Cationic amphiphilic alpha-helical peptides for the treatment of carbapenem-resistant Acinetobacter baumannii infection

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The emergence of multidrug-resistant Gram-negative bacteria, in particular Acinetobacter baumannii and Pseudomonas aeruginosa, is a critical clinical problem worldwide. Antimicrobial peptides (AMPs) have received increasing attention due to their ability to overcome multidrug-resistant microbes. We recently reported that cysteine-functionalized alpha-helical peptides LLKKLLKKC and CLLKKLLKKC effectively eradicated Gram-negative bacteria in vitro. In this study, the antibacterial properties of these peptides against carbapenem-resistant clinical isolates of A. baumannii were studied both in vitro and in vivo. The minimum inhibitory concentrations (MICs) of the peptides against 20 clinical isolates of carbapenem-resistant A. baumannii were determined in comparison with imipenem. The results showed that the A. baumannii isolates were more susceptible to [LLKK]2C than to [C(LLKK)2]C in vitro, and 90% of the 20 tested strains had an MIC of lower than or equal to 36.8 and 63.1 μM/L, respectively. However, the bactericidal effect of [C(LLKK)2]C was much faster than that of [LLKK]2C. Furthermore, these peptides also showed excellent potency in mouse models of peritonitis and pneumonia infections caused by carbapenem-resistant A. baumannii. Importantly, both peptides had a high therapeutic index (>25), but caused no significant adverse effects on the liver and kidney functions and the balance of electrolytes in the blood. These peptides can be a promising alternative treatment modality to traditional antibiotics for nosocomial bacterial infections caused by multidrug-resistant Gram-negative bacteria, especially carbapenem-resistant A. baumannii.

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1. Introduction

Acinetobacter baumannii is a nonfermentative Gram-negative bacterium, which causes a wide variety of severe nosocomial infections including pneumonia, bacteremia, urinary tract infection and wound infection in immunocompromised patients with increasing frequency and high mortality rate, especially more prominent in intensive care units [1–4]. Moreover, its remarkable ability to acquire resistance against most of commercially available antibiotics has led to global threat to human health [3–6]. Carbapenems such as imipenem and meropenem are commonly applied as the most effective and powerful agents of broad spectrum beta-lactam antibiotics against this species, however the incidence of carbapenem-resistance has risen sharply, probably as a result of increased use of antibiotics in many hospitals worldwide [7–10]. To date, therapeutic options are scarce for severe infections caused by carbapenem-resistant pathogens. In a recent investigation, Sheng and co-workers reported that patients suffering from carbapenem-resistant A. baumannii had a mortality rate of up to almost twice of those infected with the carbapenem-sensitive bacterial strains [11]. Therefore, there is an urgent need for the development of alternative antimicrobial agents against multidrug-resistant A. baumannii. Data from both the laboratory and the clinic in the last decade indicate that antimicrobial peptides (AMPs) are being evaluated as a promising solution to combat multidrug-resistance microbial
infections [12–15]. Synthetic analogues that mimic the features of natural AMPs including amphipathicity and cationic charge have been developed and shown a broad spectrum of antimicrobial activities [16–18]. It is generally believed that these structural features allow them to attach and penetrate into microbial membrane, eventually leading to cell lysis and death [16,19]. In our previous work, we synthesized self-assembled antimicrobial peptide nanoparticles (Cholesterol-conjugated G3R6TAT) with strong antimicrobial ability, especially against Gram-positive bacteria and fungi. Membrane-disrupting activity of these peptides was easily identified from morphological changes of treated bacteria [20,21]. The physical destruction nature of bacterial membrane prevents or delays the development of microbial resistance towards AMPs [12,14].

