (320 μg/mL), imipenem (16 μg/mL), polymyxin E (32 μg/mL), and hBPI23-Fcγ protein (320 μg/mL) was added to a well with biofilm formation, respectively, and incubated at 37 °C for 24 h. After the supernatant was discarded, the samples were gently rinsed three times with PBS. Following 15 min of 0.1% crystal violet (200 μL/well) staining, the samples were gently rinsed with distilled water and air dried at room temperature. Then, 200 μL of 95% ethanol was added to each well and allowed to stand at room temperature for 15 min to dissolve the crystal violet. After that, each well was measured by microplate reader (BioTek, EPCH2) at OD_{590nm}.

Scanning electron microscopy was performed to observe the biofilm. After drug-resistant *A. baumannii* (ATCC BAA1605) was diluted to 1×10^8 CFU/mL with an LB medium containing 5% glucose, 200 μL of bacterial suspension was added to a 96-well plate and cultured at 37 °C for 5 days, with the medium replaced every 24 h. After 5 days, the supernatant was discarded, and 200 μL of $hBPI_{23}$-Fcγ protein (2 mg/mL) was added to each well in the experimental groups, while normal saline was added to each well in the control group. After 24 h, the centrifuge tubes were gently rinsed three times with PBS, fixed with 2.5% glutaraldehyde solution for 4 h, and rinsed twice with PBS. Following dehydration with 30%, 50%, 70%, 90%, 100%, and 100% gradient ethanol, the samples were dried with 50%, 70%, 90%, 100%, and 100% gradient tert-butyl alcohol, sprayed with gold using an ion sputtering instrument and observed with a scanning electron microscope (Hitachi, SU8020).

Endotoxin neutralization assay of chimeric $hBPI_{23}$-Fcγ protein

In brief, 100 μL of different concentrations of $hBPI_{23}$-Fcγ protein solution (0.5 μg/mL, 1 μg/mL, and 2 μg/mL), hIgG solution (2 μg/mL), and PBS, were mixed evenly with 100 μL of the bacterial lipopolysaccharide/endotoxin standard solution (10 EU/mL) in a 1.5 mL EP tube, respectively. After holding at 37 °C for 60 min, 800 μL of PBS was added into each tube and shaken. Then, 50 μL of the mixture was taken and placed into a well of a 96-well ELISA plate. According to the instructions of the bacterial lipopolysaccharide/endotoxin Tachypleus Amebocyte Lysate (TAL) kit (BIOENDO, EC80545S), TAL and chromogenic substrate were added to each well following the instructions of the manufactories. The OD_{405nm} of each well was measured.

Detection of the *E. coli* opsonization with chimeric $hBPI_{23}$-Fcγ protein by macrophages

BALB/c mice were intraperitoneally injected with 200 μL of DMEM (Gibco, 11965092). After 2 h, 300 μL of peritoneal exudate was harvested and centrifuged for 5 min. The sedimented cells were collected and resuspended in DMEM containing 10% FBS. The resuspended peritoneal exudate cells were inoculated into a 24-well plate with cell adhesion slides (200 μL/well), and cultured in a 5% CO_2 incubator at 37 °C for 3 d to obtain peritoneal macrophages that had adhered to the slides. After the well liquid was aspirated, 200 μL of cell membrane red fluorescent probe Dilsolution (Beyotime, C1036) was added for 20 min at room temperature and washed three times with PBS. Then, 50 μL of the

E. coli pET28a-EGFP/BL21 (2×10^9 CFU/mL) was mixed evenly with 50 µL of the $hBPI_{23}$-Fcγ protein (20 µg/mL), 50 µL of hIgG (20 µg/mL), or 50 µL of PBS, respectively, and incubated at 37 °C for 10 min. The above solution was placed in a 24-well plate containing slides adhered with the Dil-labeled macrophages (100 µL/well) and incubated at 37 °C for 60 min. After the liquid in the well was aspirated, the macrophages were washed three times with PBS and fixed with 4% tissue fixative (a 1:3 mixture of acetic acid and methanol). The slides were removed, washed three times with PBS, and observed with a fluorescence microscope (Nikon, ECLIPSE). Dil-labeled peritoneal macrophages (red fluorescence) were observed at an excitation wavelength of 549 nm and an emission wavelength of 565 nm. Peritoneal macrophages adhered with *E. coli* pET28a-EGFP/BL21 (green fluorescence) were observed at an excitation wavelength of 488 nm and an emission wavelength of 510 nm.

Detection of the protective effect of chimeric $hBPI_{23}$-Fcγ protein against MLD of drug-resistant *E. coli* infection in mice

The minimal lethal dose (MLD) of *E. coli* was measured. *E. coli* pBR322/BL21 (DE3) was diluted into three different concentrations (1×10^4, 5×10^4, and 1×10^5 CFU/mL) with PBS containing 5% dried yeast. BALB/c mice were divided into three groups (10 for each group) corresponding to the *E. coli* concentrations above. Three different concentrations of *E. coli* were intraperitoneally injected into the mice (0.5 mL / mouse). Survival was observed within 72 h, and the minimal *E. coli* concentration causing 90–100% of mouse deaths was identified as the MLD of *E. coli*.

