Original Research

Synergistic antimicrobial activity of a novel cationic micelle L/D2 and imipenem against multidrug-resistant Acinetobacter baumannii

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1. Abstract

Background: The multidrug-resistant (MDR) *Acinetobacter baumannii* (*A. baumannii*) has become one of the most important pathogens of nosocomial infection due to widespread use of broad spectrum antimicrobial drugs and immunosuppressor therapy. As polymyxins resistance emerges, developing novel effective antibacterial agents capable of overcoming multidrug resistance is urgently needed. **Methods**: In this study, biodegradable triblock copolymers of polyethylene glycol (PEG), guanidinium-functionalized polycarbonate and polylactide, PEG-PGC_{20}-$PLLA_{20}$ (L2) and PEG-PGC_{20}-$PDLA_{20}$ (D2), were utilized as antibacterial agents. **Results**: The copolymers self-assemble into micellar nanoparticles (L/D2), and exhibit broad-spectrum antibacterial activity against 20 clinically isolated multidrug-resistant *A. baumannii* strains. L/D2 had more rapid killing kinetics than conventional antibiotics imipenem and ceftazidime, and exhibited potent anti-biofilm activity. Repeated use of L/D2 did not induce drug resistance. From scanning electron microscopy and nucleic acid release analyses, L/D2 showed membrane-lytic mechanism. We also demonstrated that L/D2 was synergistically active with imipenem against MDR *A. baumannii* strains. Additionally, strong synergistic antibacterial activity was also observed for the combined use of L/D2 and imipenem in a MDR *A. baumannii* abdominal infection mouse model. **Conclusions**: Therefore, the combination of L/D2 and imipenem might be an alternative option for the prevention of nosocomial infection caused by *A. baumannii*.

2. Introduction

With the widely use of broad spectrum antimicrobial drugs, immunosuppressor therapy and all kinds of invasive operations, nosocomial infection caused by multidrug-resistant (MDR) bacteria, such as *Acinetobacter baumannii* and *Staphylococcus aureus* are particularly urgent problem

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with high mortality [1–3]. Although carbapenems, including imipenem and meropenem have been proven to be sensitive and effective most of the time, multidrug-resistance *A. baumannii* strains, especially against carbapenems, is becoming increasingly prevalent in intensive care units (ICUs) [4–6]. The lack of treatment options forced clinicians to resort to the last line antibiotics including colistin and polymyxin B which may cause undesirable nephrotoxicity and neurotoxicity. However, it is of grave concern that even resistance has been reported for polymyxin B [7]. Hence, with the rapid increase in antibiotic resistance and the scarcity in antibiotic drug discovery [8, 9], it is imperative to explore new classes of antimicrobial agents.

Antimicrobial peptides (AMPs), deriving from nature world including plants, insects and humans originally, have been developing rapidly and emerging as a new generation of antimicrobial agent with its tremendous potential to overcome conventional antibiotics-resistant even multidrug-resistance infections [10, 11]. With the powerful ability to attach and pierce bacterial membrane once a critical concentration is reached, AMPs not only obtain a broad spectrum of antimicrobial activities, but also show low tendency toward drug-resistance due to the conventional mechanism such as efflux pumps, production of drug-inactivating enzymes, or target-sit modifications [12, 13]. Unfortunately, considering high systemic toxicities, poor selectivity and high manufacturing cost, it stands to reason that AMPs have not been widely used. Thus, as pioneered by Gellman [14], Tew [15], Kuroda [16], DeGrado and their colleagues [17], great efforts have been made to develop synthetic antimicrobial polymers. We previously reported a series of biodegradable guanidinium-functionalized polycarbonates with a potent bactericidal effect against a broad spectrum of multidrug resistant bacteria, especially for *A. baumannii* both *in vitro* and *in vivo* [18]. Recently, nanostructures are increasing reported as a promising delivery carrier [19–23], which mainly due to its hydrophilic corona capable of prolonging blood circulation by means of reducing interaction with serum proteins [24]. Another previously published study by our team reported a triblock copolymer self-assemble into micellar nanostructures and exhibit potent anticancer effect [25]. However, its antimicrobial activity against MDR *A. baumannii* has not been elucidated.

Therefore, in current study, we aim to assess the antimicrobial efficacy of the copolymer L/D2 against clinically isolated MDR *A. baumannii*, and compared it with imipenem and other commonly used antibiotics. Capability of the L/D2 to mitigate drug resistance onset was evaluated by repeated use of L/D2. Additionally, the synergistic effect of combined use of L/D2 and imipenem was also investigated both *in vitro* and *in vivo*.

3. Materials and method

3.1 Synthesis of copolymer

The cationic triblock copolymers, PEG-PGC20-PLLA20 (L2) and PEG-PGC20-PDLA20 (D2), were synthesized according to our previous protocols [22]. The scheme of monomer structure is shown in **Supplementary Fig. 1**.

3.2 Bacterial strains

Twenty clinically-isolated multidrug-resistant *A. baumannii* strains were gotten from blood and phlegm samples of the patients hospitalized in The First Affiliated Hospital of Medical College, Zhejiang University (Hangzhou, China). All isolates were identified and stored in 20% (v/v) glycerol at –80 °C prior to use.

3.3 Minimal inhibitory concentration (MIC) measurements

After grown overnight in Mueller-Hinton (MH) agar plate at 37 °C, the *A. baumannii* isolates were harvested in mid-exponential growth phase. The MICs of the L/D2, imipenem and ceftazidime were tested using the broth microdilution method. In Brief, the three agents were diluted serially with MH broth (MHB) to various concentrations. The L/D2 was diluted serially from 256 to 0.5 mg/L, imipenem from 512 to 0.5 mg/L, and ceftazidime from 2048 to 8 mg/L. The bacterial suspension was diluted with 0.45% NaCl solution to adjust the turbidity approximately to the standard McFarland 0.5, which corresponds to the concentration of 1×10^8 CFU/mL. Subsequently, bacterial suspension was further diluted 100-fold in MHB to a final concentration of 10^6 CFU/mL. Equal volumes (100 μL) of microbial suspension and agent solution with varied concentrations were mixed in each well of a 96-well plate. Finally, the final concentration of bacteria in each well was 5×10^5 CFU/mL and those of L/D2, imipenem and ceftazidime were 128 to 0.25 mg/L, 256 to 0.25 mg/L and 1024 to 4 mg/L, respectively. After incubated for 18 h at 37 °C, MIC values were read with unaided eyes as the concentration of the agents, at which no microbial growth was observed. Broth containing microbial cells alone was considered as the negative control, and each test was performed in 3 replicates.

