Antibacterial activity of Zunyimycin C against methicillin resistant *Staphylococcus aureus* clinical isolates

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Methicillin resistant *Staphylococcus aureus* (MRSA) has emerged since the 1960s and becomes a leading cause of bacterial infections in both healthcare and community settings in humans. MRSA can be resistant to a variety of clinical methicillin antibiotics by producing virulence factors and β-lactam resistance. The resistance to multiple antibiotics makes MRSA a very difficult treating bacterium. The average hospitalization time of patients infected with MRSA is three times more than that of other patients, while the mortality rate is five times more than that of other patients. The mortality of patients with MRSA within 30 days of infection is 34%. The patients infected with MRSA have a mortality rate of 64% comparing to non-infected patients. Therefore, it has been listed as the first of the three most difficult infectious diseases in the world. It is urgent to develop novel antibiotics to against MRSA. The objective of this study was to examine the antibacterial bioactivity of the new antibiotics, Zunyimycin C, to against MRSA. Zunyimycin C is a member of anthrabenzoxocinones antibitics family, which is isolated from streptomycetes by our research team. The antibacterial activity assay indicated that Zunyimycin C showed a good inhibitory effect on MRSA and *Enterococci*. *In vitro* antibacterial properties such as minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill of Zunyimycin C were assessed by using MRSA clinical isolated strains. *In vivo* antibacterial properties including drug half-life, killing curves, and survival rate of Zunyimycin C were assessed by using MRSA infected SPF rats. Zunyimycin C was found to have the anti-MRSA effects with MBC value of 16 μg/mL while MIC value of 0.25 μg/mL. The bactericidal power (ratio of MBC/MIC) was 64. Time-kill results showed that, after 6 h exposited in Zunyimycin C, the total number of living bacteria decreased dramatically comparing to that of normal saline control group. The half-life ($t_{1/2}$) of Zunyimyicn C in SPF rat was 6.3 h. The survival rate, whole blood bacteria, and animal weight showed no significant different ($p>0.05$) between MRSA-infected animals treated by Zunyimycin C and Vancomycin. The data analysis indicated that Zunyimycin C possessed the antibacterial potential against MRSA and could be a candidate for anti-MRSA drug development in future.

Keywords: Antibacterial activity; Zunyimycin C; Methicillin Resistant *Staphylococcus aureus*; clinical isolates.

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Introduction

Due to the broad application of intravenous antibiotics, Methicillin-resistant *Staphylococcus aureus* (MRSA), as one of the infamous superbugs, may cause many organ-specific infections [1, 2]. MRSA has been one of the main superbugs that cause hospital-acquired infections in the world, and usually associates with the increased incidence rate, mortality,

length of hospitalization, and cost burden [3, 4]. MRSA is a serious threat to human health with the average hospitalization time and mortality rate three and five times higher than that of regular in-patients, respectively. The mortality of MRSA patients within 30 days of infection is 34%. In general, MRSA infected patients demonstrated a mortality rate of 64%. MRSA has been listed as the number one of three the most difficult to treat infectious diseases in the world [5]. Physicians are facing very limited treatment choices in clinical MRSA infection because the widespread emergence of MRSA with multi-resistance has significantly undermined the efficacy of currently available antibiotic therapies [6]. Glycopeptide drugs, such as vancomycin and teicoplanin, are the first choice for MRSA infection treatment. However, the emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA) is challenging this treatment plan, which results in increasing of mortality. In addition, physicians also face the dilemma of lack of drug options while treating VRSA infection. The current gap between the emergence of drug-resistant *Staphylococcus aureus* and the development of new antibiotics is an urgent and critical issue, indicating the need for immediately development of novel antibiotics against MRSA [7].

Actinomycetes, as the most important clinical antibiotic-producing bacteria, have been considered as the most important resource for new antibiotic discovery because they can produce thousands of new natural products with different biological activities, especially antibacterial potential [8, 9]. To date, numerous actinomycete-derived natural products with antibacterial potentials have been reported. However, traditional antibiotics, such as cephalosporins, macrolides, and the glycopeptide antibiotic-vancomycin, showed no effect against superbugs [10, 11]. This situation prompted the search for novel anti-infectious active compounds against MRSA from typical habitat microbial resources [12, 13].