The protection of the chimeric $hBPI_{23}$-Fcγ protein against infection with MLD of drug-resistant *E. coli* in mice was assessed. The mice were divided into experimental group and control group. Following infection with MLD drug-resistant *E. coli* pBR322/BL21(DE3) bacteria (0.5 mL / mouse) for 10 min, experimental mice were intraperitoneally injected with 0.5 mg of $hBPI_{23}$-Fcγ protein (0.3 mL / mouse) and protein preservation solution (0.3 mL / mouse), respectively. Survival was observed within 72 h.

Statistical analysis

Statistical analyses were performed by GraphPad Prism 8. Data were compared between two groups by Student's t-test, and between more than two groups by one-way analysis of variance. Mouse survival curves were analyzed by log-rank analysis for the Kaplan–Meier test. All data are presented as mean ± standard deviation. $P<0.05$ was considered to indicate significance.

Results

Chimeric $hBPI_{23}$-Fcγ protein was expressed, purified and show longer half-life

CHO-DG44 cells were transfected with recombinant plasmid pSCm-BPI_{23}-Fcγ. After screening, the cell clones with higher expression of recombinant chimeric $hBPI_{23}$-Fcγ protein were obtained. Cell supernatants from CHO-DG44 cells with higher expression of $hBPI_{23}$-Fcγ protein were purified by cation exchange column and Protein A affinity chromatography column (supplementary Fig. 1A). As shown in supplementary Fig. 1B, the purified $hBPI_{23}$-Fcγ protein (with non-reducing protein loading buffer) were obtained and identified in the polyacrylamide gel with Coomassie brilliant blue staining (supplementary Fig. 1B). Western blotting was further used to confirm the purified $hBPI_{23}$-Fcγ protein with non-reducing protein loading buffer or with reducing protein loading buffer (supplementary Fig. 1C).

To evaluate the pharmacokinetic profile of the purified chimeric $hBPI_{23}$-Fcγ protein in vivo, the protein (10 mg/kg) was injected intravenously into the mice. We calculated half-life as follows: $t1/2=0.693/k$, $k = (Inc0-Inc)/t$ (Berg et al. 1983), where t1/2 is the half-life, 0.693 is constant, k is the first-order elimination rate constant, ln is $\log_e$, c0 is the highest concentration of the $hBPI_{23}$-Fcγ protein (23.9 µg/mL), c is the lowest concentration of the $hBPI_{23}$-Fcγ protein (6.75 µg/mL), and t is the time difference between the highest concentration and the lowest concentration (4.5 h). The recombinant protein has a $k = (\ln 23.9-\ln 6.75)/4.5=0.281$; and $t1/2=0.693/0.281=2.47$ h (148.2 min). The result showed that the half-life of $hBPI_{23}$-Fcγ protein was 148.2 min (supplementary Fig. 1D), which was significantly longer than that of 42 min for BPI and 8.2 min for $rBPI_{23}$ according to the previous reports (Fisher et al. 1994; Bauer et al. 1996). These results demonstrated that the purified chimeric $hBPI_{23}$-Fcγ protein with longer half-life was successfully obtained.

Chimeric $hBPI_{23}$-Fcγ protein showed bactericidal activity against drug-resistant GNB strains

The bactericidal activities of chimeric $hBPI_{23}$-Fcγ protein against different strains of drug-resistant *A. baumannii* and *E. coli* were further assayed. As showed in supplementary Table 1, the CFUs were 0, 0, 2.67 ± 1.15, 18.67 ± 8.33, 78 ± 12.49, 128.67 ± 64.94 and 202.67 ± 3.06 at 150 µg/mL hBPI23-Fcγ, 75 µg/mL hBPI23-Fcγ, 37.5 µg/mL hBPI23-Fcγ, 18.8 µg/mL hBPI23-Fcγ, 9.4 µg/mL hBPI23-Fcγ, 4.7 µg/mL hBPI23-Fcγ protein and the saline control, respectively. Its bactericidal rates against drug-resistant standard strain *A. baumannii* (ATCC 1605) were 100%, 100%, 98.70 ± 0.59%,

94.42 ± 2.62%, 66.46 ± 3.34%, 51.96 ± 28.00% at 150 μg/mL, 75 μg/mL, 37.5 μg/mL, 18.8 μg/mL, 9.4 μg/mL, 4.7 μg/mL $hBPI_{23}$-Fcγ protein, respectively (Fig. 1A). The bactericidal effect of $hBPI_{23}$-Fcγ protein (37.5 μg/mL) against *A. baumannii* (ATCC 1605) was also compared with imipenem and polymyxin E. As showed in supplementary Table 2, the CFUs were 222 ± 14.42, 12.00 ± 10.39, 189.67 ± 14.57 and 4.67 ± 2.51 in the saline control, $hBPI_{23}$-Fcγ, imipenem and polymyxin E treatment group, respectively. And the corresponding bactericidal rates were 94.67 ± 4.04%, 14.53 ± 6.55% and 98 ± 1.00% for $hBPI_{23}$-Fcγ, imipenem and polymyxin E, respectively (Fig. 1B).