3.4 Time-kill assay

A. baumannii 10086 isolate was grown overnight in a MH agar plate at 37 °C and then suspended in 0.45% NaCl solution to adjust the turbidity to that of a McFarland 0.5 standard (1×10^8 CFU/mL). The bacterial suspension was further diluted 100-fold in MHB to a final concentration of 10^6 CFU/mL. Various concentrations of L/D2, ceftazidime and imipenem were added to bacterial suspension to achieve final concentrations corresponding to 1 × MIC, 2 × MIC and 4 × MIC. Bacterial suspension without any treatment was used as the negative control. Samples were

incubated at 37 °C for 2 h. After each 10 min of incubation, samples were pipetted out and then diluted serially in 10-fold to various concentrations. The diluted bacterial solution (50 μL) was plated on MH agar plate and incubated at 37 °C for 24 h. Subsequently, the number of viable colonies was counted. The experiments were performed in triplicate and the results are shown as mean l g (CFU/mL) ± SD.

3.5 Biofilm assays

Antibiofilm activity of the L/D2 was evaluated according the protocols reported previously [26]. *A. baumannii* 10086 biofilm was formed after 7 days of culture and treated with the peptide at 1 × MIC, 2 × MIC, 4 × MIC, and 8 × MIC. Viability of the cells in the biofilm and biomass were tested with or without the peptide treatment.

3.6 Scanning electron microscopy (SEM)

A. baumannii 10086 in mid-exponential growth phase was acquired as described in Section 2.3. The bacterial suspension was incubated with L/D2 at a concentration of 2 × MIC and 16 × MIC for 1 h. The bacterial suspension treated with PBS was used as the negative control. The solution was then centrifuged (5000 rpm, 10 min), and the supernatant was subsequently removed. PBS containing 2.5% glutaraldehyde was added to the bacterial suspension, and incubated overnight at 4 °C for fixation. After washing with PBS three times, the bacterial suspension was subject to a post-fixing procedure for 1 h using 1% OsO_4 in PBS. The fixed samples were then washed three times with PBS, followed by dehydration using a graded ethanol series. The samples were placed on copper tape, air-dried and coated with platinum prior to observation under a field emission SEM (JSM-7400F, JEOL, Tokyo, Japan).

3.7 Outer membrane permeability and detection of LPS

Outer membrane permeability of L/D2 was tested by means of uptaking 1-*N*-phenylnaphthylamine (NPN). *A. baumannii* 10086 was suspended in PBS to a concentration of 2 × 10^9 CFU/mL, and was incubated with NPN (8 μL from a 500 μM stock in acetone) for 30 min at 25 °C. After transferred to cuvettes, fluorescence was tested using an F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm.

Similarly, L/D2 was added to the bacteria suspension to a concentration of 2 × MIC and 8 × MIC. The supernatant was harvested at 0.5, 2 and 6 h by means of centrifugating and filtering through a 0.22 μm filter. The release of LPS was tested using a chromogenic limulus amebocyte lysate assay (Xiamen Bioendo Technology, Co., Ltd., Xiamen, China).

3.8 Membrane integrity study

A. baumannii 10086 were suspended in PBS to a concentration of 2 × 10^9 CFU/mL. L/D2 was added to the bacteria suspension to a concentration of 1 × MIC, 2 × MIC, 4 × MIC, and 16 × MIC. After incubated at 37 °C for 2 h, bacteria suspension was then filtered with a 0.22 μm filter to harvest the supernatant. The supernatant was subsequently tested for its absorbance using the Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) based on UV absorption at 260 nm. The data were normalized against the absorbance of supernatant of the untreated cells in PBS. Each assay was performed in triplicates.

3.9 Resistance development

A. baumannii 10086 was exposed to L/D2, ceftazidime, and imipenem for MIC determination as previously described. Bacteria were then harvested from wells of 0.5 × MIC, washed, and grown overnight in MH agar, and then subjected to MIC determination for up to 10 similar serial passages. The development of drug resistance in the bacteria was monitored by recording the changes in the MIC.

3.10 Fractional inhibitory concentration (FIC)

Checkerboard assays were used to evaluate the synergistic effect *in vitro*. As described in Section 2.3, the plates were set up with serial doubling dilutions of L/D2, imipenem or meropenem at various concentrations. After incubated for 18 h at 37 °C, the synergistic or additive effect was evaluated by calculating the FIC indices (FICI) based on the formula: FICI = (MIC of A in combination/MIC of A) + (MIC of B in combination/MIC of B). The synergy or additive was defined based on the standard criteria (FICI $\leq$ 0.5 was defined as synergistic; 0.5 $<$ FICI $\leq$ 1 was defined as additive; 1 $<$ FICI $\leq$ 4 was defined as indifference; FICI $>$ 4 was defined as antagonism).

3.11 Animals

ICR mice (female, 8 weeks old) were used in the following *in vivo* studies. Immunosuppression was induced by intraperitoneal injection of 200 mg cyclophosphamide (Hengrui Corp, Lianyungang, China) per kg of bodyweight 4 days before the injection of bacteria. All animal studies were conducted in accordance with protocols approved by the Animal Studies Committee, China.

3.12 Evaluation of synergistic effect *in vivo*

The *in vivo* synergistic efficacy of L/D2 and imipenem was evaluated using a peritonitis mouse model. Overnight cultures of *A. baumannii* 10086 were harvested and suspended in PBS. Each of the cyclophosphamide-pretreated mice was injected intraperitoneally with 0.5 mL bacterial suspension at doses of 2.0 × 10^8 CFU/mL. With this does, mice were diagnosed to be peritonitis at which numbers of bacterial colonies were found through method of anatomy. Mice were then randomly divided into four subgroups (PBS group, L/D2 group, imipenem group, L/D2 + imipenem group). Subsequently, L/D2 and imipenem

were administered through intraperitoneally at 1h and 6 h after infection at designated dose (0.5 mg/kg, and 3.0 mg/kg for the L/D2 and imipenem respectively, 0.2 mL per injection, 15 mice per group). At 24 h post infection, five mice in each group were sacrificed to obtain peritoneal fluid, blood, and organ samples. In order to obtain peritoneal fluid sample, 3.0 mL of PBS was injected into the peritoneal cavity, and the abdomen area was gently massaged. After that, the abdomen of each mouse was opened, and the peritoneal fluid (2.0 mL per mouse) was recovered from the peritoneum. Peritoneal fluid and blood samples were diluted and plated on MH agar plates. Spleen, liver, and kidneys were then removed and homogenized in 2.0 mL of PBS. The homogenate was diluted and plated on MH agar plates. After overnight incubation at 37 °C, the number of bacterial colonies was counted. The data are presented as l g (CFU/mL of homogenate). Survival of the rest of mice was followed up for 7 days for each treatment group.