Through the data-mining of novel anti-MRSA active natural products in soil-derived actinomycetes from typical habitats, three novel chloroanthrabenzoxocinone family antibiotics including Zunyimycins A, B, and C (Figure 1) with antibacterial potentials against MRSA were identified and isolated from *Streptomyces* sp. FJS31-2. As a novel antibiotic, the application of Zunyimycin is still under the preclinical investigation stage. However, the laboratory studies of anti-MRSA and anti-tumor activities for this antibiotic showed that this antibiotic might have good clinical application potentials [14, 15].

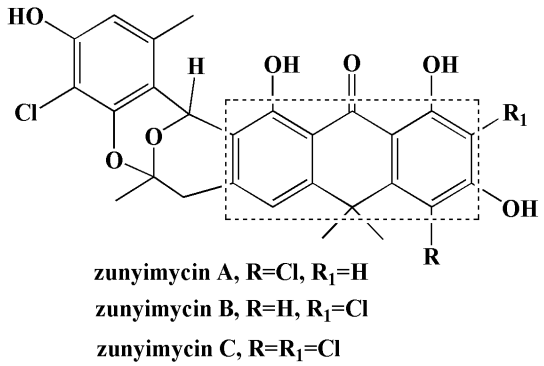

Figure 1. Chemical structures of Zunyimycins A, B, and C.

This study focused on the comprehensive investigation of anti-MRSA activities of Zunyimycin C both *in vitro* and *in vivo,* including minimum bactericidal concentration (MBC), minimum inhibitory concentration (MIC), drug half-life, time-kill studies, killing curves, and survival rate. The results of this study can be used for future anti-MRSA drug development.

Materials and methods

Determination of Zunyimycin C antibacterial properties *in vitro*

(1) MIC and MBC:

The MIC and MBC of Zunyimycin C in culture broth were determined by using the techniques of Konaté [16]. Zunyimycin C was extracted from *Streptomyces* sp. FJS31-2 under the procedures

described previously [15]. *Streptomyces* sp. FJS31-2 was isolated from Fanjing Mountain soil of Guizhou province, China by our research team and was deposited in China General Microbiological Culture Collection Center (CGMCC) under the accession number of CGMCC 4.7321. The stock solution of Zunyimycin C was prepared with 10% dimethylsulfoxide (DMSO) in water at a final concentration of 10 μg/mL followed by a serial two-fold dilution and sterilized by filtration through a 0.22 μm sterilizing Millipore express filter. The sterile discs (6 mm) were impregnated with 10 μL of sterile antibiotics. Methicillin (Sigma-Aldrich, Merck Millipore, Darmstadt, Germany) was employed as negative controls, while Vancomycin (Sigma-Aldrich, St. Louis, MO, USA) was used as positive control (10 μg/disc). In brief, MRSA clinical isolates (Clinical Microbiology Laboratory of Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou, China) bacterial suspension (10^6 colony forming unite (CFU)/mL) was coated on the base layer of Luria–Bertani (LB) agar and used to inoculate each Petri dish. 10 μL of different drug dilutions were placed on the agar. The dishes were then incubated at 37°C for 24 h. The diameters of inhibition zones were measured in millimeters (mm). Antibiotics inducing inhibition zone ≥ 3 mm around the disc were considered antibacterial. All tests were performed in triplicate, and bacterial activity was expressed as the mean of inhibition diameters (mm) produced. MIC was defined as the lowest concentration of antibiotics (Zunyimycin C or Vancomycin) at which no visible growth of microorganisms incubated under 37°C for 24 h was observed with 10-fold serially dilutions. MBC was considered the lowest concentration of chemical that produced no growth of subcultures and determined by subculturing 100 μL sample from each tube from the MIC assay onto fresh drug-free LB agar plates and incubation at 37°C for 24 h. Based on the concentration ratio of MBC/MIC (MBC/MIC range of 4 to 16), the drug was defined as bacteriostatic and the effect was considered bactericidal if the value of MBC/MIC is less than 2 [17].