In general, Gram-negative bacteria are more tolerant to antibiotics than Gram-positive bacteria, as a result of their additional structural barriers of the outer membrane and other mechanisms such as β-lactamase and multidrug efflux pumps which pump out antibiotics [22,23]. Recently, we have evaluated a series of α-helical AMPs having a general sequence of (XXYY)n for their antimicrobial activities, where X is a hydrophobic amino acid, Y is a cationic amino acid, and n is the number of repeat unit [24], and the most optimum composition was found to be (LLKK)3. However, the peptide was only selective against Gram-positive bacteria and yeast. Incorporation of thiol-bearing amino acid at the termini of (LLKK)n peptides (n = 2, 3) was demonstrated to be effective in broadening their antimicrobial spectrum towards Gram-negative bacteria [25]. Taking into account of their antimicrobial potencies and hemolytic properties, CLLKKLLKKC (i.e. C(LLKK)2C) seemed to be the optimal composition of peptide actions as illustrated in Scheme 1 [25]. The MICs of C(LLKK)2C, (LLKK)2C and imipenem (prepared with an equal dose of cilastatin, Merck & Co., Inc., U.S.A.) against clinical isolates of A. baumannii were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute method (CLSI) as described previously [26,27]. Briefly, after grown overtime in M–H broth at 37 °C under constant shaking of 250 rpm, the bacterial suspension was diluted with phosphate-buffered saline (PBS, pH 7.4) to adjust the turbidity approximately to the standard McFarland 1 (3 × 108 CFU/mL). Then the bacterial suspension was further diluted by 100 times with M–H broth to achieve an initial loading of 3 × 106 CFU/mL. Peptide and imipenem samples were prepared in M–H broth at various concentrations. The bacterial suspension (100 μL) was mixed with an equal volume of sample solution at various concentrations in each well of a 96-well plate, and incubated for 16 h at 37 °C. The MIC was defined as the lowest concentration of peptide or imipenem, at which no visible turbidity was detected with unaided eyes and a microplate reader (Bio-Rad). Broth with bacteria only was used as a negative control, and each MIC was measured in triplicate and tested twice.

2.4. In vitro time–kill curve

The time–kill curve test was performed to evaluate the bactericidal activities of peptides against A. baumannii 173. Bacterial solution was re-suspended in fresh M–H broth to achieve log (CFU/mL) values in the range of 5–6. The initial inoculum was exposed to each peptide at concentrations of 1 ×, 4 × and 8 × MIC for 0, 30, 60, 90, 120, 150, 180, 360 min, and at these time points the samples were diluted with various dilution factors. For C(LLKK)2C, time points of 10, 20, 30, 40, 50, 60 min were further tested. 50 μL of each dilution was plated on an M–H agar plate and incubated overnight. Surviving colonies were counted after incubation. An untreated inoculum group was used as a negative control. Tests were carried out in triplicate and the results were presented as mean log (CFU/mL) ± SD.

2.5. Transmission electron microscopy (TEM)

The morphology of A. baumannii 173 before and after the treatment with the C(LLKK)2C peptide was observed under a JEM-12300 transmission electron microscope (JEOL, Japan) using an acceleration voltage of 80 kV. After grown overnight, the bacterial suspension (1.5 mL) was incubated in the presence of the peptide at 2 × MIC for 1 h. The suspension was centrifuged at 5000 rpm for 10 min, and the supernatant was removed. To fix the bacterial cells, PBS (pH 7.0, 0.5 mL) containing 2.5% glutaraldehyde was added at 4 °C overnight. After washing with PBS three times, the A. baumannii and Pseudomonas aeruginosa strains are carbapenem-resistant clinical isolates extracted from patients' phlegm and obtained from The First Affiliated Hospital of Medical College, Zhejiang University (Hangzhou, China). All isolates had been identified by routine laboratory methods and stored in 20% (v/v) glycerol at −80 °C. They were grown in Mueller–Hinton (M–H) broth (Oxoid) at 37 °C prior to use.

2.3. Minimum inhibitory concentration (MIC) measurement

Peptides were synthesized through a Fmoc-solid phase protocol at GL Biochem (Shanghai, China) as reported previously [25]. Their molecular weights were measured via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS, Model: Autoflex II, Bruker Daltonics Inc., USA), and 0.5 mg peptide in 1 mL de-ionised (DI) water and 0.5% 4-hydroxy-2-phenylamino benzoic acid (Sigma–Aldrich, Singapore) matrix solution (saturated in acetonitrile/water mixture at 1:1 volume ratio) were pre-mixed and spotted onto a MALDI ground steel target plate. The peptide solution was also run through a reverse phase (RP)-HPLC (with C-18 column as the stationery phase and the mixture of acetonitrile and water as the mobile phase), and the purity of the peptides was determined to be more than 95%.