3.13 *In vivo* toxicity

To assess the toxicity of L/D2 toward the major organs in peritonitis mouse model, blood samples were extracted from the periorbital plexus of anesthetized mice at 72 h after the treatment of PBS and L/D2. Each mouse in the L/D2 group was injected intraperitoneally with L/D2 at designated dose (4 mg/kg of body weight, once daily i.p. injection for 3 consecutive days). Analysis of aspartate transaminase (AST), alanine transaminase (ALT), creatinine, ureanitrogen, sodiumions and potassium ions was made through blood biochemistry.

3.14 Statistical analysis

Analyses for difference between the control and treatment arms were conducted using one-way analysis of variance (ANOVA) and Student's *t*-test. Statistically significant differences were set as a p value < 0.05.

4. Results

4.1 L/D2 exerted rapid bactericidal activity against MDR *A. baumannii*

To assess the antimicrobial ability of L/D2, the MICs of L/D2 against 20 clinical isolates of MDR *A. baumannii* were tested firstly. Simultaneously, in order to compare with the first-line antimicrobial agents in clinic, the MIC values of ceftazidime and imipenem against these *A. baumannii* strains were also determined (Table 1). All of the *A. baumannii* strains could be inhibited by L/D2 and imipenem, with MICs ranging from 16 to 64 mg/L and 16 to 128 mg/L, respectively. The bactericidal effect of L/D2 was convincingly superior to ceftazidime, with 100% of strains inhibited at a much lower concentration. Moreover, against a particular MDR strain *A. baumannii* 10086, L/D2 exhibited superior efficacy with dramatical lower MIC values (16 mg/L) in comparison with both imipenem (128 mg/L) and ceftazidime (256 mg/L) (Table 2).

The time-killing curves of L/D2 were subsequently plotted against *A. baumannii* 10086, in comparison with antibiotics imipenem and ceftazidime. As shown in Fig. 1, L/D2 exhibited a more rapid bactericidal kinetics against *A. baumannii* 10086 than imipenem and ceftazidime. At 1 × MIC concentration, L/D2 could completely eradicate all bacteria within 40 min, while viable bacteria were still observed with imipenem or ceftazidime-treated samples within the same time frame. Importantly, even at higher concentrations of up to 4 × MIC, ceftazidime or imipenem couldn't obviously eradicate *A. baumannii* 10086 within 1 h. Additionally, the killing kinetic of L/D2 against *A. baumannii* 10086 was also showed to be dose dependent. It was noted that the killing time was halved (40 to 20 min) when L/D2 concentration quadrupled from 1 × to 4 × MIC (Fig. 1D). Taken together, these results demonstrated that L/D2 was able to eradicate the *A. baumannii* rapidly and effectively.

4.2 L/D2 exerted efficient antibiofilm activity against MDR *A. baumannii*

To study the antibiofilm activity of L/D2, *A. baumannii* 10086 biofilm was formed after 7 days of culture and treated with L/D2 for 24 h at different concentrations. As a result, the L/D2 showed a dose-dependent antibiofilm efficacy (Fig. 2). The viability of *A. baumannii* 10086 in the biofilms decreased to ~20%, and the amount of biomass reduced to ~32% after a single treatment at 8 × MIC. These results showed that L/D2 was able to effectively eradicate the bacteria in the biofilm and disperse the biofilm matrix.

4.3 L/D2 eradicated MDR *A. baumannii* via membrane lytic activity

In order to ascertain if membrane-lysis of action was operative for L/D2, *A. baumannii* 10086 was treated with L/D2 at concentration of 2 × MIC or 16 × MIC for 1 h while phosphate-buffered saline (PBS, pH 7.4) was utilized as a negative control. Pretreated samples were subsequently observed under scanning electron microscope (SEM). No obvious membrane lysis was seen after treatment of L/D2 at 2 × MIC, as compared with the smooth and integrated surface of control (Fig. 3A). However, after increasing the treatment concentration from 2 to 16 × MIC, membrane corrugation and lysis were observed on the surface of bacteria. As shown in Fig. 3B, a significant increase in the fluorescence intensity was observed 30 min post incubation, which indicate that outer membrane integrity was damaged by L/D2 in a time-dependent manner. Moreover, the LPS release was tested to further study the effect of L/D2 on the outer membrane of *A. baumannii* 10086. As a result, a significant increase of LPS was detected in the supernatant at 2 h post incubation at dose of 2 × MIC (Fig. 3C). In addition, a rapid release of LPS could also be detected when L/D2 concentration quadrupled from 2 × MIC to 8 × MIC. Subsequently, the bacterial membrane integrity was investigated after treatment with L/D2 by detecting leak-

Table 1. Cumulative distribution of MIC values (μg mL^{-1}) of copolymers (L/D2) against clinically isolated multidrug-resistant *A. baumannii* (n = 20).

Antimicrobial agents	Cumulative % of 20 *A. baumannii* strains at indicated MICs							
	4	8	16	32	64	128	256	≥512
L/D2			15	80	100			
Imipenem [a]			5	40	85	100		
Ceftazidime [a]					10	40	65	100

[a] According to the CLSI (Clinical Laboratory Standards Institute), drug resistant of bacteria is defined when MICs of Imipenem and Ceftazidime were ≥8 and 32 μg mL^{-1}, respectively.