(2) The time-kill curve:

The time-kill measurement was performed by the actual reduction in viable counts of MRSA clinical isolates at 12 h. After incubation at 37°C for 24 h, freshly cultured MRSA was resuspended in 10 mL of LB broth with the final concentration of 10^6 CFU/mL. The bacterial cultures were then incubated at 37°C for 0, 1, 4, 8, and 12 h, respectively. After centrifugation at 10,000 x g for 10 min at 4°C, the supernatants were removed, and the bacterial pellets were resuspended in 10 mL of LB broth at a final concentration of 10^6 CFU/mL. 100 μL of each bacterial suspension was applied to coat on the agar plates that contained 2×MBC, MBC, and MIC antibiotics followed by incubation at 37°C for 24 h. The viability of MRSA clinical isolates was determined by the presence of colonies on the LB agar plates. The bactericidal effect was defined as a 3 Log decrease in CFU/mL.

Determination of Zunyimycin C antibacterial properties *in vivo*

(1) MRSA infected animal model:

Specific pathogen-free (SPF) rats were obtained from Tengxin Biological Technology Co., Ltd (Chongqing, China) with animal production license [SCXK(Army) 2012-0011]. The animal experiment was approved by the ethics committee of the Third Affiliated Hospital of Zunyi Medical University (No.2018-012). A total of 60 male SPF rats were randomly divided into four groups including vehicle group (negative control, with DMSO only), Vancomycin group (positive control, 30 mg/kg), Zunyimycin C low-dose group (5 mg/kg), and Zunyimycin C high-dose group (10 mg/kg). Each experimental group had three replicates with five rats in each replicate. After 1 h of intraperitoneally injection of MRSA (1.0×10^9 CFU), Zunyimycin C low- and high-dose and Vancomycin were administrated intraperitoneally to each animal every 12 h for 4 times. During the experiment, 200 μL of blood was drawn from the rat tail vein every 12 h to determine the number of bacteria in the blood.

PCR amplification followed by DNA sequencing were employed to confirm that the pathogen presenting in infected animal blood was the

injected MRSA. MRSA mecA gene specific primers (mecA-RE: CTGGAACTTGTTGAGCAGAG and mecA-FO: TGGCTATCGTGTCACAATCG) were applied following the standard methods described by Sambrook [20] or the manufacturer's instructions of each kit. Genomic DNAs of standard strain *Staphylococcus aureus* (ATCC: 29213) (CGMCC) and MRSA clinical isolates 170402019 and 08301 (Clinical Microbiology Laboratory of Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou, China) were extracted directly from the liquid bacterial cultures by using DNA extraction kit (Takara Biotechnology, Dalian, Liaoning, China). PCR reaction was performed by using PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). In brief, 20 µg of genomic DNA from each bacterial strain was used for PCR reaction under the following program: 95°C for 3 min, followed by 30 cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s. The PCR product was then sequenced by Life Technologies (Shanghai, China), and the results were blasted with online tools of BLASTN (https://blast.ncbi.nlm.nih.gov)

(2) Drug killing curve and animal survival rate:

The killing curve determination was carried out by subculturing 20 µL of fresh drawn blood from each rat onto the LB agar plates and incubating at 37°C for 24 h. Killing curves were calculated based on the total number of bacterial colonies on the plates. Survival rates and therapeutic efficacy were calculated after 96 h according to the numbers of surviving animal in each group.

(3) Drug half-life:

