

Combination of ASI01 and Mefloquine Inhibits Carbapenem-Resistant *Pseudomonas aeruginosa* in vitro and in vivo

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Background: In recent years, carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) has spread around the world, leading to a high mortality and close attention of medical community. In this study, we aim to find a new strategy of treatment for CRPA infections.

Methods: Eight strains of CRPA were collected, and PCR detected the multi-locus sequence typing (MLST). The antimicrobial susceptibility test was conducted using the VITEK@2 compact system. The minimum inhibitory concentration (MIC) for ASI01 and mefloquine was determined using the broth dilution method. Antibacterial activity was tested in vitro and in vivo through the chessboard assay, time killing assay, and a mouse model. The mechanism of ASI01 combined with mefloquine against CRPA was assessed through the biofilm formation inhibition assay, electron microscopy, and detection of reactive oxygen species (ROS).

Results: The results demonstrated that all tested CRPA strains exhibited multidrug resistance. Moreover, our investigation revealed a substantial synergistic antibacterial effect of ASI01-mefloquine in vitro. The assay for inhibiting biofilm formation indicated that ASI01-mefloquine effectively suppressed the biofilm formation of CRPA-5 and CRPA-6. Furthermore, ASI01-mefloquine were observed to disrupt the bacterial cell wall and enhance the permeability of the cell membrane. This effect was achieved by stimulating the production of ROS, which in turn hindered the growth of CRPA-3. To evaluate the therapeutic potential, a murine model of CRPA-3 peritoneal infection was established. Notably, ASI01-mefloquine administration resulted in a significant reduction in bacterial load within the liver, kidney, and spleen of mice after 72 hours of treatment.

Conclusion: The present study showed that the combination of ASI01 and mefloquine yielded a notable synergistic bacteriostatic effect both in vitro and in vivo, suggesting a potential clinical application of this combination in the treatment of CRPA.

Keywords: ASI01, mefloquine, carbapenem resistance, *Pseudomonas aeruginosa*, synergy, biofilm

Introduction

Pseudomonas aeruginosa is one of the common G⁻-opportunistic pathogens in hospital-acquired infection. It may cause a variety of infectious diseases, including pneumonia, septicemia, and urinary tract infection. Frequently used antibiotics for the treatment of *P. aeruginosa* include β -lactams such as cefepime, ceftazidime, piperacillin-tazobactam, aztreonam, aminoglycosides (gentamicin, tobramycin), and fosfomycin.¹ However, due to the irrational use of antibiotics, the spread of resistant plasmids, and various other factors, multidrug resistance (MDR) and extensively drug-resistant (XDR) *P. aeruginosa* have emerged and widely spread across the world,^{2,3} posing a serious threat to public health. According to the report on antibiotic resistance issued by the United States Centers for Disease Control and Prevention (CDC) in 2019,⁴ anti-microbial pathogens caused more than 2.8 million infections and more than 35000 deaths every year. In fact, as early as 2017, the World Health Organization (WHO) listed CRPA as a “key” group in urgent need of new antibiotics.⁵ Recently, the CDC reported an outbreak of XDR-*P. aeruginosa*

carrying VIM-GES-CRPA, which involved 18 states and resulted in various types of infections, including eyes, bloodstream, urinary tract, and respiratory system.⁶ This situation has drawn great concerns not only to the medical community but also to the governments around the world. Thus, a novel treatment strategy is urgently needed. In recent years, researchers have tried to explore additional therapeutics for CRPA for clinical use. Several new combinations of β -lactamase inhibitors have been introduced for clinical applications, including meropenem-vaborbactam, imipenem-relebactam, and ceftazidime-avibactam. These antibiotics might be toxic, especially to the patients with renal failure. At the same time, long-term use of antibiotics might expand the development of drug resistance and dysbiosis of homeostasis.⁷ In addition, new antibiotics have been investigated to treat refractory bacteria including CRPA. However, the development of new drugs requires a lot of manpower and material resources and has a long cycle. Explorations for treatment options besides antibiotic therapy, including inhibition of quorum sensing and biofilm formations as well as bacteriophage therapy^{8,9} are under investigations. On the other hand, the reuse of old drugs has been tried to solve the current problems.^{10–12} A small molecule of AS101 is a fully synthesized organic compound containing tellurium,¹³ which has the activity of immune regulation and can induce the secretion of cytokines such as interleukins and cluster stimulating factors in human lymphocyte proliferation.^{14,15} It could also be used to treat malignant tumors, autoimmune diseases, viral infections, and other disorders.^{16–18} In addition, AS101 showed antibacterial activity. In the sepsis mouse model of cecal ligation and puncture, AS101 could improve the survival rate of animals.¹⁹ Furthermore, recent studies demonstrated that AS101 had promising antibacterial activity against a variety of bacteria in vitro and in vivo, including *Enterobacter cloacae*, carbapenem-resistant *Acinetobacter baumannii*, colistin-resistant *Klebsiella pneumoniae*, etc.,^{20–22} but the antibacterial effect of CRPA is still unclear. Although the above research showed a potential choice of AS101 for carbapenem-resistant bacteria, we noted that the minimum inhibitory concentration (MIC) of AS101 to CRPA was as high as 128 $\mu\text{g/mL}$, which was not far from its 50% cytotoxicity level (145 $\mu\text{g/mL}$).²³ A high dose of AS101 will bring unsafe results. Therefore, further improvement of the antibacterial activity of AS101 would be needed.

Mefloquine is an antimalarial drug. It has been noted that mefloquine has a strong antibacterial activity against G^+ -bacteria but not against G^- -bacteria.^{24,25} Interestingly, mefloquine can significantly improve the efficacy of antibiotics against G^- -bacteria when used in combination with antibiotics. For example, mefloquine might enhance the activity of colistin against colistin-resistant *Enterobacter* and *P. aeruginosa*.^{26,27} In addition to common antibiotics, mefloquine possesses a synergistic effect on inhibitory drugs and improves the efficacy of anti-tuberculosis drugs such as isoniazid, pyrazinamide, and fluoroquinolones.²⁸ Thus, we postulate that the combination of AS101 with mefloquine may enhance the antibacterial activity through synergistic effect.

Bacterial biofilm is composed of bacteria and their secreted extracellular matrix, including extracellular polysaccharides, DNA, and/or proteins.²⁹ Additionally, planktonic cells could diffuse from the biofilm to establish new biofilm colonies in the distance.³⁰ This special microenvironment forms a barrier to resist the impact of external environment on bacteria, allowing pathogens to escape host defense and immune clearance and cause persistent infection, and drug resistance. The biofilm has higher resistance to most antimicrobial compounds, and the antibiotic concentration required to inhibit the growth of biofilm would be about 4–1000 times noted in planktonic bacteria.³¹ Therefore, the development of new biofilm inhibitory agents is an alternative strategy for the control of antibiotic-resistant bacterial infections.

The present study aims to evaluate the synergistic antibacterial activity, biofilm effect, and the mechanism of impact of AS101 and mefloquine combination on CRPA in vitro and in murine model.

Materials and Methods

Bacterial Isolates and Culture

Eight CRPA strains were collected as part of routine hospital procedure. They were isolated from the sputum of clinical patients of the First Hospital of Anhui Medical University in 2022 and were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, BioMerieux Inc, France). All strains were stored in LB broth (Solarbio Science&Technology Co., Ltd. China) containing 30% glycerol at -80°C . *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 (purchased from the National Clinical Laboratory Center (NCCL)) were used as quality control strains.