For clinically isolated strain of drug-resistant *A. baumannii* (31781), the CFUs were 184 ± 12.49, 146.67 ± 12.58, 40.67 ± 2.31, 12.33 ± 5.86, 175.67 ± 0.58 and 13 ± 1.73 in the saline control, 18.8 μg/mL $hBPI_{23}$-Fcγ, 37.5 μg/mL $hBPI_{23}$-Fcγ, 75 μg/mL $hBPI_{23}$-Fcγ, imipenem and polymyxin E treatment group, respectively (supplementary Table 3). And the corresponding bactericidal rates were 22 ± 6.56%, 78.67 ± 1.16%, 92.67 ± 3.21%, 2.33 ± 0.58% and 93.33 ± 1.15% for 18.8 μg/mL $hBPI_{23}$-Fcγ, 37.5 μg/mL $hBPI_{23}$-Fcγ, 75 μg/mL $hBPI_{23}$-Fcγ, imipenem and polymyxin E, respectively (Fig. 1C). These results suggest that 37.5 μg/mL − 75 μg/mL chimeric $hBPI_{23}$-Fcγ protein exhibited powerful bactericidal properties, which was similar with polymyxin E on both standard and clinically isolated strains of drug-resistant *A. baumannii.*

Similarly, the bactericidal effects of the chimeric $hBPI_{23}$-Fcγ protein against drug-resistant standard strain *E. coli* (ATCC 2469) and clinically isolated strain *E. coli* (41578) were compared with imipenem and polymyxin E. For standard strain of drug-resistant *E. coli* (2469), the CFU were 100 ± 8.19, 80.00 ± 8.00, 22.33 ± 10.59, 5.33 ± 5.86, 83.67 ± 4.16 and 0.67 ± 1.15 in the saline control, 18.8 μg/mL $hBPI_{23}$-Fcγ, 37.5 μg/mL $hBPI_{23}$-Fcγ, 75 μg/mL $hBPI_{23}$-Fcγ, imipenem and polymyxin E treatment group, respectively (supplementary Table 4). And the corresponding bactericidal rates were 20 ± 8%, 77.67 ± 10.60%, 95 ± 5.29%, 14.67 ± 1.53% and 99.33 ± 1.15% for18.80 μg/mL $hBPI_{23}$-Fcγ, 37.5 μg/mL $hBPI_{23}$-Fcγ, 75 μg/mL $hBPI_{23}$-Fcγ, imipenem and polymyxin E, respectively (Fig. 2A).