The animal plasma half-life of Zunyimycin C was analyzed by using liquid–liquid extraction technique described by Akhlaq [18] with slight modification. After Zunyimycin C (40 mg/kg) was injected for three times with 30 min interval, 200 µL of animal plasma was drawn from the tail vein at 0, 0.5, 1, 2, 4, 6, 8, 10, and 12 h. Equal volume of trichloromethane was added to the plasma and vortexed for 30 min. After centrifugation at 12,000 rpm for 10 min at 4°C, the upper layer of the solvent was transferred to a sterile vial. Then, 200 µL of ethyl acetate was added to the remaining aqueous phase, and mixed by vortex for 30 min. After centrifugation at 12,000 rpm for 10 min at 4°C, the upper layer of the mixed liquid was transferred to a sterile vial. The drug-containing trichloromethane and ethyl acetate in the upper layers were mixed and vaporized completely followed by dissolving in 100 µL of methanol. The Zunyimycin C samples were spotted by using glass spotters onto silica gel (UV 254) pre-coated thin layer chromatography (TLC) (GE Healthcare, Tokyo, Japan) plates. The TLC plates were placed in a solvent tank containing 15 mL mixture of trichloromethane/methanol (15:1) and run for several seconds. The plates were then removed from the tank, dried, and viewed under UV light. The Zunyimycin C concentration in rat plasma was calculated in accordance with the standard curve. The pharmacokinetic constraint $t_{½}$ was calculated from the plasma concentration time profile of the drug.

Statistical analysis

The *in vivo* and *in vitro* experiments were repeated independently three times. All data were statistically analyzed by using SPSS 19.0 (IBM, Ammon, New York, USA). The data were expressed as mean ± SD, and t-test was used for the comparison between controls and Zunyimycin C. $P \leq 0.05$ indicated a statistically significant difference between the two groups at a specific concentration.

Results and discussion

In vitro* antibacterial activity of Zunyimycin C on human-isolated *S. aureus

The i*n vitro* effects of Zunyimycin C on methicillin-susceptible *Staphylococcus aureus* (MSSA), MRSA, and VRSA were shown in Table 1. The chemically inhibited growth of bacteria was observed at 0.25, 1, and 4 mg/mL, while the MBC values were 2, 6, 16 µg/mL, and the MBC/MIC ratios were 1.5, 2, 64 for MSSA, MRSA, and VRSA, respectively. The positive control, Vancomycin, showed the same effects as Zunyimycin C in MIC, MBC, and the MBC/MIC values for MSSA, MRSA, and VRSA.

Table 1. Bacteriostatic (-) and bactericidal (+) effects of Zunyimycin C.

Strains	MIC (μg/mL)		MBC (μg/mL)		MBC/ MIC		Effect	
	Zun	Van	Zun	Van	Zun	Van	Zun	Van
MSSA standard strain (ATCC: 29213)	1±0.0	1±0.0	2±0.0	1±0.0	2	1	+	+
MRSA clinical isolates (08301)	4±0.0	2±0.0	6±0.0	4±0.0	1.5	2	+	+
MRSA clinical isolates (161222330)	8±0.0	2±0.0	16±0.0	4±0.0	2	2	+	+
MRSA clinical isolates (161231380)	4±0.0	2±0.0	6±0.0	4±0.0	1.5	2	+	+
MRSA clinical isolates (170108317)	4±0.0	2±0.0	6±0.0	4±0.0	1.5	2	+	+
MRSA clinical isolates (161231350)	4±0.0	2±0.0	6±0.0	4±0.0	1.5	2	+	+
VRSA clinical isolates (170402019)	0.25±0.0	1±0.0	16±0.0	48±0.0	64	48	-	-

Note: The results were the means of number of the colonies ± standard deviations. **+**: bactericidal effect; **-**: bacteriostatic effect; Zun: Zunyimycin C; Van: Vancomycin.