Multi-Locus Sequence Typing (MLST)

The sequence types of CRPA were determined by MLST. The internal parts of seven different housekeeping genes (*acsA*, *aroE*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, *trpE*) of *P. aeruginosa* were amplified with specific primers (the primer sequences have been shown in Table 1) after extracting *E. coli* DNA by the thermal lysis. The amplification product was sequenced and compared with the database (https://pubmlst.org/bigsub?db=pubmlst_paeruginosa_seqdef) to determine the sequence type.

Antimicrobial Susceptibility Test (AST)

The AST was conducted by the VITEK@2 compact system (BioMerieux Inc, France). The tested antibiotics included gentamicin, tobramycin, amikacin, ceftazidime, cefepime, ciprofloxacin, levofloxacin, piperacillin/tazobactam, imipenem, and meropenem. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as control strains. The interpretation of sensitivity results was based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2022).³²

Sensitivity of the Bacteria to AS101 and Mefloquine

The MIC to AS101 (Selleck Chemicals Co., Ltd., USA) and mefloquine (Selleck Chemicals Co., Ltd., USA) was determined by the broth dilution method.³³ Briefly, AS101 and mefloquine were dissolved in DMSO (Sigma-Aldrich, Saint Louis, MO, USA), and the two drugs were continuously twofold diluted to 0.25–128 µg/mL in 96-well microtitration plates (Solarbio Science&Technology Co., Ltd. China) by brain-heart infusion (BHI) broth. The final bacterial concentration of each well was about 5×10^5 CFU/mL. The absorbance near OD600nm was measured by a microplate reader (TECAN Infinite F50, Switzerland) after incubation in a 37 °C incubator for 18 h. The tests were performed twice for all isolates.

Chessboard Assay

Eight strains of bacteria were detected by chessboard assay, and some modifications were made.³⁴ Briefly, AS101 and mefloquine were diluted into a series of concentrations based on the MIC of each test strain through BHI broth in 96-well microtiter plates. The overnight colonies were diluted to 0.5 McFarland standard in sterile saline and then diluted in BHI broth at 1:150. The final bacterial concentration of each well was about 5×10^5 CFU/mL. The results were observed after incubation at 37 °C for 18 h. The experiment was divided into three replicates.

The synergy effect was evaluated by calculating the fractional inhibition concentration index (FICI), and the calculation and interpretation of FICI referred to the document standard.³⁵ FIC_A was obtained by dividing the

Table 1 The Sequences of Primers and Corresponding PCR Programs Used in This Study

Genes	Function	Primers ^a	DNA Sequences (5'-3')	Annealing Temperature (°C)	Amplicon Size (bp)
<i>acsA</i>	Acetyl coenzyme A synthetase	<i>acsA</i> -F <i>acsA</i> -R	ACCTGGTGTACGCCTCGCTGAC GACATAGATGCCCTGCCCTTGAT	56	842
<i>aroE</i>	Shikimate dehydrogenase	<i>aroE</i> -F <i>aroE</i> -R	TGGGGCTATGACTGGAAACC TAACCCGGTTTTGTGATTCCTACA	56	1056
<i>guaA</i>	GMP synthase	<i>guaA</i> -F <i>guaA</i> -R	CGGCCTCGACGTGTGGATGA GAACGCCTGGCTGGTCTTGTTGGTA	56	940
<i>mutL</i>	DNA mismatch repair protein	<i>mutL</i> -F <i>mutL</i> -R	CGCGACCTGTTCTTCAACAC CAGGGTGCCATAGAGGAAGTC	58	490
<i>nuoD</i>	NADH dehydrogenase I chain C, D	<i>nuoD</i> -F <i>nuoD</i> -R	ACGGCGAGATACGAGGACTAC GGCTTCCTTGGTGTGACT	56	590
<i>ppsA</i>	Phosphoenolpyruvate synthase	<i>ppsA</i> -F <i>ppsA</i> -R	GGTCGCTCGGTCAAGGTAGTGG GGGTTCTCTTCTCCGGCTCGTAG	56	989
<i>trpE</i>	Anthralite synthetase component I	<i>trpE</i> -F <i>trpE</i> -R	GCGGCCAGGGTGTGAG CCCGCGCTTGTGATGGTT	56	811

Notes: ^aPrimers, The "F" meant the forward primer and the "R" meant the reverse primer.

concentration of drug A when used in combination (C_A) by the concentration when used alone (MIC_A). Similarly, FIC_B was obtained by dividing the concentration of drug B when used in combination (C_B) by the concentration when used alone (MIC_B), $FICI = FIC_A + FIC_B = [C_A/MIC_A] + [C_B/MIC_B]$. The explanation of the interaction was as follows: $FICI \leq 0.5$ was synergism, $0.5 < FICI \leq 1$ was additive, $1 < FICI \leq 2$ was irrelevant, and $FICI > 2$ was antagonistic.

Time Killing Assay

The time killing assay was based on the results of the chessboard experiment. The modified time killing assay was carried out as described earlier.³⁶ Briefly, the colonies cultured overnight were diluted with BHI broth, and the final concentration of bacteria was about 1×10^6 CFU/mL. Then, AS101 and mefloquine were added individually or jointly, and the control group was not added with drugs. The bacterial suspension (with or without drugs) was incubated at 37 °C and shaken moderately for 0, 2, 4, 6, 12, and 24 h, respectively. The CFU was then counted after incubation at 37 °C overnight. Next, we draw the time-kill curve with \log_{10} CFU/mL as the ordinate and the time as the abscissa. Point t_0 was the time point when the antibacterial drug solution was added to the bacterial solution under test. Each strain was tested three times. The bactericidal activity was defined as the reduction of CFU/mL in 24 h $\geq 3 \log_{10}$, and the synergetic activity was defined as the reduction of CFU/mL in 24 h $\geq 2 \log_{10}$ ³⁷ when two drugs were used together compared with any one drug alone.

Biofilm Formation Inhibition Assay

The biofilm formation inhibition assay was carried out as described previously and had been slightly modified.³⁸ Adjusted the bacteria growing overnight to 0.5 McFarland standard, and then distributed AS101 and mefloquine (alone or in combination) in 96-well microtiter plates. The selection of the concentration of the two drugs was selected based on FICI equal to 0.5 (the concentrations of mefloquine and AS101 for strain CRPA-4 were 64 and 16 $\mu\text{g/mL}$, respectively, and the remaining seven bacterial strains exhibited concentrations of 64 and 32 $\mu\text{g/mL}$, respectively). The plates were then washed twice by 200 μL 1 \times PBS and inverted after incubation at 37 °C for 24 h, dried at room temperature. Next, 200 μL 99% methanol (Jiangsu Qiangsheng Functional Chemical Co., Ltd.) was used to fix for 15 min, then removed methanol and dried. Subsequently, each well was added 200 μL 0.4% crystal violet (CV) solution (Solarbio Science&Technology Co., Ltd. China) for 15 min, and then it was washed twice by 200 μL 1 \times PBS (Sigma-Aldrich, Saint Louis, MO, USA) to remove the excess CV thoroughly and dried at room temperature. The combined CV was dissolved by 200 μL 33% glacial acetic acid (Tianjin Damao Chemical Reagent Factory). The absorbance was read at 595 nm using a multi-function microplate reader (TECAN Infinite F50, Switzerland). The experiment was made in triplicate and repeated three times.