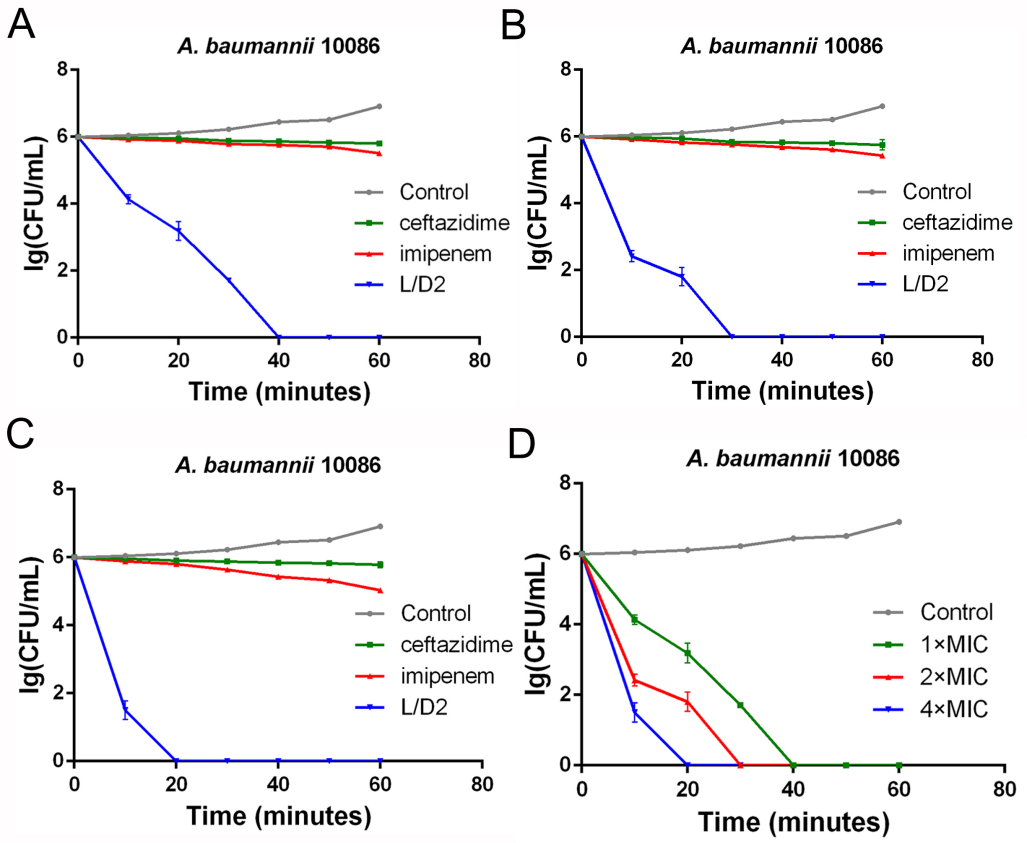

Fig. 1. *In vitro* killing kinetics of L/D2, ceftazidime, and imipenem. CFUs of *A. baumannii* 10086 from the different treatment arms were compared at varying concentrations (A) 1 × MIC, (B) 2 × MIC, and (C) 4 × MIC. (D) Effect of varying concentrations of L/D2 on killing kinetics. Error bars indicate respective standard deviations.

age of cytoplasmic materials. As shown in Fig. 3D, the release of the cytoplasmic materials was L/D2 concentration-dependent, and it increased rapidly after L/D2 treatment at 4 × MIC. Taken together, these results convincingly indicated that L/D2 served membrane-disruption antibacterial mechanism.

4.4 L/D2 could effectively mitigate drug resistance onset

As known commonly, prolonged repeated exposure of bacteria to non-lethal doses of antibiotic could induce acquisition of drug resistance. To determine if L/D2 could inhibit drug resistance development of MDR *A. baumannii*, the representative strain *A. baumannii* 10086 was serially passaged in the presence of L/D2, imipenem and ceftazidime at sub-lethal doses for 10 passages. As shown in Fig. 3E, MIC value of L/D2 stayed unchanged till the last 10 passage. Nevertheless, MIC value of imipenem started to increase by the 6th passage, and increased by 16 times. Astonishingly, by the 10 passage, MIC value of ceftazidime increased by 128 times.

4.5 L/D2 acts synergistically with imipenem against MDR *A. baumannii in vitro* and *in vivo* with negligible toxicity

With the promising bactericidal phenomenon in vitro presented above, we proceed to assess the synergistic effect of L/D2 and carbapenems by using the checkerboard method. All fractional inhibitory concentration (FIC) in-

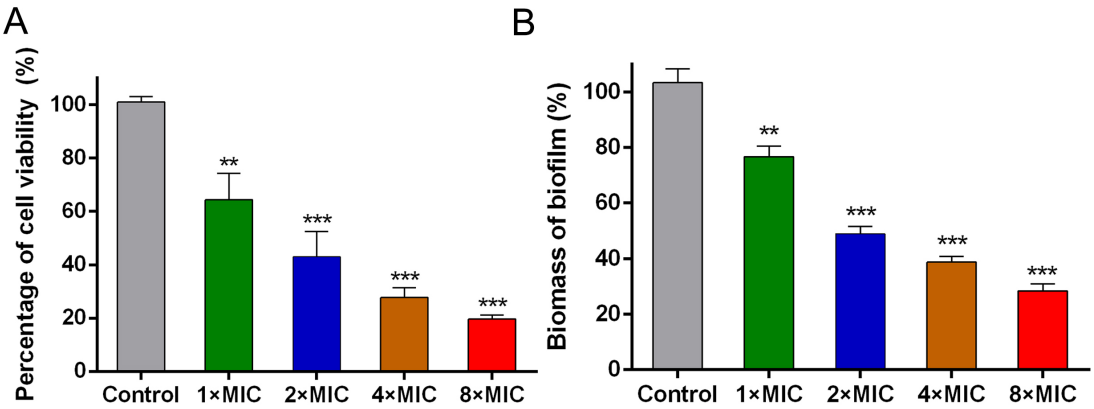

Fig. 2. Antibiofilm activity of L/D2. (A) Cell viability and (B) biomass of *A. baumannii* 10086 biofilm after L/D2 treatment for 24 h at various concentrations. NS, not significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 2. MIC values (μg mL^{-1}) of L/D2 and antibiotics against clinically isolated multidrug-resistant *A. baumannii* 10086.

Strain	Antimicrobial agents	MIC
A. baumannii 10086	L/D2	16
	Ceftazidime [a]	256
	Imipenem [a]	128

[a] According to the CLSI (Clinical Laboratory Standards Institute), drug resistant of bacteria is defined when MICs of Imipenem and Ceftazidime were $\geq$8 and 32 μg mL^{-1}, respectively.

dices were $\leq$1 for six representative *A. baumannii* strains, indicating that L/D2 served synergistic or additive effect with imipenem or meropenem (Fig. 4A). The Fig. 4B,C indicated that, the antimicrobial activity of imipenem and meropenem against *A. baumannii* 10086 was restored in the presence of sub-MIC L/D2, with the MIC value decreased from 128 to 16 mg/L. Combined usage of L/D2 and imipenem/meropenem exhibited dramatical bactericidal capacities against *A. baumannii* 10086 at concentration much lower than MIC when mono-administered (Fig. 4D, E). The synergistic antibacterial activity of L/D2 and imipenem/meropenem was further investigated in *A. baumannii* 10086-induced peritonitis mouse models. As shown in Fig. 5A, combined usage of L/D2 and imipenem was able to effectively remove bacteria from the peritoneal fluid, blood, and organs with >99.0% efficiency as compared with the control as well as L/D2/imipenem alone. Furthermore, combined usage of L/D2 and imipenem increased the survival rates from 0% for the control group and 20% for the imipenem or L/D2 group to 80% at 7 days post-treatment (Fig. 5B).