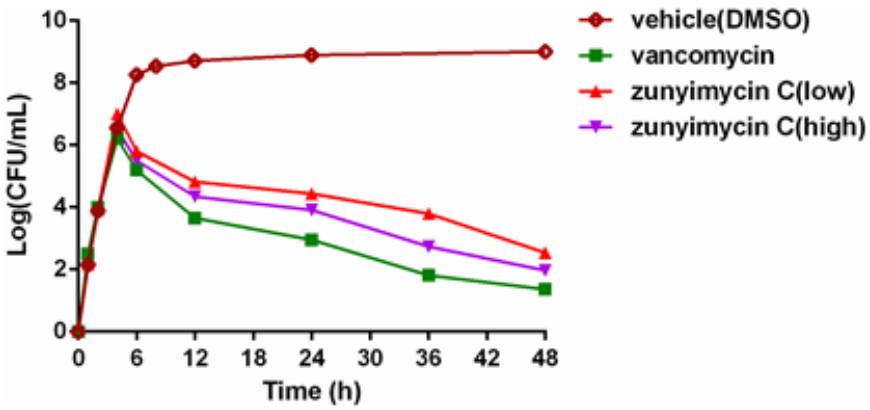

Figure 2. Time–kill curves of Zunyimycin C and Vancomycin. Vancomycin: 30 μg/mL; Zunyimycin C (low): 5μg/mL; Zunyimycin C (high): 10 μg/mL.

Time-kill effect of Zunyimycin C

The change patterns of MRSA growth and killing by Zunyimycin C and Vancomycin at different concentrations were shown in Figure 2. In general, the growth curves for MRSA without antibiotic treatment (DMSO and blank) reached a plateau after 6 h of inoculation, while the three antibiotic-treated groups showed sharp decreases in bacterial population comparing to that of the control groups after a short lag phase. All antibiotic-treated groups exhibited reduction to the lowest CFU counts after incubation for 48 h. The analysis of each individual antibiotic assay result indicated that Vancomycin had the best effect against MRSA after incubation for 48 h, with time-kill kinetic tests showing that Vancomycin was more effective than Zunyimycin C (at low and high concentrations) against MRSA. No significant difference was observed among the antibiotic-treated groups (P>0.05). However, a significant difference (P<0.01) was recorded between the antibiotic-treated and control groups, which indicated that Zunyimycin C shared the same kill rate as Vancomycin against MRSA.

Half-life and *in vivo* bactericidal activity of Zunyimycin C

The plasma concentration of Zunyimycin C was calculated in accordance with the standard curve, and the half-life (t½) was then determined. The results showed that the blood concentration of Zunyimycin C reached the peak level at 2 h with the t½ of 6.3 h (Figure 3).

Given that Vancomycin and Teicoplanin remain the first choices of MRSA treatment, Vancomycin was employed as the positive control in this

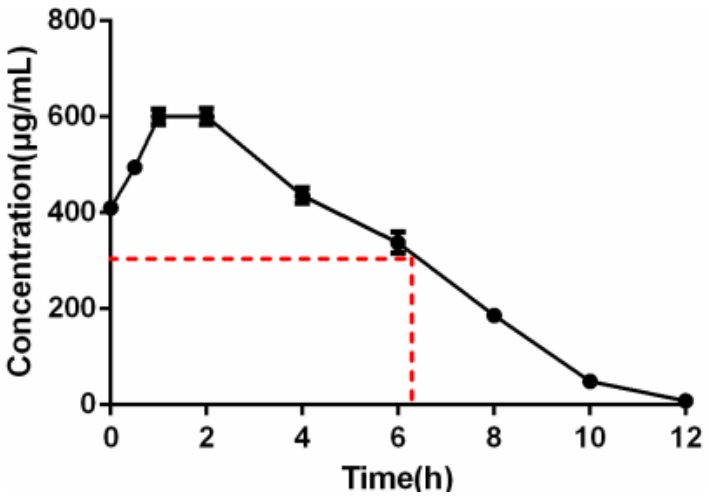

Figure 3. The animal plasma half-life of Zunyimycin C.

Table 2. *in vivo* bactericidal activity of Zunyimycin C.

Group	Bacterial number (CFU/mL)			
	12 h	24 h	36 h	48 h
Vehicle (DMSO)	5.51×10^{8}	2.78×10^{9}	6.31×10^{9}	9.41×10^{9}
Vancomycin (30 mg/kg)	3.31×10^{6}	$9{,}24\times10^{5}$	3.06×10^{4}	5.36×10^{4}
Zunyimycin C (5 mg/kg)	2.17×10^{8}	2.27×10^{7}	6.49×10^{6}	3.09×10^{5}
Zunyimycin C (10 mg/kg)	1.06×10^{8}	8.14×10^{6}	5.35×10^{5}	5.34×10^{4}

study. The numbers of blood bacteria were counted in the interval of every 12 h. The results demonstrated that the lowest bacterial CFU (< 1.0×10^{4} CFU) was achieved in antibiotic-treated animal groups after 48 h incubation comparing to that of 0.994×10^{10} CFU in vehicle groups (Table 2).