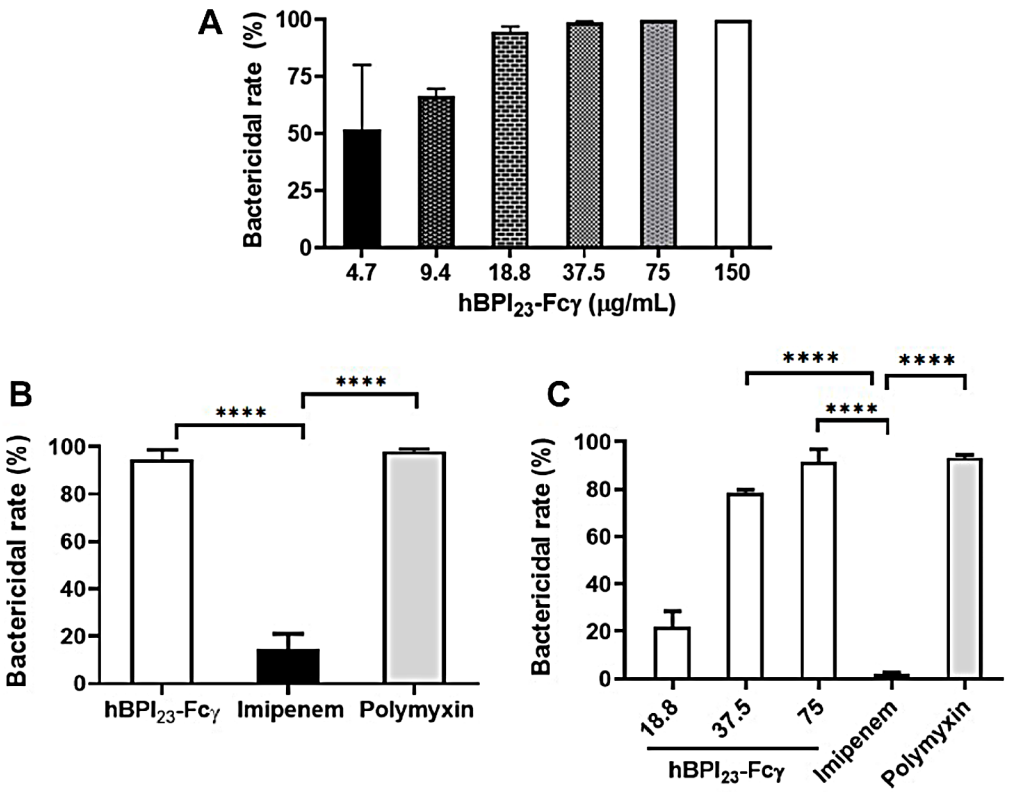


Fig. 1 The bactericidal rates of chimeric $hBPI_{23}$-Fcγ protein against drug-resistant *A. baumannii* (**A**) The standard strain of drug-resistant *A. baumannii* (ATCC BAA-1605) was incubated with indicated concentrations of the $hBPI_{23}$-Fcγ protein ($n = 3$). The (**B**) standard strain *A. baumannii* (ATCC BAA-1605) and (**C**) clinically isolated strain (31781) of drug-resistant *A. baumannii* was incubated with the $hBPI_{23}$-Fcγ protein with indicated concentrations ($n = 3$), bacteria-resistant antibiotic imipenem (16 μg/ml) ($n = 3$), bacteria-sensitive antibiotic polymyxin E (8 μg/ml) ($n = 3$) or saline ($n = 3$), respectively. The CFU was counted by pouring plate method. And the bactericidal rate was calculated with the formula: bactericidal rate = (CFU in saline group − CFU in experimental groups) / (CFU in saline group) ×100%. Results are presented as the mean ± SD. **** represent $P < 0.0001$. All the results shown are representative of two or three independent experiments

For clinically isolated strains of drug-resistant *E. coli* (41598), the CFUs were 192.33 ± 17.62, 162.67 ± 11.60, 110.00 ± 5.29, 4.33 ± 2.52, 160.33 ± 11.72 and 8 ± 1 in the saline control, 9.4 μg/mL $hBPI_{23}$-Fcγ, 18.8 μg/mL $hBPI_{23}$-Fcγ, 37.5 μg/mL $hBPI_{23}$-Fcγ, imipenem and polymyxin E treatment group, respectively (supplementary Table 5). And the corresponding bactericidal rates were 14.67 ± 6.11%, 43 ± 2.65%, 97.67 ± 1.53%, 16.33 ± 5.86% and 95.67 ± 0.58% for 9.4 μg/mL $hBPI_{23}$-Fcγ, 18.8 μg/mL $hBPI_{23}$-Fcγ, 37.5 μg/mL $hBPI_{23}$-Fcγ, imipenem and polymyxin E treatment group, respectively (Fig. 2B). These results also demonstrated that 37.5 μg/mL −75 μg/mL chimeric $hBPI_{23}$-Fcγ protein exerted similar antibacterial effects with polymyxin E against both standard and clinically isolated strains of drug-resistant *E. coli*.

Chimeric $hBPI_{23}$-Fcγ protein destructed and eliminated biofilm derived from drug-resistant *A. baumannii*

Biofilms are major contributors to chronic infections, which are highly resistant to antimicrobial therapies and are a major concern in hospitals worldwide (Kostakioti et al. 2013). Biofilms are estimated to be 10 to 1000-times more resistant to conventional antibiotics than planktonic (free-swimming) bacteria. AMPs can interfere in the early stages of biofilm formation to prevent the initial adhesion of bacteria to surfaces (Batoni et al. 2016). As shown in Fig. 3, biofilm of *A. baumannii* could be formed in the control group. And the hIgG (320 μg/mL) or imipenem (16 μg/mL) treatment could not eliminate the biofilm. However, $hBPI_{23}$-Fcγ protein (320 μg/mL) or polymyxin E (32 μg/mL) treatment significantly diminished the amount of biofilm (Fig. 3A). The bacterial morphology and biofilm were further observed by scanning electron microscopy. We found that *A. baumannii*. gathered into clusters to form a compact and dense biofilm with abundant extracellular matrix in the saline treatment group. However, the biofilm was destroyed, and the *A. baumannii*. were distributed as scattered and small patches with significantly decreased extracellular matrix after $hBPI_{23}$-Fcγ protein treatment (Fig. 3B). These findings indicated that the $hBPI_{23}$-Fcγ protein could destruct and decrease the biofilm derived from drug-resistant *A. baumannii*, which was similar with polymyxin E.