To further evaluate whether the L/D2 might induce any side effect toward the major organs in the peritonitis mouse models, the liver and kidney functions, and the balance of electrolytes in blood were tested through blood biochemistry. As shown in Table 3, the levels of ALT, AST, urea nitrogen, creatinine, and sodium ion in the blood samples of the L/D2 treatment group exhibited no significantly changes compared with the control group treated with PBS. Taken these results together, it can be concluded that L/D2 showed synergistic effects with imipenem against MDR *A. baumannii in vitro* and *in vivo* with negligible toxicity.

5. Discussion

Recently, carbapenems remain the first treatment choice for *A. baumannii*. However, inappropriate antibiotic usage leads to the emergence of MDR *A. baumannii* strains that serves as a common cause of nosocomial infection, especially in immunocompromised patients [27]. Carbapenem-resistant *A. baumannii* was now listed by WHO as top critical-priority for investment in new drugs [28]. As the emergence of polymyxin resistance in *A. baumannii* in recent years [7], efficient antimicrobial treatments were urgently needed. Accumulating studies have demonstrated that several antimicrobial polymers show potent activities against MDR *A. baumannii* with no detectable resistance [29–31]. Here, we proposed copolymers L/D2 as a promising antimicrobial agent to combat MDR *A. baumannii* while mitigating drug resistance onset. Our study also confirmed the synergistic effect between L/D2 and imipenem *in vitro* and *in vivo*. Our results implied that L/D2 could be utilized as a promising synergistic agent to improve the antimicrobial efficiency of the carbapenems against MDR *A. baumannii*, and reduce its therapeutic dose, thus minimizing toxic side effects.

Our study demonstrated that copolymer L/D2 had potent antimicrobial activity against MDR *A. baumannii* with relatively lower MIC value than imipenem and ceftazidime. In addition to its potent antimicrobial efficacy, L/D2 also showed a rapid bactericidal kinetics against *A. baumannii*. L/D2's superior bactericidal kinetics certainly stands it in good stead as a promising modality of treatment for MDR infections, especially for the management

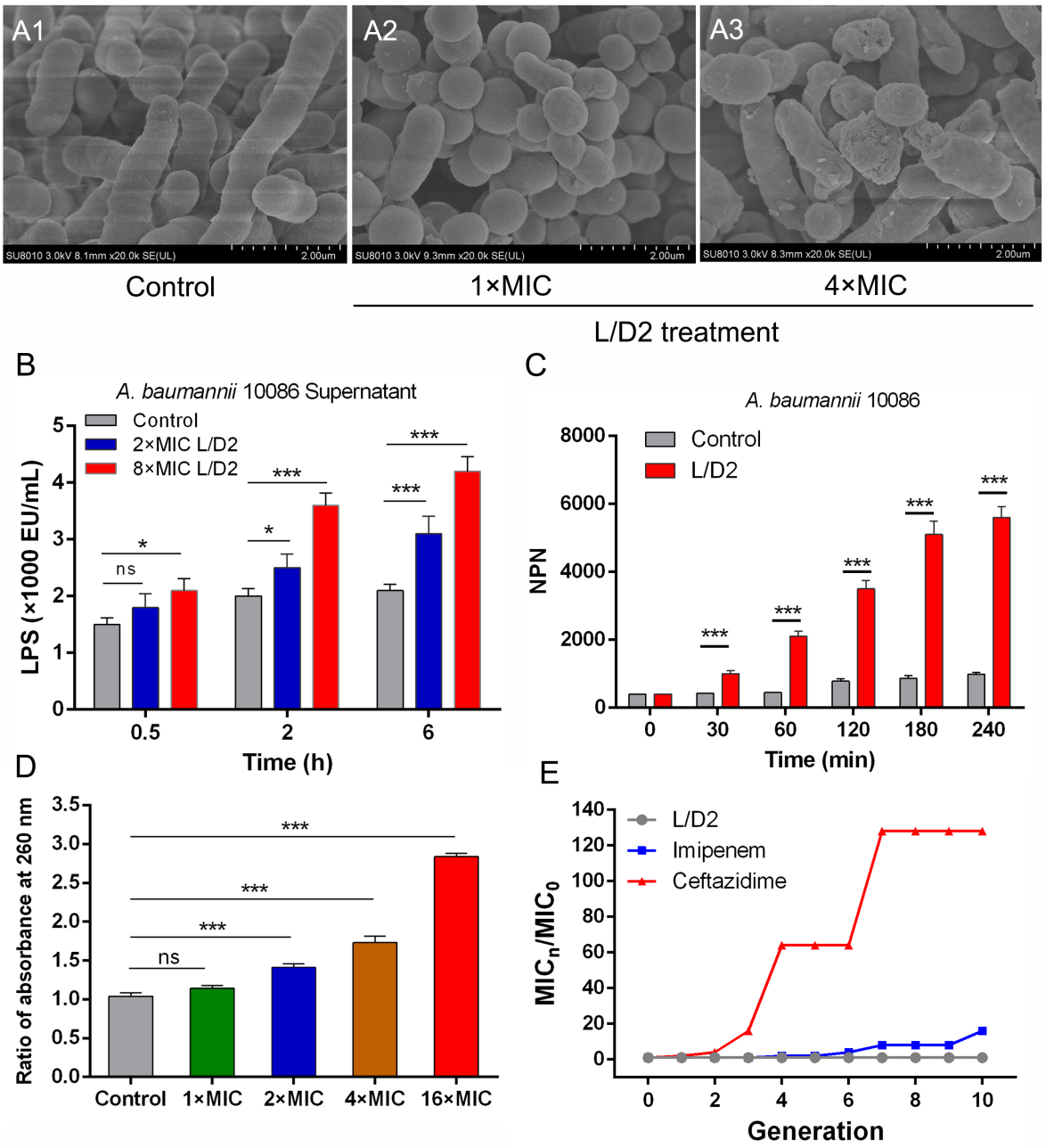

Fig. 3. Membrane-lytic mechanism of L/D2. (A) SEM images of *A. baumannii* 10086. (B) LPS levels in the supernatants was detected at 0.5, 2 and 6 h after 2 × MIC or 8 × MIC L/D2 treatment. (C) Outer membrane permeabilization of *A. baumannii* 10086 after L/D2 treatment was measured by detecting the fluorescence intensity of NPN. (D) Concentration of nucleic acids in the supernatants was detected after L/D2 treatment at doses of 1 × MIC, 2 × MIC, 4 × MIC and 16 × MIC. (E) *In vitro* evolution of antimicrobial resistance in *A. baumannii* 10086. NS, not significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

of sepsis. Its rapid bactericidal capacity potentially limits the secretion and circulation of bacterial endo and exotoxins, thus preventing septic shock and other complications. Biofilms formed from *A. baumannii* are notorious for causing chronic and persistent infections, which may be attributed to the presence of a dense matrix formed from extracellular polymeric substances that limit antibiotic penetration [32]. Hence, conventional antibiotics are less effective in treating biofilm bacteria than planktonic bacteria. However, as expected, copolymer L/D2 was proved to be effective in eradicating biofilm bacteria as well as inhibiting biofilm formation.