There was no significant difference observed between the groups of Vancomycin and Zunyimycin C treated MRSA infected animals. However, a significant difference ($P < 0.01$) was observed between the vehicle group and the antibiotic-treated groups, which indicated that Zunyimycin C demonstrated the similar anti-bacterial effect against MRSA as Vancomycin. The therapeutic effect of Zunyimycin C suggested that it might have the same therapeutic effect on MRSA infection as Vancomycin, and, therefore, Zunyimycin C might have a potential clinical application in the treatment of MRSA infection.

After infected animals with MRSA for 6 h, animals in the vehicle groups became weak and faint with two animals died. All animals in vehicle groups died after 48 h of infection without treatment, while there was no animal death observed in the Vancomycin treated groups. In the groups treated with low-dose Zunyimycin C, two animals died after 12 h of infection, followed by two additional deaths within 24 h. In the group treated with high-dose Zunyimycin C, only two animals died within 24 h (Figure 4).

The bacteriostatic and bactericidal effects of Zunyimycin C were calculated according to the ratio of MBC/MIC. For MSSA and MRSA, the effect of Zunyimycin C was considered bactericidal while, for VRSA, the effect of the chemical was bacteriostatic. The antibacterial activity of Zunyimycin C in this study was relatively low, and the effective concentrations at *in vitro* experiments were hardly reached at *in vivo* animal experiments, which were probably

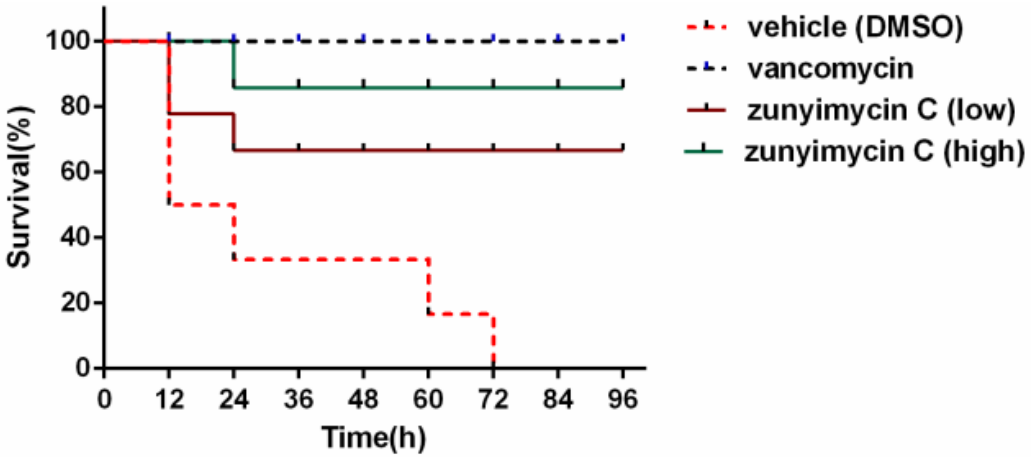

Figure 4. Rats survival rate until to 96 h.

due to the poor responses of antibiotic treatment to *Staphylococcus aureus*. In the groups treated with low- and high-dose Zunyimycin C, four and two animals died within 24 h of infection, respectively. However, there was no animal death in the Vancomycin-treated groups, which suggested that the therapeutic effect of Zunyimycin C was insufficient under the concentrations of 5 or 10 mg/kg bodyweight, and the drug dosage must be further optimized to improve the therapeutic effect of Zunyimycin C.

Conclusions

In summary, the *in vitro* and *in vivo* bactericidal activity, time-kill assay, and half-life of the novel chloroanthrabenzoxocinone antibiotic, Zunyimycin C, against MRSA in rats were determined. The results indicated that Zunyimycin C might present a good therapeutical effect in the treatment of MRSA infection and offer a good perspective for clinical use.

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