The presence of free-thiol group in the purified lyophilized peptides was verified with the standard Elmann’s test.

In this work, the antimicrobial properties of (LLKK)2C and C(LLKK)2C against carbapenem-resistant clinical isolates of A. baumannii were studied. The minimum inhibitory concentrations (MICs) of the peptides against 20 clinical isolates of carbapenem-resistant A. baumannii strains were determined in comparison with imipenem. The bactericidal effects of the peptides against an A. baumannii strain were further investigated by time-killing kinetic studies. Furthermore, the in vivo antibacterial activities of the peptides were assessed in mouse models of peritonitis via an infection process [16,19]. It is generally believed that these structural features allow them to attach and penetrate into microbial membrane, eventually leading to cell lysis and death [16,19]. In our previous work, we synthesized self-assembled antimicrobial peptide nanoparticles (Cholesterol-conjugated G3R6TAT) with strong antimicrobial ability, especially against Gram-positive bacteria and fungi. Membrane-disrupting activity of these peptides was easily identified from morphological changes of treated bacteria [20,21]. The physical destruction nature of bacterial membrane prevents or delays the development of microbial resistance towards AMPs [12,14].

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times, the samples were post-fixed with 1% OsO₄ in PBS (pH 7.0) for 1 h. The fixed samples were washed three times with PBS again, followed by dehydration in a graded ethanol series. After placing in 1:1 mixture of absolute acetone and Spurr resin for 1 h, the samples were transferred to 1:3 mixture of absolute acetone and Spurr resin for 3 h, followed by transferring to the Spurr resin for overnight. Ultrathin sections (70–90 nm), acquired with a Reichert-Jung Ultracut E Ultramicrotome, were post-stained with uranyl acetate and lead citrate for 15 min before examination.

### 2.6. Microbial membrane integrity test

The leakage of cytoplasmic constituents such as nucleic acids with absorbance at 260 nm (i.e. 260 nm absorbing materials) was proposed to detect the integrity of the microbial membrane upon treatment with membrane-lytic agents [28]. To further probe that the loss of membrane integrity of A. baumannii 173 after being treated with the peptide C(LLKK)₄C, the presence of the 260 nm absorbing materials in PBS was evaluated. Roughly, the bacterial suspension was first adjusted to ~ 3 × 10⁸ CFU/ml using PBS (similar colony number as that used for MIC determination). Equal volume (500 µl) of bacterial suspension (~ 3 × 10⁸ CFU/ml) and peptide solution (at MIC, 2 × MIC, and 4 × MIC) or PBS solution (as a negative control) were mixed and incubated for 1 h. At the end of the incubation, the suspension was filtered with 0.22 µm filter to separate the bacteria from the supernatant, after which the supernatant was measured for its absorbance at 260 nm. The experiment was done in triplicate for each condition, and the data were normalized against the absorbance of supernatant of the untreated cells in PBS at the same wavelength.

### 2.7. Animals

ICR mice (male, 4–6 weeks, 25 ± 2 g) were used in all experiments for the in vivo studies. Immunosuppression was induced by intraperitoneal injection of 200 mg cyclophosphamide (Hengrui Corp, Jiangsu Province, P. R. China) per kg of body weight 4 days before infection. Mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine (Hengrui Corp, Jiangsu Province, P. R. China) with 10 mg/kg xylazine (Sigma–Aldrich) prior to any invasive operations. Mice were randomly marked to permit individual identification. Animal studies were performed in accordance with protocols approved by the Animal Studies Committee, P. R. China.