The dominating bactericidal mechanism of natural antimicrobial peptide (AMP) was identified as the rapid perturbation and destruction of microbial membranes, which eventually lead to the leakage of cytoplasmic constituent, such as nucleic acids and proteins, and bacteria death [33, 34]. We hypothesized that a similar antimicrobial

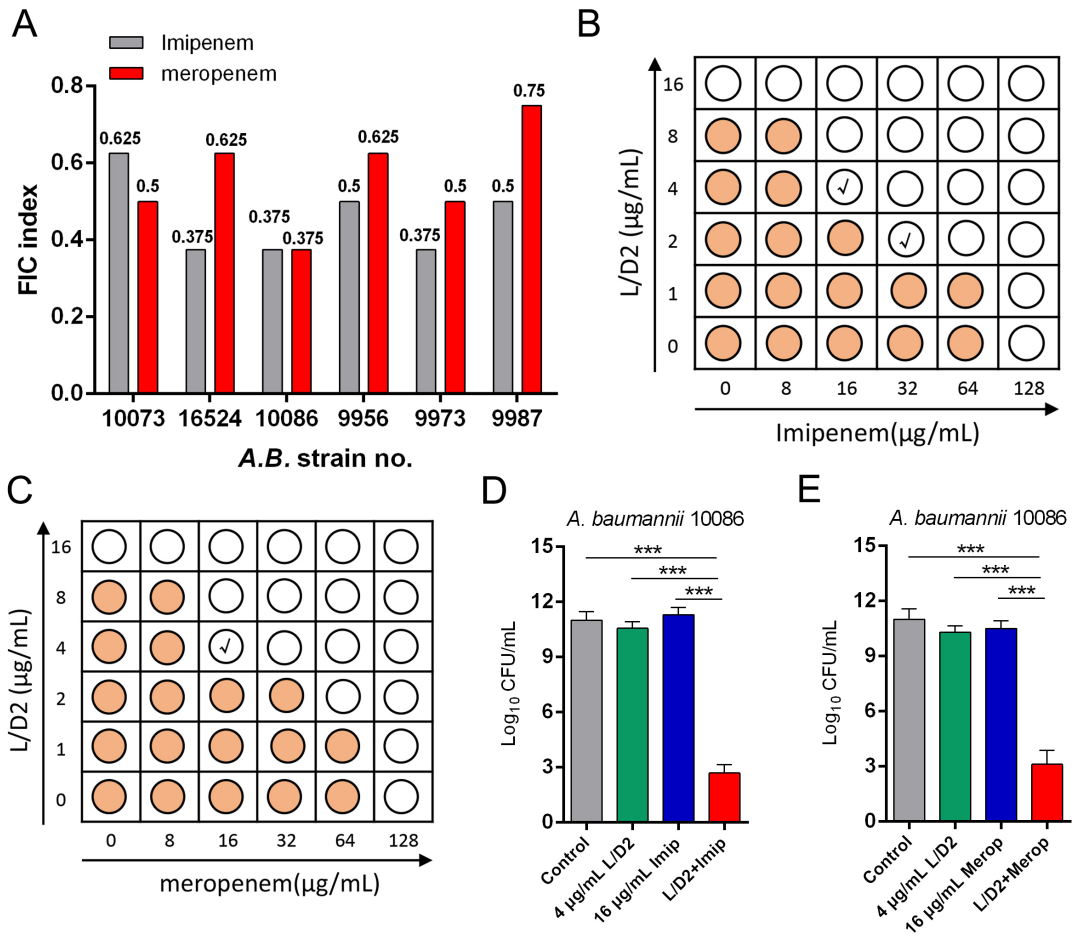

Fig. 4. Synergistic effect between L/D2 and carbapenems *in vitro*. (A) The fractional inhibitory concentration index (FICI) of the L/D2/carbapenems combination against the various *A. baumannii* strain. (B) Microdilution checkerboard analysis of the combined effect of L/D2 and imipenem against *A. baumannii* 10086. (C) Microdilution checkerboard analysis of the combined effect of L/D2 and meropenem against *A. baumannii* 10086. (D) CFUs of *A. baumannii* 10086 after combined use of L/D2 and imipenem as well as its monotherapy. (E) CFUs of *A. baumannii* 10086 after combined use of L/D2 and meropenem as well as its monotherapy. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

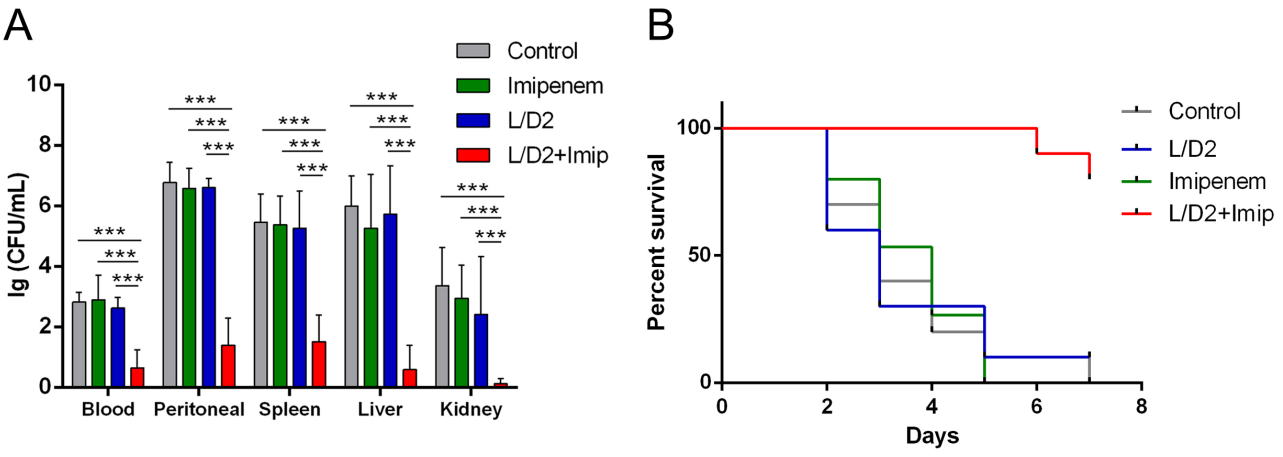

Fig. 5. Synergistic effect between L/D2 and imipenem *in vivo*. (A) CFUs of *A. baumannii* 10086 in blood, peritoneal cavity, spleen, liver, and kidney at 24 h post infection. (B) Mice survival. Tracked for up to 7 days post infection. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 3. Liver and kidney functions as well as blood sodium/potassium ion concentration of mice (n = 6 in each group) after L/D2 treatment (4 mg/kg of body weight, once daily i.p. injection for 3 consecutive days).