Chimeric $hBPI_{23}$-Fcγ protein neutralized bacterial endotoxin

Endotoxins (LPS) is an important pathogenic factor for GNB. Our previous results revealed that Ad5-BPI_{23}-Fcγ could not only directly sterilizes bacteria, but also neutralizes endotoxin to reduce the incidence of septic shock (Wang et al. 2024). Thus, the endotoxin-neutralizing activity of $hBPI_{23}$-Fcγ protein was also evaluated. As shown in Fig. 4, the OD_{405nm} values for 0.5 μg/mL, 1 μg/mL and 2 μg/mL $hBPI_{23}$-Fcγ protein treatment groups were 0.394 ± 0.251, 0.087 ± 0.006, and 0.030 ± 0.030, respectively, while those for the PBS control group and hIgG control group were 1.338 ± 0.021 and 1.023 ± 0.015. These results indicated that the $hBPI_{23}$-Fcγ protein has significant endotoxin-neutralizing activity with dose-dependent manner in vitro.

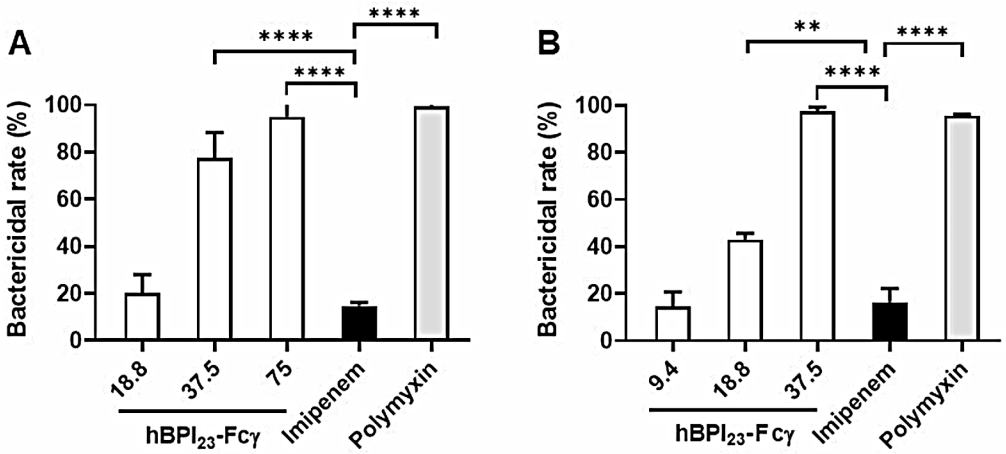


Fig. 2 The bactericidal rates of chimeric $hBPI_{23}$-Fcγ protein against drug-resistant *E. coli* (**A**) The standard strain (ATCC BAA-2469) and (**B**) clinically isolated strain (41578) of drug-resistant *E. coli* were incubated with the $hBPI_{23}$-Fcγ protein with indicated concentrations ($n = 3$), bacteria-resistant antibiotic imipenem (16 μg/ml) ($n = 3$), bacteria-sensitive antibiotic polymyxin E (8 μg/ml) ($n = 3$) or saline ($n = 3$), respectively. The CFU was counted by pouring plate method and the bactericidal rate was calculated. The bactericidal rate in each group was calculated with the formula: bactericidal rate = (CFU in saline group − CFU in experimental groups) / (CFU in saline group) ×100%. Results are presented as the mean ± SD. **, **** represent $P < 0.01$, $P < 0.0001$, respectively. All the results shown are representative of three independent experiments