<table>
<thead>
<tr>
<th>Organism (number of strains)</th>
<th>Antimicrobial agent</th>
<th>MIC (µmol/L</th>
<th>Rangea</th>
<th>50%b</th>
<th>90%c</th>
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<tr>
<td>A. baumannii (20)</td>
<td>C(LLKK)₄C</td>
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<td>42.1 [50]</td>
<td>63.1 [75]</td>
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<td></td>
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<td>27.6 [30]</td>
<td>36.8 [40]</td>
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<tr>
<td>Imipenem</td>
<td></td>
<td>50.4–403.3</td>
<td>100.8 [32]</td>
<td>201.6 [64]</td>
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</table>

- a: Range of the MIC values of the antibacterial agents against 20 tested strains of A. baumannii.
- b: 50% of MIC values against 20 strains of A. baumannii are at this concentration or lower.
- c: 90% of MIC values against 20 strains of A. baumannii are at this concentration or lower.
- d: C(LLKK)₄C has a molecular weight of 1188.7 Da.
- e: (LLKK)₂C has a molecular weight of 1085.5 Da.
- f: For imipenem, the molecular weight is 317.4 Da, the resistant classification is ≥ 50.4 µmol/L (based on the CLSI guideline).

The molecular weights of C(LLKK)₄C and (LLKK)₂C was 1188.7 Da and 1085.5 Da respectively.

**Fig. 1.** Dose-dependent growth inhibition of carbapenem-resistant clinical isolates of A. baumannii 173 (a, b) and P. aeruginosa (c, d) in the presence of C(LLKK)₄C (a, c) or (LLKK)₂C (b, d) for 16 h. The data are expressed as mean ± standard deviations of at least three replicates. The standard deviations are shown by the error bars. OD, optical density. The molecular weights of C(LLKK)₄C and (LLKK)₂C was 1188.7 Da and 1085.5 Da respectively.
2.8. Systemic infection and ED$_{50}$ determination

The in vivo efficacy of peptides was first determined using a mouse model of peritonitis. Overnight cultures of A. baumannii 173 were harvested and suspended with PBS. Each of the cyclophosphamide-pretreated mice was injected intraperitoneally with 0.5 mL of the bacterial suspension at designated doses (i.e. $1.3 	imes 10^7$, $6.0 	imes 10^6$, $2.8 	imes 10^6$, $1.3 	imes 10^6$, $6.6 	imes 10^5$ CFU/mL; 0.5 mL, six mice per group). The minimum lethal dose, which was sufficient to cause 100% mortality in untreated animals, was determined from the survival rate of mice at 48 h post-infection by the BLISS method. The bacterial suspension with the minimum lethal dose (0.5 mL) was introduced to mice intraperitoneally, then peptides and imipenem were administered intraperitoneally at 1 and 6 h after infection at designated doses (i.e. 1.44, 2.4, 4.0, 6.67, 11.1 mg/kg for the peptides, 12.96, 21.6, 36.0, 60.0, 100 mg/kg for imipenem, 0.2 mL/20 g, six mice per group). The survival of the infected mice was recorded over 7 days to estimate the 50% effective dose (ED$_{50}$) by the method of BLISS.

2.9. Pulmonary infection

The therapeutic efficacy of C(LLKK)$_2$C was further tested with a mouse model of pneumonia caused by A. baumannii 173. Fifteen mice were assigned to the PBS control group and the treated group. Each of the cyclophosphamide-pretreated mice was anesthetized and infected intranasally with 0.02 mL of a bacterial suspension ($1.5 	imes 10^7$ CFU/mouse). The peptide C(LLKK)$_2$C was administered at the dose of ED$_{50}$ value (0.2 mL/20 g) intraperitoneally twice daily for 3 days starting at 1 h after infection. The survival of the infected mice was monitored for 72 h after infection and the surviving animals were sacrificed at 72 h. Blood samples were obtained from the porcine plexus and 0.05 mL aliquots plated on M–H agar plates for quantitative culture analysis. The lungs were removed and homogenized in 2 mL PBS aseptically, the homogenates were serially diluted, and 0.02 mL dilutions were plated on M–H agar plates. The plates were incubated overnight, and the number of viable microbes (CFU/g of lung) was counted. The data were expressed as mean lg (CFU/g of lung) ± SD and % (CFU/mL of blood).