Morphological Alterations Following AS101 and/or Mefloquine Treatment

The effects of AS101 and mefloquine separately and in combination with the morphology of bacteria were observed by electron microscopy. The steps of the scanning electron microscopy (SEM) were referred to previous literature.²² The logarithmic growth phase strain CRPA-3 was incubated with AS101 and mefloquine (alone or in combination) at 37 °C for 1 h, and the control group was not added with drugs. The samples were immersed in a 2.5% glutaraldehyde fixation solution (Solarbio Science&Technology Co., Ltd. China) and kept at 4 °C for 4 h. Subsequently, they were washed and soaked by 1 \times PBS at room temperature for 3 times, each time for 20 min. Then dehydrated the sample by increasing the concentration of ethanol (Sinopharm Chemical Reagent Co., Ltd.) (30%, 50%, 70%, 80%, 90%, and 100% (twice) [vol/vol], 4 °C, 15 min each). All samples were dried at 37 °C for 15 min and observed by SEM (ZEISS GeminiSEM 300, Germany). The sample preparation conditions of transmission electron microscopy (TEM) were the same as those of SEM, and then fixation, staining, and preparation were carried out according to the procedure of TEM (Thermo scientific Talos L120C G2, USA).³⁹

Cell Membrane Permeability Test

The detection of cell membrane permeability with propidium iodide (PI) staining was performed as described before with slight modifications.⁴⁰ CRPA-3 at logarithmic phase was incubated with AS101 or/and mefloquine (AS101 32 $\mu\text{g/mL}$; mefloquine 64 $\mu\text{g/mL}$) at 37 °C for 2 h, and the bacteria in the non-drug group served as the control group. The samples

were then washed with normal saline at room temperature and incubated with PI (50mg/mL) (Sigma-Aldrich, Saint Louis, MO, USA) for 10 min. The samples were analyzed by flow cytometry (BD FACS Calibur, USA).

Reactive Oxygen Species (ROS) Detection

ROS detection followed previously established method and was appropriately modified,⁴¹ and 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Biyuntian Biotechnology Co., Ltd., China) was used to detect the ROS level. In short, the CRPA-3 in logarithmic period was mixed with drugs (AS101, mefloquine alone or in combination) and incubated at 37 °C for 2 h, and the bacteria in the non-drug group were used as the control. Then the bacteria were adjusted to 0.5 McFarland standard and incubated with DCFH-DA at 37 °C for 20 min (the final concentration was 100 μM). The bacteria treated with or without DCFH-DA were collected and washed with normal saline and resuspended. 200 μL for each sample was transferred to the 96-well fluorescence microplate well plat (Solarbio Science&Technology Co., Ltd. China). The fluorescence intensity was measured with a microplate reader (enspire, USA) under excitation and emission at 488 nm and 525 nm, respectively. The final bacterial solution was continuously diluted for 10 times, and then CFU was counted. For normalization, the net fluorescence value was divided by the number of viable bacteria number. All experiments were repeated three times.

In vivo Evaluation of Synergy in the Mouse Model

Five-week-old ICR (CD1) female mice (Jiangsu Huachuang Xinnuo Pharmaceutical Technology Co., Ltd.) were selected for the in vivo experiment. All animal experiments were approved by the Experimental Animal Ethics Committee of Anhui Medical University. The procedures complied with the ARRIVE guidelines and were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, and associated guidelines.

The mouse abdominal infection models were established using previously experimental methods.^{22,26} Mice were intraperitoneally injected with 100 μL 1×10^7 CRPA-3 isolate, and intraperitoneally injected with 1 × PBS, AS101 and mefloquine (alone or in combination) 30 min after infection. The daily doses of AS101 and mefloquine were 3.33 mg/kg and 10 mg/kg, respectively. The mice were killed after 72 h of continuous treatment, and the liver, kidney, and spleen of the mice were collected with sterile PBS. Then they were homogenized with homogenizer (Xavier Biotechnology Co., Ltd., China). The bacterial burden was quantified by the CFU count of visceral homogenate. The bacterial count was normalized by organ weight. There were 3 mice in each group.

Statistical Analysis

GraphPad Prism 8.0.1 software program was used for statistical analysis. Student's *t*-test and Log rank test were used to determine the significance, $P < 0.05$ was considered significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

CRPA Exhibits Multidrug Resistance Profiles

Eight CRPA strains were distributed in different sequence types, including ST242 (n=1), ST9 (n=1), ST645 (n=2), ST708 (n=1), ST1835 (n=1), ST1437 (n=1), and ST274 (n=1). The drug sensitivity of strains to 10 clinical antibiotics is shown in Table 2. Eight strains of *P. aeruginosa* were resistant to carbapenem antibiotics (meropenem or imipenem) and basically sensitive or intermediary to six antibiotics (gentamicin, tobramycin, amikacin, ceftazidime, cefepime, and piperacillin/tazobactam), of which 6 strains were resistant to quinolones (ciprofloxacin and levofloxacin). The data showed that these strains were all multidrug resistant *P. aeruginosa*.

Synergistic Activity Test by the Checkerboard Assay

The results of chessboard assay are shown in Table 3. The MIC₉₀ of mefloquine was ≥ 256 μg/mL, MIC₉₀ of AS101 was 128 μg/mL. The combined use of AS101 and mefloquine could reduce the MIC of the two drugs by 4–8 times, and the FICI values were between 0.375 and 0.5, indicating that AS101 and mefloquine have synergistic antibacterial activity.

Table 2 The Drug Sensitivity of Eight CRPA Strains to Ten Kinds of Antibiotics

Bacterial Strains	Gentamicin	Tobramycin	Amikacin	Ceftazidime	Cefepime	Ciprofloxacin	Levofloxacin	Piperacillin/Tazobactam	Imipenem	Meropenem
CRPA-1	I	S	I	S	I	S	R	S	R	I
CRPA-2	S	S	S	S	S	I	R	S	R	S
CRPA-3	S	S	S	S	S	S	S	I	R	R
CRPA-4	S	S	S	S	S	R	R	S	R	S
CRPA-5	S	S	S	I	I	S	S	I	R	R
CRPA-6	S	S	S	R	I	I	R	I	R	R
CRPA-7	R	R	I	R	I	R	R	I	R	R
CRPA-8	S	S	S	I	I	R	R	I	R	R

Abbreviations: S, susceptible; I, intermediate; R, resistant.

Table 3 Summary of MIC Value and FICI of Eight CRPA Strains Treated with AS101 and Mefloquine

Strains	Monotherapy ($\mu\text{g/mL}$)		Combination ($\mu\text{g/mL}$)		FICI	Interpretation
	Mefloquine	AS101	Mefloquine	AS101		
CRPA-1	≥ 256	128	64	32	0.5	Synergistic
CRPA-2	≥ 256	128	64	16	0.375	Synergistic
CRPA-3	≥ 256	128	64	32	0.5	Synergistic
CRPA-4	≥ 256	64	64	16	0.5	Synergistic
CRPA-5	≥ 256	128	64	32	0.5	Synergistic
CRPA-6	≥ 256	128	64	16	0.375	Synergistic
CRPA-7	≥ 256	128	64	32	0.5	Synergistic
CRPA-8	≥ 256	128	64	16	0.375	Synergistic

Synergistic Activity Test by the Time Killing Assay

The time killing curves of eight CRPA strains are shown in [Figure 1](#). In contrast, after all strains were treated with AS101 and mefloquine combination for 24 h, the colonies of viable bacteria decreased significantly, with a decrease of more than $2\log_{10}$ (CFU/mL) times, compared with any single agent.