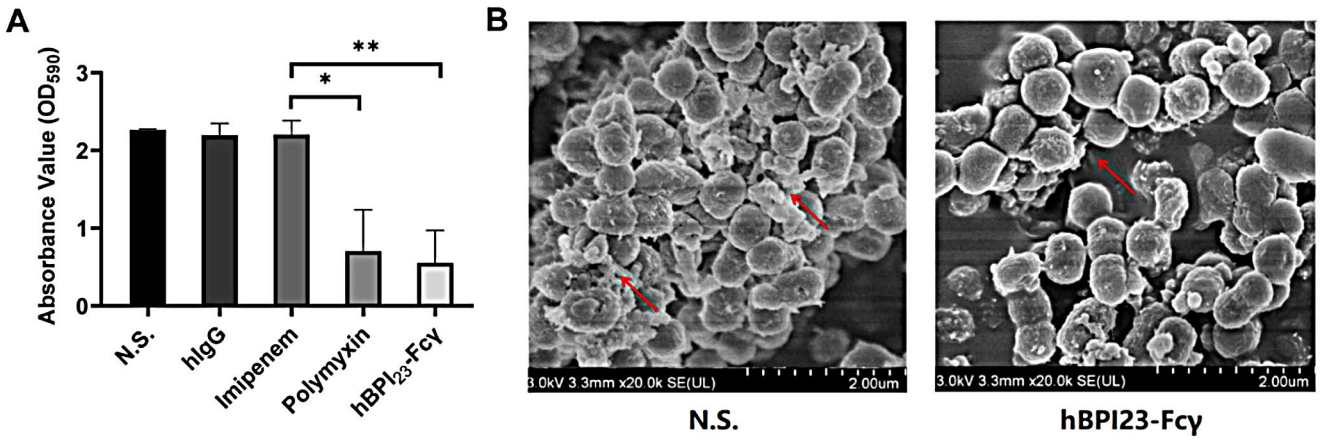


Fig. 3 The chimeric hBPI$_{23}$-Fcγ protein showed destruction and elimination effect on biofilm formed by drug-resistant *A. baumannii* The biofilm formed by cultured drug-resistant *A. baumannii* (ATCC BAA 1605) was detected by crystal violet staining ($n=3$) (**A**) and scanning electron microscopy (**B**), respectively. Results are presented as the mean ± SD. *, ** represent $P<0.05$, $P<0.01$, respectively. N.S.: Normal saline. The red arrow shows the biofilm was destroyed. All experiments were performed three times

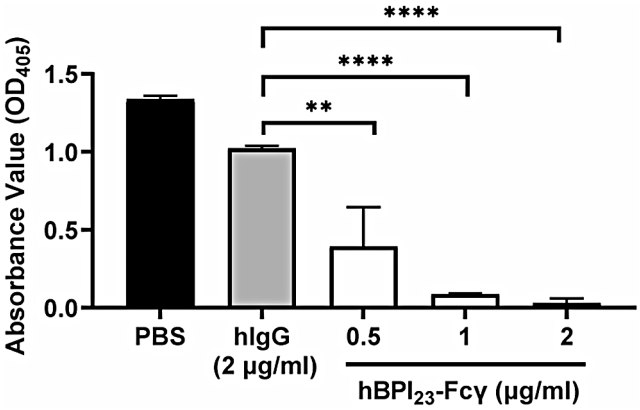


Fig. 4 The chimeric hBPI$_{23}$-Fcγ protein neutralized LPS in vitro with dose-dependent Bacterial lipopolysaccharide/endotoxin standard solution were mixed with different concentration of hBPI$_{23}$-Fcγ protein ($n=3$), hIgG ($n=3$), PBS ($n=3$), respectively. After incubating, unneutralized LPS was detected by Tachypleus Amebocyte Lysate kit. Results are presented as the mean ± SD. **, **** represent $P<0.01$, $P<0.0001$, respectively. All experiments were performed three times

Chimeric hBPI$_{23}$-Fcγ protein enhanced the phagocytosis of macrophages

The designed Fc segment of hBPI$_{23}$-Fcγ protein could bind with Fc receptors on macrophages and mediate opsonophagocytosis. To assess the effect of hBPI$_{23}$-Fcγ protein on opsophagocytosis, the uptake of the EGFP-positive *E. coli* by macrophages (labeled with Dil) was monitored and quantified by fluorescence microscopy. As shown in Fig. 5, EGFP-positive macrophages accounted for 14.3% and 25% of the total macrophages in PBS and hIgG control group, respectively. In contrast, the percentages of EGFP-positive macrophages were significantly increased to 89.7% in hBPI$_{23}$-Fcγ protein group. These results suggest that the hBPI$_{23}$-Fcγ protein could enhanced the phagocytosis of *E. coli* by macrophages.

Chimeric hBPI$_{23}$-Fcγ improved the survival rate of mice infected with MLD of drug-resistant *E. coli*.

We also investigated whether chimeric hBPI$_{23}$-Fcγ protein could resist to the MLD of drug-resistant *E. coli* infection in vivo. The MLD of drug-resistant *E. coli* was established by infecting BALB/c mice with indicated CFU for 72 h. As shown in supplementary Table 6, the mortalities were 100%, 90% and 90% with the infective dose of 5×10^4 CFU, 2.5×10^4 CFU and 0.5×10^4 CFU of *E. coli* at day 3 post infection, respectively. The dose of 2.5×10^4 CFU was established as the MLD of drug-resistant *E. coli* infection to mice. Following infection with the MLD of drug-resistant *E. coli* for 10 min, the mice in the experimental and control groups were intraperitoneally injected with hBPI$_{23}$-Fcγ protein and vehicle, respectively. And the survival rates were observed within 72 h. As shown in Fig. 6, the mice in control group began to die at 20 h post infection and the survival rate was 0% in 40 h post infection. In contrast, the survival rate of hBPI$_{23}$-Fcγ treatment group was significantly increased to 80% (Fig. 6). These results suggest that the hBPI$_{23}$-Fcγ protein significantly improved the survival rate of mice after infection with the MLD of drug-resistant *E. coli*.