2.10. In vivo toxicity studies

The acute systemic toxicities of the peptides to the mice were evaluated by determination of the in vivo lethal concentration (LC$_{50}$) (six mice per group). After being dosed in PBS, peptides were given to mice intraperitoneally at designated doses for each group (i.e. 98.3, 122.9, 153.6, 192, 240 and 300 mg/kg, 0.2 mL/20 g). The number of mice surviving at each group was monitored for up to a period of 7 days after treatment, and the values of LC$_{50}$ were calculated by the method of BLISS.

To assess their toxicities to the major organs such as liver and kidney, mice were divided randomly into the PBS control group and the treated groups (ten mice per group). Each mouse in the treated groups received intraperitoneal injection of C(LLKK)$_2$C or [LLKK]$_2$C at an ED$_{50}$ dosage (0.2 mL/20 g) twice daily for 3 days. Blood samples were obtained from the porcine plexus of anesthetized animals at 72 h after the first administration for visual inspections of any signs of abnormality, as well as for analysis of ALT, AST, creatinine, urea nitrogen and sodium ion levels.

2.11. Statistical analysis

The acute toxicity of A. baumannii 173, LD$_{50}$ and ED$_{50}$ of peptides were calculated by the method of BLISS [29]. Between the treated group and the control group, levels of alanine transaminase (ALT), aspartate transaminase (AST), creatinine, urea nitrogen and sodium ion in the blood samples and the mean log bacterial concentrations of lung were tested using Student’s t-test. The two-tailed Fisher’s exact test was performed to compare survival (%) and bacterial counts of blood (%). Differences were considered significant with a p value < 0.05.

3. Results and discussion

3.1. In vitro antimicrobial activities

The MICs of the peptides against 20 clinical isolates of carbapenem-resistant A. baumannii strains were first determined and summarized in Table 1. The highest MIC values of C(LLKK)$_2$C, (LLKK)$_2$C, and imipenem, at which the growth of 90% clinical isolates was inhibited, were 63.1, 36.8 and 201.6 mg/L, respectively. The A. baumannii isolates were more susceptible to (LLKK)$_2$C than to C(LLKK)$_2$C, with MICs ranging from 18.4 to 46.1 mg/L versus 33.6–63.1 mg/L. According to the CLSI breakpoints for A. baumannii, imipenem–resistance (R) was defined as MIC $>16$ mg/L (i.e. 50.4 mg/L) [27]. Since imipenem had an MIC above 50.4 mg/L against all the 20 isolates, these isolates were resistant to imipenem.

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Minimum lethal dose$^a$ (CFU/mouse)</th>
<th>Antimicrobial agent</th>
<th>MIC$^b$ (mg/L)</th>
<th>LD$_{50}^c$ (mg/kg) (95% confidence limits)</th>
<th>ED$_{50}^d$ (mg/kg) (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. baumannii 173</td>
<td>$1 	imes 10^6$</td>
<td>C(LLKK)$_2$C</td>
<td>42.1</td>
<td>173.0 (146.7–204.1)</td>
<td>5.05 (2.72–14.56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(LLKK)$_2$C</td>
<td>36.8</td>
<td>192.4 (162.5–230.8)</td>
<td>6.75 (4.14–23.36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Imipenem</td>
<td>403.3</td>
<td>ND$^e$</td>
<td>40.63 (27.59–61.34)</td>
</tr>
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</table>

$^a$ Minimum lethal dose defined as initial inoculum size of a particular bacterial strain required to induce 100% mortality at 48 h post-infection.

$^b$ The MIC was tested with the standard protocol at an initial inoculum size of $10^5$ CFU/mL.

$^c$ Peptides dissolved in PBS were given to mice (n = 6 in each group) intraperitoneally at designated doses.

$^d$ Six cyclophosphamide-pretreated mice in each group received two doses of the peptides or imipenem at 1 and 6 h after the bacterial challenge.