Treatment	ALT (U/L)	AST (U/L)	Creatinine (μmol/L)	Urea nitrogen (mmol/L)	Sodium ion (mmol/L)	Potassium ion (mmol/L)
PBS	27.4 ± 1.3	84.2 ± 6.2	18.3 ± 6.3	6.8 ± 2.2	141.7 ± 1.9	4.5 ± 0.5
L/D2	32.6 ± 4.2	86.7 ± 5.3	18.7 ± 7.3	7.1 ± 3.3	146.2 ± 2.8	4.3 ± 0.6

U/L, international units per liter.

mechanism existed for copolymer L/D2. As expected, an obvious membrane-disruptive activity was observed under the SEM microscope. The bacterial OMs serve as a barrier to the uptake of antibiotics due to the presence of teichoic acid and lipopolysaccharides [35, 36]. Our result demonstrated that L/D2 significantly increased the permeability of the OMs of *A. baumannii* in a dose dependent manner. Furthermore, quantitative evaluation of cytoplasmic materials convincingly indicated that L/D2 exerted bactericidal effects *via* membrane lytic mechanism.

Antibiotics resistance was reported to occur *via* a variety of mechanism, such as metabolic alteration, production of β-lactamases, efflux pumps and outer membrane modifications [37]. Prolonged repeated exposure to sublethal doses of antibiotics inevitably lead to development of antibiotic resistance. In our study, continuous growth in resistance was seen in *A. baumannii* treated with imipenem and ceftazidime, with MICs remaining unchanged till the 10th passage. However, negligible resistance was observed in L/D2 treated group, which might be due to its membrane-lysis antimicrobial mechanism.

Combined use of antimicrobial agents remains a routine method to deal with MDR infections clinically. We hypothesized that membrane-lysis mechanism may lead to synergistic effect between L/D2 and conventional antibiotics. As expected, our results convincingly demonstrated that L/D2 acted synergistically with imipenem both *in vitro* and *in vivo*, largely because of the fact that L/D2-mediated disruption of membrane integrity could efficiently facilitate the uptake of carbapenems into bacterial cells, thus causing a relatively high drug concentration to eradicate bacteria. In addition, compared with the broadly used polymyxin or colistin which associated with nephrotoxicity and neurotoxicity, L/D2 showed not only potent antimicrobial efficacy but also negligible toxicity towards major organs in mice model. Therefore, L/D2 exerted as a safe antimicrobial agent and might has potential to combat clinical infections caused by MDR *A. baumannii*.

6. Conclusions

In summary, the present study demonstrated that L/D2, a triblock copolymer, has a strong bactericidal activity against MDR *A. baumannii* strains. Compared with conventional antibiotics, L/D2 exhibited various advantages including rapid bactericidal activity, low tendency of resistance onset, and synergistic effect with carbapenems. Importantly, combined usage of L/D2 and imipenem had a promising therapeutic effect in the *A. baumannii* induced peritonitis mouse models with negligible toxicity. Based on these results, L/D2 exerted as a promising alternative treatment choice against clinical infection caused by multidrug-resistant *A. baumannii* strains.

7. Author contributions

GZ and YZ contributed to the idea and design. GZ, YZ, YJZ, KY, LW contributed to the manuscript writing and revision. GZ, LW, HL, YZ contributed to the data acquisition and analysis. All authors have read and approved the final version of this manuscript.

8. Ethics approval and consent to participate

This study was approved by the independent Ethical Committee/Institutional Review Board of the First Affiliated Hospital, Zhejiang University. The ethical approval code is 202024.

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11. Conflict of interest

The authors declare no conflict of interest.

12. References

[1] Vázquez-López R, Solano-Gálvez SG, Juárez Vignon-Whaley JJ, Abello Vaamonde JA, Padró Alonzo LA, Rivera Reséndiz A, *et al*. *Acinetobacter baumannii* Resistance: A Real Challenge for Clinicians. Antibiotics. 2020; 9: 205.

[2] Noval M, Banoub M, Claeys KC, Heil E. The Battle Is on: New Beta-Lactams for the Treatment of Multidrug-Resistant Gram-Negative Organisms. Current Infectious Disease Reports. 2020; 22: 1.

[3] Dhankhar P, Dalal V, Kotra DG, Kumar P. *In-silico* approach to identify novel potent inhibitors against GraR of S. *aureus*. Frontiers in bioscience (Landmark edition). 2020; 25: 1337–1360.

[4] Su CH, Wang JT, Hsiung CA, Chien LJ, Chi CL, Yu HT, *et al*. Increase of carbapenem-resistant Acinetobacter baumannii infection in acute care hospitals in Taiwan: association with hospital antimicrobial usage. PLoS ONE. 2012; 7: e37788.

[5] Ayenew Z, Tigabu E, Syoum E, Ebrahim S, Assefa D, Tsige E. Multidrug resistance pattern of Acinetobacter species isolated from clinical specimens referred to the Ethiopian Public Health Institute: 2014 to 2018 trend anaylsis. PLoS ONE. 2021; 16: e0250896.

[6] Kishk R, Soliman N, Nemr N, Eldesouki R, Mahrous N, Gobouri A, *et al*. Prevalence of Aminoglycoside Resistance and Aminoglycoside Modifying Enzymes in *Acinetobacter baumannii* Among Intensive Care Unit Patients, Ismailia, Egypt. Infection and Drug Resistance. 2021; 14: 143–150.

[7] Park J, Kim M, Shin B, Kang M, Yang J, Lee TK, *et al*. A novel decoy strategy for polymyxin resistance in *Acinetobacter baumannii*. Elife. 2021; 10: e66988.

[8] Butler MS, Cooper MA. Antibiotics in the clinical pipeline in 2011. The Journal of Antibiotics. 2011; 64: 413–425.

[9] Blaskovich MA, Zuegg J, Elliott AG, Cooper MA. Helping Chemists Discover New Antibiotics. ACS Infectious Diseases. 2015; 1: 285–287.

[10] Annunziato G, Costantino G. Antimicrobial peptides (AMPs): a patent review (2015–2020). Expert Opinion on Therapeutic Patents. 2020; 30: 931–947.

[11] Yang Z, He S, Wu H, Yin T, Wang L, Shan A. Nanostructured Antimicrobial Peptides: Crucial Steps of Overcoming the Bottleneck for Clinics. Frontiers in Microbiology. 2021; 12: 710199.