Discussion

The global spread of bacterial resistance poses a major threat to world public health. With the increase of antibiotic resistance globally, drug-resistant GNB infections are a worldwide serious threat that shows no sign of abating due to limited therapeutic options (Sommer et al. 2017;

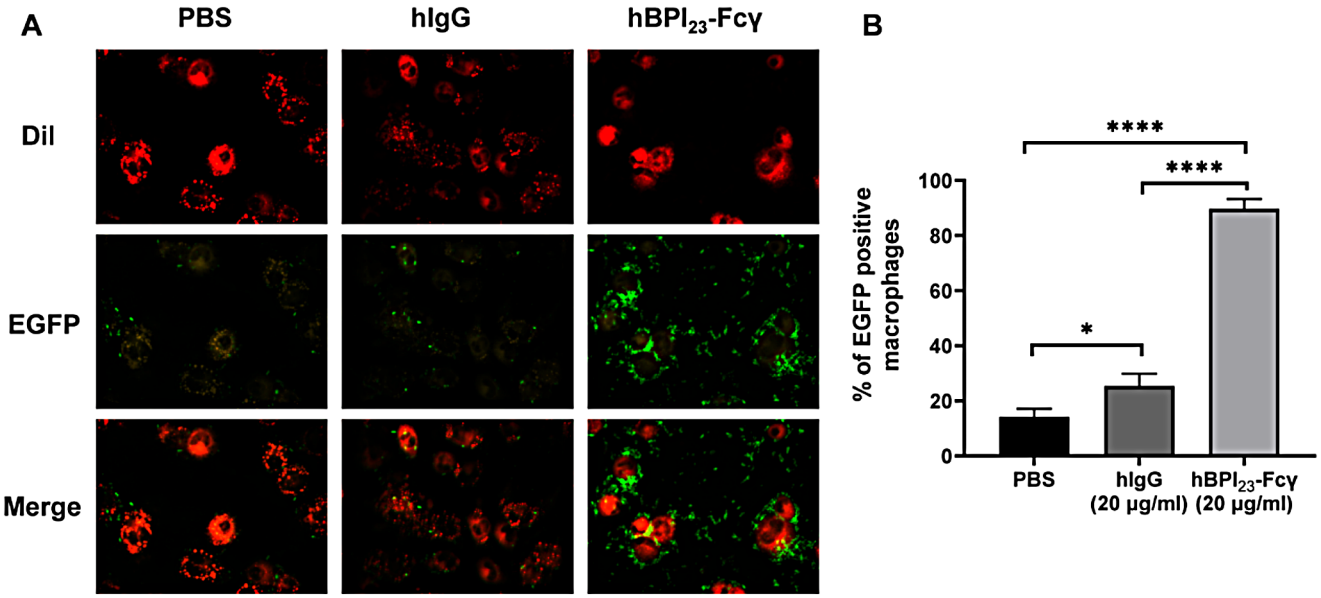


Fig. 5 The macrophages opsonophagocytosis of EGFP positive *E. coli* mediated bychimerichBPI_{23}-Fcγ protein (**A**) The isolated peritoneal macrophages labeled with Dil (red) were incubated with EGFP positive *E. coli* in the presence or absence of hBPI_{23}-Fcγ protein. The opsonophagocytosis was detected by fluorescence microscopy. (**B**) The histogram showed the proportion of macrophages engulfing *E. coli*. Dil indicates red fluorescence-labeled macrophages. EGFP indicates green fluorescence positive *E. coli*. Results are presented as the mean ± SD ($n=3$). *, **** represent $P<0.05$, $P<0.0001$, respectively. All experiments were performed three times

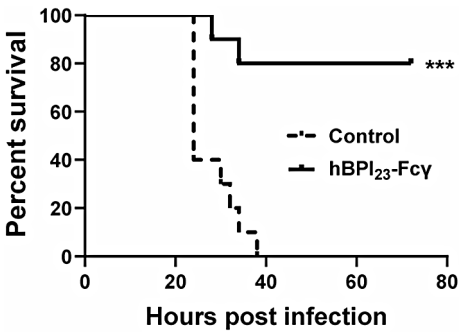


Fig. 6 The chimeric hBPI_{23}-Fcγ improved the survival rate of mice with MLD of drug-resistant *E. coli* infection Mice were treated intraperitoneally with hBPI_{23}-Fcγ protein or vehicle for 10 min and challenged intraperitoneally with MLD of drug-resistant *E. coli*. The survival rate was calculated ($n=10$). This experiment was repeated for four times, and the representative one was shown. *** represent $P<0.001$

Oliveira et al. 2020). Innate immunity plays critical roles in eliminating bacterial pathogens. Antimicrobial peptides are kinds of important components of anti-bacterial innate immunity (Luong et al. 2020). BPI is a potent antimicrobial protein that released by neutrophil azurophilic granules. BPI potentially represents a central component to directly combat microbes and modulate subsequent innate/adaptive immune responses (Chen et al. 2017; Theprungsirikul et al. 2021; Wang et al. 2024). The N-terminus of BPI contains a cluster of positively charged amino acids, which shows selectivity and high affinity with the negatively charged lipid A region of LPS of GNB (Bersier and Sugihara 1997).