$^e$ ND, not determined.
The representative strain, *A. baumannii* 173 was employed to continue the subsequent studies, and the MIC of (LLKK)$_2$C, C(LLKK)$_2$C and imipenem against this strain was 36.8, 42.1 and 403.3 μmol/L, respectively (Fig. 1 and Table 2). To further confirm if the peptides are effective to inhibit the growth of other carbapenem-resistant Gram-negative bacteria, MICs of the peptides against carbapenem-resistant *P. aeruginosa* were measured. These peptides were also effective in suppressing the growth of *P. aeruginosa* (Fig. 1). Similar to *A. baumannii*, *P. aeruginosa* was more susceptible to (LLKK)$_2$C than to C(LLKK)$_2$C, with MICs of 69.1 and 126.2 μmol/L, respectively.

The bactericidal effects of the peptides against *A. baumannii* 173 were further evaluated by determining their time-kill curves. The peptides showed a dose-dependent rapid bactericidal effect (Fig. 2). Interestingly, C(LLKK)$_2$C killed the bacteria much faster than (LLKK)$_2$C. For example, the bacteria were completely killed by C(LLKK)$_2$C after an hour of exposure at MIC, and this occurred even in less than 10 min at higher concentrations (4 × and 8 × MIC). However, for (LLKK)$_2$C, it took 2–6 h to eradicate the same amount of bacteria. It is worth noting that cysteine increased the bactericidal potency of AMPs, which was consistent with our previous study [25]. Although the MIC of C(LLKK)$_2$C was slightly higher than that of (LLKK)$_2$C, the bactericidal effect of the peptide with two cysteine residues (C(LLKK)$_2$C) was stronger than that of the peptide with one cysteine ([LLKK]$_2$C). Such relatively fast killing mechanism is generally observed for membrane-active antimicrobials [30–32] as they interact with microbial membranes, which eventually lead to membrane disruption and rapid cell death upon contact. In a previous study, it was found that C(LLKK)$_2$C induced trans-membrane pores on the surface of Gram-negative *Escherichia coli*, allowing entries of macromolecular dyes through passive diffusion, and could further damage the membrane integrity upon a longer contact time [25].

Considering that carbapenem-resistant *A. baumannii* 173 strain might have undergone membrane composition alterations from the wild-type strain [33], the functional mechanism of C(LLKK)$_2$C against the carbapenem-resistant strain was further studied by...
3.2. In vivo efficacy against infections caused by carbapenem-resistant A. baumannii

The in vivo anti-infective activities of the peptides were first studied by a mouse model of pneumonia caused by A. baumannii 173. Mice were pretreated with cyclophosphamide to induce a severe immunosuppression condition in accordance with clinical context of nosocomial infections caused by this opportunistic pathogen mostly in immunocompromised patients. Different doses of A. baumannii 173 strain were introduced intraperitoneally to determine the minimum lethal dose. This was calculated from the survival rate of mice at 48 h post-infection, and it was about 2 x 10^7 CFU/mL (with 0.5 mL injection volume). For the subsequent experiments, mice were injected intraperitoneally with this inoculum size, which was sufficient to cause 100% mortality in the untreated mice population. The peptides had comparable anti-infective potency in vivo, the ED50 of the peptides at which half of the infected mice survived, were 5.05 mg/kg and 6.75 mg/kg for C(LLKK)2C and (LLKK)2C, respectively. Similar to the in vitro activity, both the peptides were more effective against carbapenem-resistant A. baumannii 173 in vivo as compared to imipenem (ED50: 40.63 mg/kg). Table 2 summarizes the ED50, 95% confidence limit, and MIC values of the peptides and imipenem.