AS101 Add Mefloquine Can Inhibit the Biofilm Formation

The experiments studied the ability of AS101 and mefloquine alone or in combination to inhibit the formation of biofilm. As shown in [Figure 2](#). Compared to the drug-free control group, treatment with mefloquine alone resulted in decreased biofilms for CRPA-5 and CRPA-7, whereas biofilms for CRPA-2, CRPA-4, and CRPA-8 increased. Biofilms for CRPA-1, CRPA-3, and CRPA-6 remained unchanged. Furthermore, when treated with AS101 alone, biofilms for CRPA-1, CRPA-3, CRPA-5, and CRPA-7 decreased, while biofilms for CRPA-8 increased. Biofilms for CRPA-2, CRPA-4, and CRPA-6 remained unchanged. When the two drug groups were combined, biofilms for CRPA-5 and CRPA-6 were significantly lower than those in the control group and the monotherapy group ($P < 0.05$), while the remaining six strains of *P. aeruginosa* biofilms did not show a significant reduction compared to the monotherapy group and the control group.

Destructive Effect of AS101 and Mefloquine on Bacterial Morphology

In order to further study the mechanism of action of AS101 and mefloquine on CRPA, we used SEM and TEM to observe the changes of bacterial morphology ([Figures 3](#) and [4](#)). It could be seen that at the $15,000\times$ magnification, the bacteria in the blank group and AS101 treatment group showed complete and smooth cell morphology ([Figure 3A](#) and [C](#)). The bacteria in the mefloquine treatment group began to distort and the cell wall perforated, and the bacteria in the AS101-mefloquine combined treatment group shriveled and further distorted and perforated ([Figure 3B](#) and [D](#)). The results of TEM were consistent with those of SEM. No alterations of the bacteria in blank control and AS101 treatment group were seen ([Figure 4A](#) and [C](#)), while breakings of the bacteria following mefloquine and AS101-mefloquine combined treatments were observed ([Figure 4B](#) and [D](#)).

Increased Cell Membrane Permeability Induced by AS101-Mefloquine Treatment

Cell wall and cell membrane are imperative for maintaining the complete structure and normal physiological function of bacteria. As described above, the combined use of AS101-mefloquine could destroy the cell morphology, and might increase the permeability of bacterial cell membrane. As shown in [Figures 5](#) and [6](#), compared with the single drug group, the bacterial particles carrying PI increased notably, and the results were statistically different.

Combination of AS101 and Mefloquine Promoted ROS Production

The result of ROS is shown in [Figure 7](#). Compared with the blank group, ROS level slightly increased when AS101 or mefloquine was used alone. However, a significant elevation of ROS level was noted when the two drugs were used together.

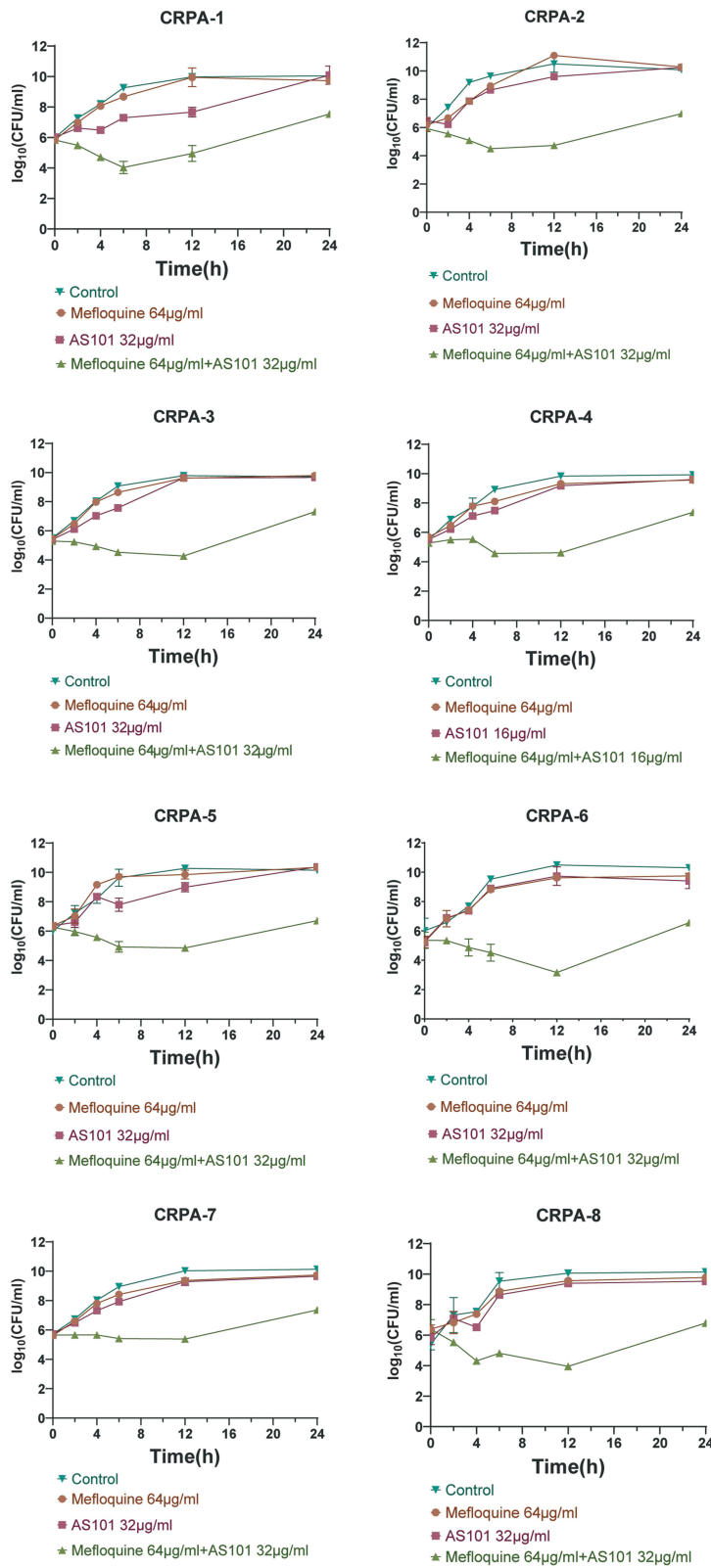


Figure 1 Time killing curves of AS101 and mefloquine acting alone or jointly on eight CRPA strains.

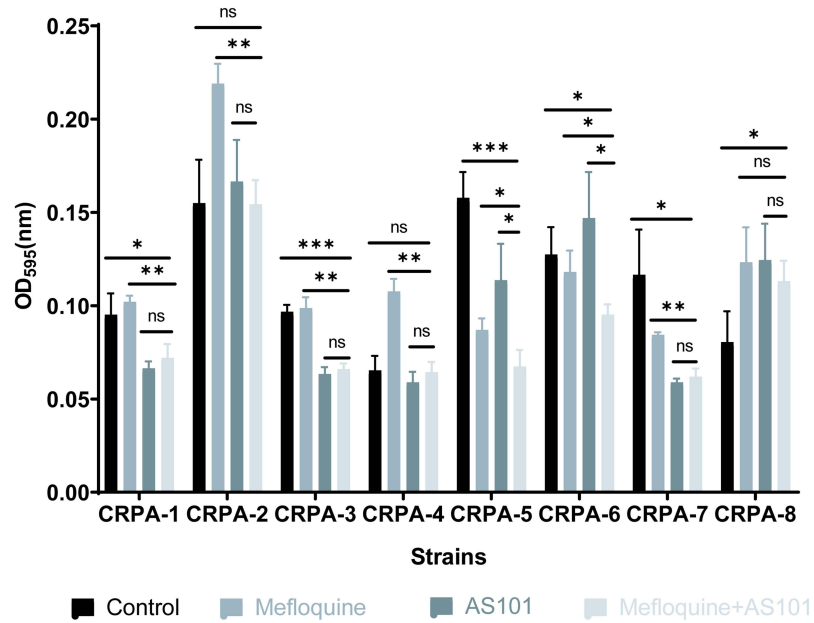


Figure 2 AS101 and mefloquine used separately or jointly on the inhibition of biofilm formation of CRPA. Bars of different colors represented different treatment groups. The data were analyzed by Student's *t* test; ns, no statistical significance; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The experiment was conducted three times. Data was expressed in mean±standard deviation; OD595, optical density at 595 nm.