Given the relatively conserved nature of lipid A, the activities of BPI can extend to many GNB regardless of its different drug resistance. The BPI/LL-37 fusion protein enhanced the bacterial clearance and LPS neutralization (Li et al. 2020). Our previous results demonstrated that Ad5-BPI_{23}-Fcγ virus transfection protected mice from multidrug-resistant *A. baumannii* infection induced pneumonia not only by direct bactericidal effect, but also by promoting the recruitment, phagocytosis and maturation of neutrophils (Wang et al. 2024). The chimeric hBPI_{23}-Fcγ is protein contained the LPS binding fragment of BPI and the Fc segment of human immunoglobulin G1. There are several characteristics and advantages of hBPI_{23}-Fcγ in the future clinical application. Firstly, the hBPI_{23}-Fcγ protein is an IgG-like bactericidal fusion protein, having longer half-life in peripheral blood, similar with other IgG-like fusion protein (such as TNFR-Fcγ fusion protein) (Baldo 2015; Lagassé et al. 2019). Secondly, BPI_{23} fragment and the Fc segment are all originated from human, which may be naturally tolerated by human. Thus, it could be repeatedly used for a long time without losing its clinical efficacy due to immune tolerance. Thirdly, BPI_{23} and IgG Fcγ are self-substances and can be eliminated through normal physiological metabolic pathways. So, the chimerichBPI_{23}-Fcγ protein may have low toxic side effects. Fourthly, the chimeric hBPI_{23}-Fcγ protein recognizes and binds to LPS, which is an indispensable pathogen-associated molecular pattern for the survival and pathogenicity of GNB. Therefore, it is unlikely for GNB to develop resistance to the hBPI_{23}-Fcγ protein. Lastly, the

chimeric $hBPI_{23}$-Fcγ protein can control drug-resistant and non-drug-resistant GNB infections by direct sterilization, neutralizing endotoxins and mediating opsonophagocytosis. Therefore, the chimeric $hBPI_{23}$-Fcγ protein may be applied for the clinical treatment of drug-resistant or non-drug-resistant GNB infections, as well as severe endotoxemia. We will focus on the bactericidal mechanisms, pharmacokinetics, and toxicology of the chimeric $hBPI_{23}$-Fcγ protein in our subsequent research.

Antibiotics are common treatments for bacterial infections. Although antibiotics possess bactericidal effect by different mechanisms, they lack LPS neutralization properties to prevent or neutralize septic shock. The chimeric $hBPI_{23}$-Fcγ protein we designed can not only directly or indirectly eliminate bacteria, but also neutralize LPS (Fig. 4).

The molecular weight of chimeric hBPI23-Fcγ protein is 48 kDa (supplementary Fig. 1C) is larger than that of polymyxin E (1.352 kDa). Although the mass concentration of the $hBPI_{23}$-Fcγ protein (75 μg/mL) was higher than the polymyxin E (8 μg/mL), the molar concentration of the $hBPI_{23}$-Fcγ protein (75 μg/mL/48 kDa = 1.563 nmol/L) was lower than that of polymyxin E (8 μg/mL/1.352 kDa = 5.92 nmol/L). Our data suggest that the chimeric $hBPI_{23}$-Fcγ protein has stronger bactericidal effects on drug-resistant GNB than polymyxin E based on the molar concentration. In the present study, several type of standard and/or clinically isolated drug-resistant *A. baumannii* and *E. coli*, which pose great threat to human health according to the WHO (Organization 2014), were selected to investigate the biological effects of the $hBPI_{23}$-Fcγ protein. Chimeric $hBPI_{23}$-Fcγ might be as a novel non-antibiotic biological agent against multiple drug-resistant GNB.

Short half-life (<45 min) and high cost of recombinant full-length or N-terminal fragment of BPI are the main obstacles for the application of recombinant BPI in clinical trials (Iovine et al. 1997; Levin et al. 2000). The half-life of chimeric $hBPI_{23}$-Fcγ protein was significantly longer than that of rBPI or $rBPI_{21}$ in BALB/c mice in *vivo* (Supplement Fig. 1D) (Fisher et al. 1994; Bauer et al. 1996).

In summary, our results demonstrated that chimeric $hBPI_{23}$-Fcγ protein has the bactericidal activity against four kinds of multiple drug-resistant GNB strains and ameliorates survival rate of MLD of drug-resistant *E. coli* infection. The chimeric $hBPI_{23}$-Fcγ may be a promising drug candidate of non-antibiotic biological agent against drug-resistant GNB infection.

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Author contributions The study was designed by QK, YA, and BC. Experiments were performed by TM, YW, CH, and QK. Results were analyzed by TM, XZ and QK. TM, XZ, YA and QK wrote the paper. All authors read and revised the paper. All authors contributed to the article and approved the submitted version.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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