Considering nosocomial pneumonia and bacteremia are the most common and life-threatening infections caused by A. baumannii [3,37], the mouse pulmonary infection was chosen to further assess the in vivo therapeutic effect of C(LLKK)2C against A. baumannii 173 infection. As shown in Table 3, among all the immunocompromised mice developing pneumonia, seven out of fifteen mice in the control group died within 3 days post-infection, while one mouse died in the group treated with C(LLKK)2C; the survival rate was 53.33% and 93.33% (p < 0.05) for the untreated and treated mice, respectively. The mean number of bacteria from the lungs of the surviving mice in the control group was approximately 2.14 x 10^8 CFU/g of lung. Treatment with C(LLKK)2C at a dose of 5.05 mg/kg twice daily for 3 days induced a remarkable reduction of more than 3 log10 (CFU/g of lung) in the viable bacterial counts when compared to the control group (Table 3). In addition, the peptide also showed excellent potency in killing bacteria in the blood and the mean number of bacteria in the blood of the treated group decreased by 79.8% compared with that of the untreated control group (Table 3). These data suggested that the peptide not only improved the survival of mice with pulmonary infection but also reduced the bacterial overall burden within the body of these mice. The therapeutic effect of the peptide in the pneumonia model may be attributed to its strong bactericidal potency in vivo and excellent biodistribution in lung tissues and blood after intraperitoneal injection.

3.3. In vivo evaluation of acute toxicity

Median lethal dose (LD50), a dose at which half the mice are killed, was determined to evaluate the in vivo acute toxicity. The LD50 value of (LLKK)2C was slightly higher than that of C(LLKK)2C (192.4 mg/kg versus 173.0 mg/kg) via a single dose of intraperitoneal injection (Table 2). However, taking their ED50 values into consideration, C(LLKK)2C had a higher in vivo therapeutic index (LD50/ED50) than (LLKK)2C (34.3 versus 28.5) against carbapenem-resistant A. baumannii infection. In addition, compared with other membrane-lytic cationic antimicrobial agents such as polymyxin B with an LD50 level of 8–10 mg/kg [38], both of the peptides appear to be safer and better tolerated due to much higher LD50 values.

To further evaluate whether the peptides might have induced any damages to the major organs under the current treatment regimen, liver and kidney functions, as well as the balance of electrolytes in the blood were investigated by comparing levels of alanine transaminase (ALT), aspartate transaminase (AST), creatinine, urea nitrogen, and sodium ion in the blood samples of the control group with those of the groups treated with the peptides (administered intraperitoneally at the ED50 dose twice a day for 3 days), and the results are summarized in Table 4. Change in the levels of the functional parameters of the liver and kidney or fluctuation of sodium ion concentration in the blood was not significant after the peptide treatment. This observation implies that the peptides neither adversely affect the liver and kidney functions nor disturb the balance of electrolytes in the blood under the tested regimen. In addition, all the mice treated with the peptides were found in good condition, without observable weight loss.

Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>Creatinine</th>
<th>Urea nitrogen</th>
<th>Sodium ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.1 ± 0</td>
<td>85.3 ± 10</td>
<td>1.2</td>
<td>10.4</td>
<td>149.0 ± 2.6</td>
</tr>
<tr>
<td>C(LLKK)2C</td>
<td>30.1 ± 0</td>
<td>85.3 ± 10</td>
<td>1.2</td>
<td>10.4</td>
<td>149.0 ± 2.6</td>
</tr>
<tr>
<td>(LLKK)2C</td>
<td>30.1 ± 0</td>
<td>85.3 ± 10</td>
<td>1.2</td>
<td>10.4</td>
<td>149.0 ± 2.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice (n = 10 in each group) treated with PBS or the peptides (ED50 dosages) twice daily for 3 days.

<sup>b</sup> ALT, alanine transaminase; AST, aspartate transaminase; U/L, international units per litre.

4. Conclusion

In this study, we have demonstrated the potent antimicrobial activities of (LLKK)2C and C(LLKK)2C against carbapenem-resistant Gram-negative clinical isolates of A. baumannii and P. aeruginosa in vitro, with (LLKK)2C having one thiol group being stronger. However, the peptide C(LLKK)2C with two thiol groups kills the...
bacteremia more rapidly, and has a higher therapeutic index in the immunocompromised mouse peritonitis model. In the mouse pneumonia model, the peptide C(LLKK)2C treats the infected mice efficiently, yielding a significantly greater survival rate as compared to the untreated control group. Importantly, at the dose level that is effective in treating the infected mice, the treatments with both peptides do not induce liver and kidney dysfunctions or sodium ion imbalance. Therefore, the peptide C(LLKK)2C would be a promising candidate for treating nosocomial infections caused by carbapenem-resistant A. baumannii.

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