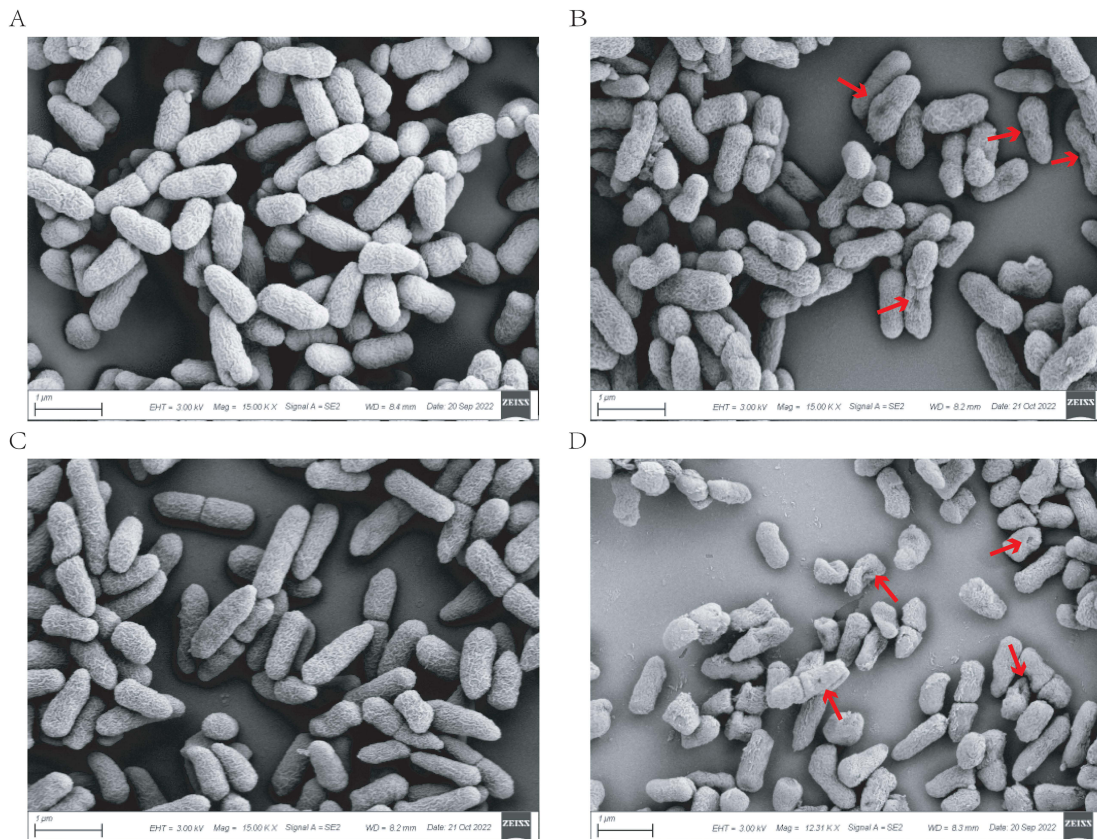


Figure 3 SEM images of CRPA-3 processed by AS101 and mefloquine. (A) BHI blank control at 15 000x magnification (B) Mefloquine at 15 000x magnification (C) AS101 at 15 000x magnification (D) Mefloquine-AS101 combination at 15 000x magnification. The red arrows indicate the holes in the bacterial cell wall.

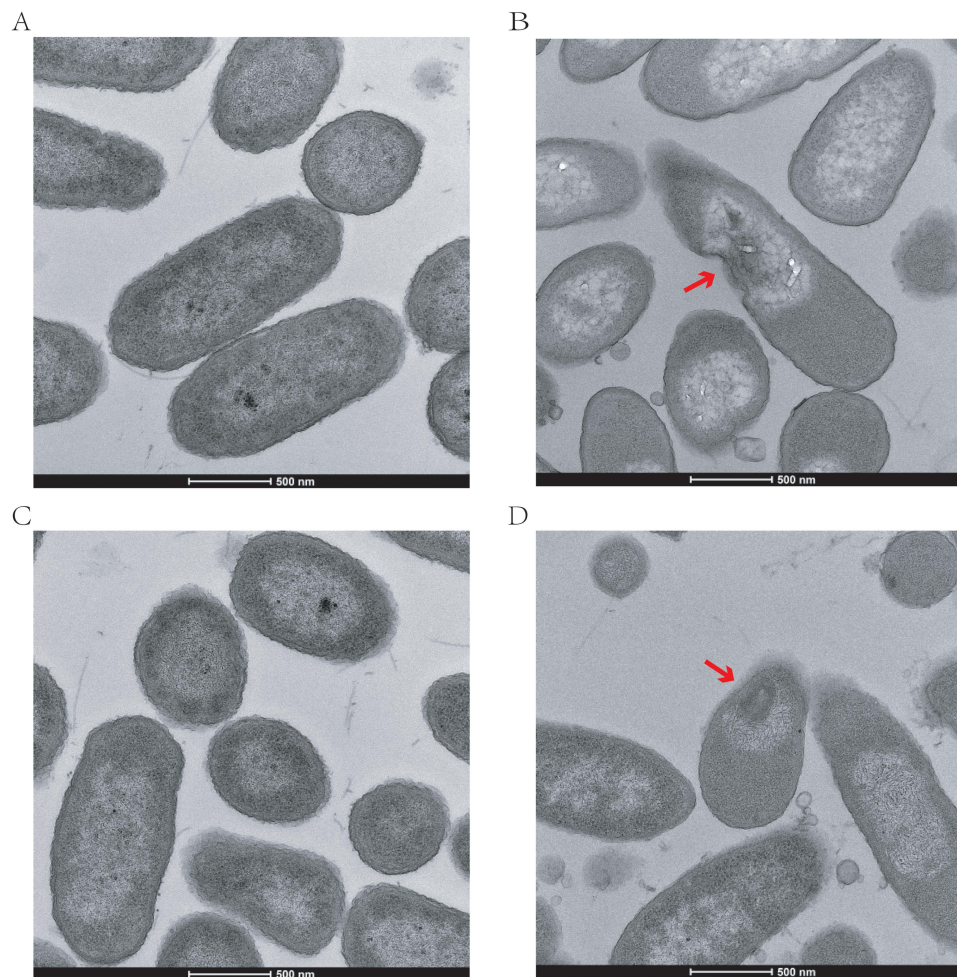


Figure 4 TEM images of CRPA-3 processed with AS101 and mefloquine. (A) BHI blank control at 22 000x magnetization (B) Mefloquine at 22 000x magnetization (C) AS101 at 22 000x magnetization (D) Mefloquine–AS101 combination at 22 000x magnetization. The red arrows indicate cell wall ruptured.

In vivo Treatment Verification

In order to verify the therapeutic effect of AS101-mefloquine combination on CRPA *in vivo*, we set up a mouse infection model. Figure 8 indicates the microbial load of mouse liver, kidney, and spleen after PBS, AS101, and mefloquine administration alone or in combination. After 72 h, the combined use of AS101 and mefloquine could significantly reduce the bacterial burden in the organs of mice compared with the control and the single-drug treatment.