[12] Lima PG, Oliveira JTA, Amaral JL, Freitas CDT, Souza PFN. Synthetic antimicrobial peptides: Characteristics, design, and potential as alternative molecules to overcome microbial resistance. Life Sciences. 2021; 278: 119647.

[13] Browne K, Chakraborty S, Chen R, Willcox MD, Black DS, Walsh WR, *et al*. A New Era of Antibiotics: The Clinical Potential of Antimicrobial Peptides. International Journal of Molecular Sciences. 2020; 21: 7047.

[14] Mowery BP, Lee SE, Kissounko DA, Epand RF, Epand RM, Weisblum B, *et al*. Mimicry of antimicrobial host-defense peptides by random copolymers. Journal of the American Chemical Society. 2007; 129: 15474–15476.

[15] Ilker MF, Nüsslein K, Tew GN, Coughlin EB. Tuning the hemolytic and antibacterial activities of amphiphilic polynorbornene derivatives. Journal of the American Chemical Society. 2004; 126: 15870–15875.

[16] Palermo EF, Kuroda K. Chemical structure of cationic groups in amphiphilic polymethacrylates modulates the antimicrobial and hemolytic activities. Biomacromolecules. 2009; 10: 1416–1428.

[17] Tew GN, Scott RW, Klein ML, Degrado WF. De novo design of antimicrobial polymers, foldamers, and small molecules: from discovery to practical applications. Accounts of Chemical Research. 2010; 43: 30–39.

[18] Chin W, Zhong G, Pu Q, Yang C, Lou W, De Sessions PF, *et al*. A macromolecular approach to eradicate multidrug resistant bacterial infections while mitigating drug resistance onset. Nature Communications. 2018; 9: 917.

[19] Xiong XB, Falamarzian A, Garg SM, Lavasanifar A. Engineering of amphiphilic block copolymers for polymeric micellar drug and gene delivery. Journal of Controlled Release. 2011; 155: 248–261.

[20] Hwang D, Ramsey JD, Kabanov AV. Polymeric micelles for the delivery of poorly soluble drugs: from nanoformulation to clinical approval. Advanced Drug Delivery Reviews. 2020; 156: 80–118.

[21] Thipparaboina R, Chavan RB, Kumar D, Modugula S, Shastri NR. Micellar carriers for the delivery of multiple therapeutic agents. Colloids and Surfaces. Biointerfaces. 2015; 135: 291–308.

[22] Lategan KL, Walters CR, Pool EJ. The effects of silver nanoparticles on RAW 264.7. Macrophages and human whole blood cell cultures. Frontiers in bioscience (Landmark edition). 2019; 24: 347–365.

[23] Trimukhe AM, Pofali PA, Vaidya AA, Koli UB, Dandekar P, Deshmukh RR, *et al*. Pulsed plasma surface functionalized nanosilver for gene delivery. Frontiers in bioscience (Landmark edition). 2020; 25: 1854–1874.

[24] Pelegri-O'Day EM, Lin E, Maynard HD. Therapeutic Protein-Polymer Conjugates: Advancing beyond PEGylation. Journal of the American Chemical Society. 2014; 136: 14323–14332.

[25] Zhong G, Yang C, Liu S, Zheng Y, Lou W, Teo JY, *et al*. Polymers with distinctive anticancer mechanism that kills MDR cancer cells and inhibits tumor metastasis. Biomaterials. 2019; 199: 76–87.

[26] Wang Y, Ke XY, Khara JS, Bahety P, Liu S, Seow SV, *et al*. Synthetic modifications of the immunomodulating peptide thymopentin to confer anti-mycobacterial activity. Biomaterials. 2014; 35: 3102–3109.

[27] Ramirez MS, Bonomo RA, Tolmasky ME. Carbapenemases: Transforming *Acinetobacter baumannii* into a Yet More Dangerous Menace. Biomolecules. 2020; 10: 720.

[28] Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, *et al*. Discovery, research, and development of new antibiotics: the who priority list of antibiotic-resistant bacteria and tuberculosis. The Lancet Infectious Diseases. 2018; 18: 318–327.

[29] Barman S, Konai MM, Samaddar S, Haldar J. Amino Acid Conjugated Polymers: Antibacterial Agents Effective against Drug-Resistant *Acinetobacter baumannii* with no Detectable Resistance. ACS Applied Materials & Interfaces. 2019; 11: 33559–33572.

[30] Li X, Gui R, Li J, Huang R, Shang Y, Zhao Q, *et al*. Novel Multifunctional Silver Nanocomposite Serves as a Resistance-Reversal Agent to Synergistically Combat Carbapenem-Resistant *Acinetobacter baumannii*. ACS Applied Materials & Interfaces. 2021; 13: 30434–30457.

[31] Uppu DSSM, Samaddar S, Ghosh C, Paramanandham K, Shome BR, Haldar J. Amide side chain amphiphilic polymers disrupt surface established bacterial bio-films and protect mice from chronic Acinetobacter baumannii infection. Biomaterials. 2016; 74: 131–143.

[32] Li Z, Ding Z, Liu Y, Jin X, Xie J, Li T, *et al*. Phenotypic and Genotypic Characteristics of Biofilm Formation in Clinical Isolates of *Acinetobacter baumannii*. Infection and drug resistance. 2021; 14: 2613–2624.

[33] Mwangi J, Hao X, Lai R, Zhang Z. Antimicrobial peptides: new hope in the war against multidrug resistance. Zoological research. 2019; 40: 488–505.

[34] Chen CH, Lu TK. Development and Challenges of Antimicrobial Peptides for Therapeutic Applications. Antibiotics. 2020; 9: 24.

[35] Mohanram H, Bhattacharjya S. Resurrecting Inactive Antimicrobial Peptides from the Lipopolysaccharide Trap. Antimicrobial Agents and Chemotherapy. 2014; 58: 1987–1996.

[36] Hemeg HA. Nanomaterials for alternative antibacterial therapy. International Journal of Nanomedicine. 2017; 12: 8211–8225.

[37] Varela MF, Stephen J, Lekshmi M, Ojha M, Wenzel N, Sanford M, *et al*. Bacterial Resistance to Antimicrobial Agents. Antibiotics. 2021; 10: 593.

Supplementary material: Supplementary material associated with this article can be found, in the online version, at https://www.fbscience.com/Landmark/articles/10.52586/5002.

Abbreviations: MDR, multidrug drug resistance; PEG, polyethylene glycol; ICUs, intensive care units; AMPs, Antimicrobial peptides; MIC, minimal inhibitory concentration; SEM, scanning electron microscopy; FIC, fractional inhibitory concentration.

Keywords: Antimicrobial polymer; Micelle; Multidrug resistance; *Acinetobacter baumannii*; Synergistic antimicrobial activity

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