Discussion

In recent years, the prevalence and spread of CRPA have brought great challenges to healthcare institutions around the world. Previous reports in different countries of Latin America from 2002 to 2013 exhibited that the resistance rate of *P. aeruginosa* to carbapenems was as high as 66%.⁴² In a monitoring study in the United States, 3881 isolates of *P. aeruginosa* were collected from medical related infections from 2011 to 2014, and the resistance rate to carbapenems was 23.7–28.4%.⁴³ According to the CARSS report of China in 2021, the drug resistance rate of *P. aeruginosa* to carbapenems was 17.7% on average nationwide, with 25.8% in some regions.⁴⁴ Here, the results of drug sensitivity showed that all eight strains of *P. aeruginosa* were multidrug resistant, which limited the choice of antibiotics available for CRPA. The increase of bacterial resistance limited the treatment plan of *P. aeruginosa* and increased the hospital costs of patients and even led to deaths. Among them, there were 32,600 inpatients in MDR-*P. aeruginosa*, of which 2700 died in 2017⁴⁵ according to the data from the CDC. However, irrational use of antibiotic treatment would aggravate the spread of bacterial resistance and result in dysbiosis of gut

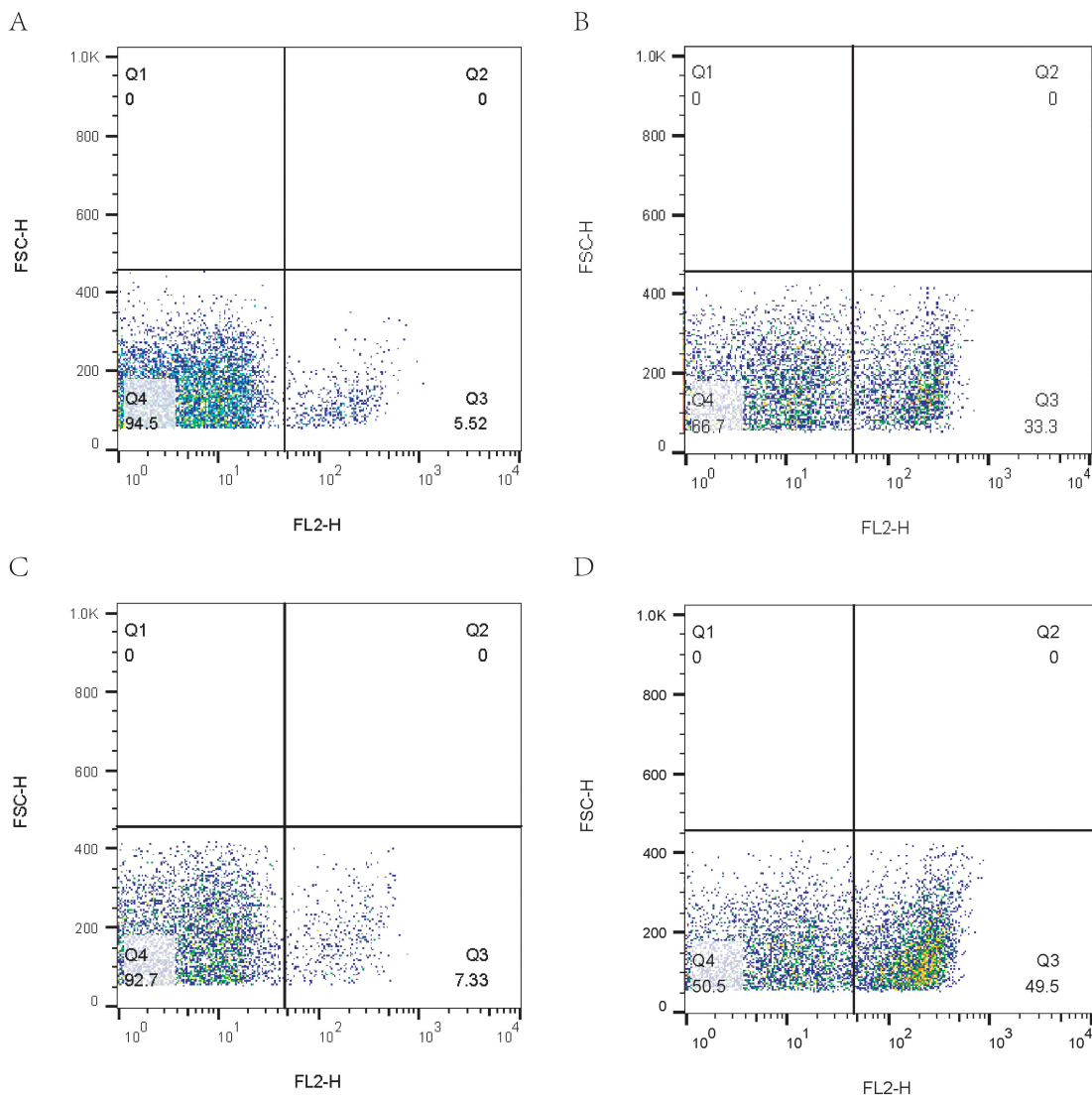


Figure 5 The flow cytometry picture of cell membrane permeability of CRPA-3 treated with AS101 and mefloquine. Compared with the control group (**A**) and the monotherapy group (**B** and **C**), the bacterial particles generally moved to the right after the combination of AS101 and mefloquine (**D**), that was, the fluorescence intensity increased and the permeability of cell membrane increased.

microbiota.⁴⁶ Therefore, rational use of antibiotics and control of the spread of multidrug resistant bacteria are important tasks of medical and healthcare institutions.

It has been recognized that the destructive effect of antimicrobial resistance (AMR) is a significant public health problem around the world. In Europe and the United States alone, at least 50,000 people died of anti-microbial infections each year, and hundreds of thousands more died in other parts of the world. 10 million people would die each year, and the world production would be reduced by 2% to 3.5% by 2050.⁴⁷ In this case, reuse of approved drugs as alternative treatments is increasingly important in view of the long-term use of new antibiotics development. Fortunately, promising achievements have been made in this regard. A variety of drugs have been found to be able to stop the growth of drug-resistant bacteria, including multidrug resistant *Acinetobacter baumannii*, colistin-resistant *E. coli*, and methicillin-resistant *Staphylococcus*.^{48–50}

It has been reported that AS101 has a strong antibacterial activity against various drug-resistant bacteria, including refractory colistin resistant G^- bacteria and multidrug resistant *A. baumannii*.^{20–22} However, in the study conducted by Katip et al, it was observed that while long-term high-dose monotherapy could effectively decrease the 30-day mortality rate of patients and enhance clinical as well as microbial responses, it also resulted in an increase in nephrotoxicity.^{51,52} Antimicrobial combinations hold the potential to enhance outcomes through broadening the spectrum of antimicrobial

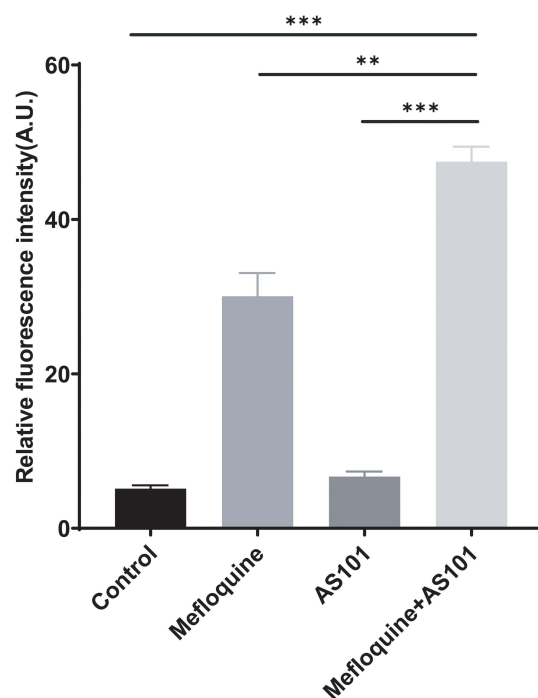


Figure 6 The statistical results of cell membrane permeability of the CRPA-3 under the action of AS101 and mefloquine by flow cytometry. Compared with the control group and the monotherapy group, the fluorescence intensity after the combination of AS101 and mefloquine was significantly increased. The data were analyzed by Student's *t* test; ns, no statistical significance; ***P* < 0.01; ****P* < 0.001. The experiment was conducted three times. Data was expressed in mean ± standard deviation.

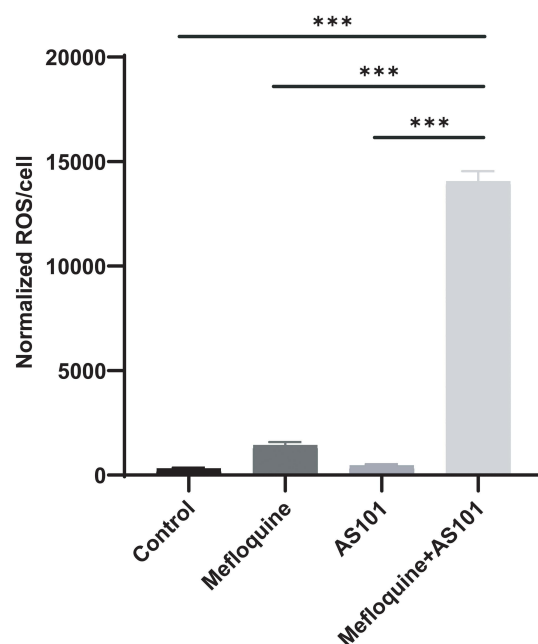


Figure 7 ROS production after the CRPA-3 treated with AS101 and mefloquine. Compared with the control group and the monotherapy group, the combined use of AS101 and mefloquine significantly increased ROS. The data were analyzed by Student's *t* test; ****P* < 0.001. The experiment was conducted three times. Data was expressed in mean ± standard deviation.

activity, diminishing the likelihood of antimicrobial resistance, and generating a more robust antimicrobial effect via synergy.⁵³ Moreover, we discovered that AS101 exhibited antibacterial activity in isolation, however, its MIC was notably elevated, reaching 128 µg/mL, which is closely proximate to its 50% cytotoxicity threshold. This high MIC implies that AS101 might not effectively inhibit the activity of *P. aeruginosa*. Notably, in a separate study, Sung et al

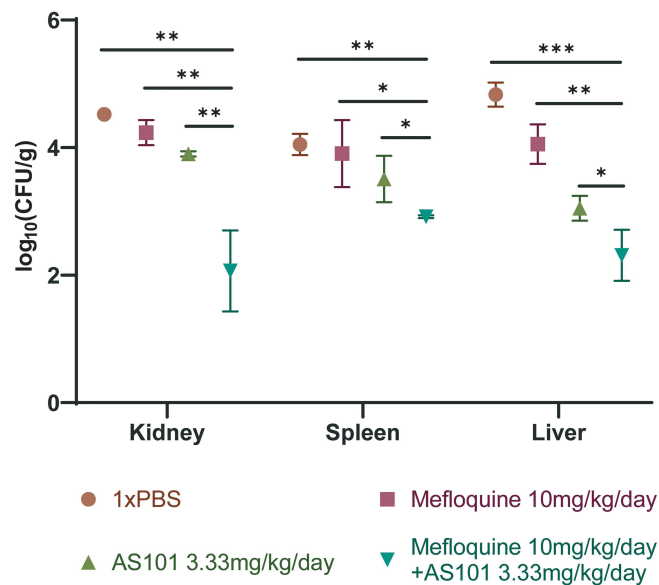


Figure 8 Log₁₀ changes of liver, kidney and spleen of CRPA-3 infected mice after 72 h of treatment with placebo (1 × PBS), single drug (mefloquine 10 mg/kg/day, AS101 3.33 mg/kg/day) or combination (mefloquine 10 mg/kg/day + AS101 3.33 mg/kg/day). The data were analyzed by Student's t-test; *P < 0.05; **P < 0.01; ***P < 0.001. Three mice in each group. Data was expressed in mean ± standard deviation.

reported a synergistic effect resulting from the combination of AS101 and azidothymidine in the treatment of carbapenem-resistant *K. pneumoniae*.⁵⁴ We did not find any significant synergistic effect after using AS101 and azidothoracil on CRPA. According to reported literature, the antimalarial drug mefloquine has demonstrated the ability to significantly enhance antibacterial activity when utilized in combination with other antibacterial agents, such as colistin and anti-tuberculosis drugs.^{26,27} Based on this premise, the study employed a combination of AS101 and mefloquine for the treatment of CRPA. The chessboard assay showed that when AS101 and mefloquine were combined, the MIC of the two drugs decreased by 4–8 times. The results of the time killing experiment were consistent with those of the chessboard experiments. All of these indicated that AS101 and mefloquine had synergistic inhibition activity on CRPA. Meanwhile, we found that mefloquine alone had no antibacterial effect on CRPA, which is consistent with previous results.²⁴

Bacterial biofilm is a form of biofilm that develops on the surfaces of bacteria during their growth process, characterized by a structure composed of bacteria and an extracellular matrix secreted by the bacteria themselves. Biofilms serve to shield bacteria from unfavorable environmental conditions, enhance their resistance to drugs, and naturally establish a favorable barrier for bacterial proliferation.⁵⁵ The formation of biofilm is one of the important reasons for difficult treatment of *P. aeruginosa*, leading to high frequency of complications and mortality.⁵⁶ The formation of bacterial biofilms primarily involves adhesion, maturation, and dispersal. Gram-negative bacteria can adhere to surfaces using flagella, pili, or fimbriae. Biofilm maturation is governed by a range of bacterial mechanisms, including quorum sensing (QS) systems, the RetS/LadS and GacS/GacA two-component regulatory systems, and c-di-GMP-mediated polysaccharide regulation. QS coordinates interconnected signaling pathways that guide bacterial social behavior through multiple channels. This enables bacterial communities to regulate the essential processes and resilience required for bacterial adaptation across various organisms. *P. aeruginosa* exhibits intricate QS network structures, with one type being the N-acyl-homoserine lactone (AHL)-dependent QS system, encompassing the las and rhl QS systems. These systems primarily consist of rhlI-rhlR and lasI-lasR, respectively, which oversee the production of key autoinducers, namely C4-HSL and 3-oxo-C12-HSL. Subsequently, these autoinducers govern the sequential regulation of downstream gene expression. Another type of signaling molecule is the *Pseudomonas* quinolone signal (PQS), specifically 2-heptyl-3-hydroxy-4(1H)-quinolone. Notably, the function of this signaling molecule operates independently of AHL. Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) serves as a prominent second messenger within bacterial systems. The pelA-G pathway of the c-di-GMP system becomes active upon interaction with the C4-HSL molecule, produced by the rhl system associated with AHL. Elevated c-di-GMP levels foster the biosynthesis of bacterial extracellular polysaccharides, playing a pivotal role in biofilm formation. Conversely, reduced c-di-

GMP concentrations augment bacterial motility by facilitating flagella development and bacterial dispersion. Furthermore, in pulmonary infections, *P. aeruginosa* can exhibit overproduction of c-di-GMP, resulting in the formation of small colony variants (SCVs). This particular state is characterized by sustained low toxicity and exceptional biofilm-forming capabilities. The two-component regulatory systems constitute essential signaling pathways for bacteria, enabling them to perceive alterations in the environment, such as pH, osmotic pressure, temperature, and nutritional changes, and respond with adaptive adjustments. Among these pathways, the GacS/GacA system assumes a pivotal role as an upstream regulator of the QS systems, governing the synthesis of diverse virulence factors and the formation of biofilms. Meanwhile, RetS and LadS modulate the phosphorylation of GacA by exerting control over the phosphorylation levels of GacS, thereby fine-tuning the function of the GacS/GacA system.⁵⁷ In this study, we noted the synergistic inhibitory effects of AS101-mefloquine on CRPA-5 and CRPA-6 biofilms. Prior research by Daniel Hoffmann et al demonstrated AS101's ability to reduce biofilm formation by inhibiting the virulence factors responsible for the movement and attachment of *Enterobacter cloacae*.²⁰ Furthermore, Wong et al reported that mefloquine acts as a protein synthesis inhibitor, primarily through its binding to the 80S ribosome of *Plasmodium falciparum*, thereby inhibiting protein synthesis.⁵⁸ Additionally, Zhang et al conducted a study that revealed the synergistic antibacterial and anti-biofilm effects of combining mefloquine and colistin on *Escherichia coli*. Their findings suggest that colistin alters cell membrane permeability, facilitating the entry of mefloquine into bacterial cells, resulting in effective antibacterial properties. Notably, this combination treatment was observed to inhibit biofilm formation, and SEM images displayed the disruption of biofilm structures. Consequently, they hypothesized that, in the presence of colistin, mefloquine may inhibit the synthesis of biofilm proteins, contributing to the disintegration of biofilm structures. Furthermore, the combination of mefloquine-colistin might also reduce the expression of the QS system, thereby inhibiting biofilm formation.²⁷ In our present study, the combination of mefloquine and AS101 exhibited a significant reduction in CRPA-5 and CRPA-6 biofilms. We postulate that this effect is potentially attributed to the notable increase in bacterial cell membrane permeability and enhanced drug influx during concurrent administration. These mechanisms collectively hinder the synthesis of biofilm-related proteins, disrupt biofilm structures, and potentially influence the signaling molecules associated with biofilm formation mentioned earlier, ultimately leading to a substantial decrease in biofilm formation.

Bacterial cell membrane is a highly permeable barrier that allows liposoluble drugs to pass through, but water-soluble drugs need the help of channel proteins to enter the bacteria.⁵⁹ The results demonstrated that AS101 and mefloquine, when used alone, had no, if any, effect on the cell membrane of bacteria, but the combined use of both could significantly increase the permeability of the cell membrane. Previous study showed that the oxidation-reduction reaction mediated by antibacterial drugs was also an important part of its lethality.⁶⁰ In this study, we noted that the combined administration of AS101 and mefloquine could significantly increase the ROS level to facilitate inhibition of bacterial growth. Previous study has also confirmed the association between the antibacterial activity of AS101 and the accumulation of ROS.²² Hence, in this study, mefloquine inhibited bacterial growth by destabilizing cell walls and enhancing cell membrane permeability. This augmentation facilitated the entry of AS101 into the intracellular space, leading to the generation of a substantial quantity of ROS. Prior research has established that both mefloquine and AS101 have the potential to influence cell membrane stability. AS101, for instance, has been shown to disrupt the cell membrane and affect the Na⁺/K⁺ pump.^{20,61} In the present study, we observed that while AS101 alone had a relatively minor impact on cell membrane permeability, mefloquine significantly altered the cell membrane permeability of *P. aeruginosa*. The precise mechanism by which mefloquine acts on various bacteria remains unclear in previous reports. In a study conducted by Martín Galiano et al on *Streptococcus pneumoniae*, it was observed that mefloquine could impact the activity of the membrane-associated ATPase, F0F1 H-ATPase. This enzyme primarily functions in *Streptococcus pneumoniae* to generate proton gradients and uphold intracellular pH through the extrusion of protons powered by ATP hydrolysis. The strains that exhibited mefloquine inhibition possessed specific amino acid substitutions within the c subunit of F0F1 H-ATPase. Mutation in the c or a subunit of the F0 complex induces alterations in the cell membrane's transmembrane helix, disrupting proton translocation and resulting in mefloquine resistance.⁶² Furthermore, Dos Santos et al identified that mefloquine displayed synergistic effects when combined with isoniazid, pyrazinamide, and multiple fluoroquinolone drugs. This synergy arises from the potential positioning of mefloquine between the lipid polarity region and the initial chain methylene, inducing disruption in these areas. This perturbation results in lipid anomalies and heightened membrane permeability.²⁸ Hence, the synergistic effect of AS101 and mefloquine on cell membrane permeability may

be attributed to mefloquine's role as a spacer, increasing cell membrane disorder while simultaneously affecting cell membrane ATPase activity, ultimately leading to enhanced cell membrane permeability. Subsequently, upon entering the compromised cell membrane, AS101 further impairs the Na^+/K^+ pump, resulting in a substantial increase in cell membrane permeability.

Finally, we further evaluated the therapeutic effect of AS101 and mefloquine in vivo by establishing mouse peritoneal infection model. Compared with the control and the single treatment group, the combined treatment of AS101 and mefloquine could remarkably reduce the bacterial load in the liver, kidney, and spleen of mice, indicating that AS101 together with mefloquine are bacteriostatic and promising for the treatment of CRPA.

The combination of AS101 and mefloquine showed excellent synergistic effects against CRPA in vitro and in vivo. However, there are some limitations in our study. In this study, we focused on a limited pool of eight bacterial strains as our research subjects, thereby representing a subset of CRPA. To comprehensively validate the antibacterial efficacy of this combination, a diverse range of strains should be incorporated. Moreover, our in vivo experiments were confined to CRPA-3 and CD1 mice, potentially introducing experimental variability. The concentration range of mefloquine and AS101 employed in our in vivo experiments was constrained, rendering it challenging to ascertain the optimal dosage combination. As such, it is imperative to expand both the scope of our experimental animal model and the range of dosage combinations. This expansion will facilitate the verification of in vivo antibacterial effectiveness and toxicity, ultimately leading to the identification of the most effective combination strategy. Furthermore, we have observed that combination drugs possess the ability to impede biofilm formation and augment cell membrane permeability. However, a comprehensive understanding of the precise mechanisms underlying the inhibition of biofilm formation and disruption of cell membranes remains lacking. Consequently, further experimental investigations are imperative to delve deeper into the intricacies of the drug action mechanisms. Besides, its potential clinical application value needs further research.

Conclusions

In general, this is the first report on the synergistic activity of AS101 and mefloquine combination in carbapenem-resistant *P. aeruginosa* although no, if any, antibacterial activity takes place when used alone for each of them. The possible mechanisms include oxidative injury, enhanced permeability of bacterial membrane as well as inhibition of biofilm formation.

Data Sharing Statement

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

Ethics Approval and Consent to Participate

Animal experiments were approved by the Experimental Animal Ethics Committee of Anhui Medical University. The animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, and associated guidelines. The clinical isolates used in the study have been approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no competing interests to declare for this work.

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