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Full paper

Potentiation activity of multiple antibacterial agents by Salvianolate from the Chinese medicine Danshen against methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Salvianolate (SAL) is a prescribed medicine from the Chinese herb Danshen (*Salvia miltiorrhiza* Bunge). It has been widely used in treatment of coronary and other diseases with significant effects. The *in vitro* antimicrobial activities of SAL against infectious pathogens were assayed and its combined effects on 10 clinical isolates of SCCmec III type methicillin-resistant *Staphylococcus aureus* (MRSA) with ten antibiotics were evaluated. Susceptibility to each agent alone was tested using a broth microdilution method, and the chequerboard and time-kill experiments were used for the combined activities. The results showed MIC was 128–256 mg/L for SAL used alone against MRSA. Significant synergies were observed for SAL/Ampicillin (Fosfomycin, Erythromycin, Piperacillin-tazobactam or Clindamycin) combination against over half of the isolates, with their MICs reduced by times of dilution (TOD) to 4–32 (FICIs 0.375–0.5), respectively. SAL/AMP combination showed the best combined effect of synergy on bacteriostatic and bactericidal activities, while SAL/AMK combination reversed the resistance of MRSA to AMK. The results demonstrated that SAL enhanced widely the *in vitro* anti-MRSA efficacy of the ten antibacterial agents, which had potential for combinatory therapy of patients infected with MRSA and warrants further investigations.

1. Introduction

Since the clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in 1961, its current incidence is still high worldwide. MRSA could be amount to over 50% strains of all *S. aureus* in clinical infected patients, leading to increased morbidity and mortality, length of stay and healthcare burden (1, 2). MRSA is resistant not only to β-lactams, but also to macrolides, clindamycin, quinolones and other antibacterial agents. MRSA has become one of the important pathogens in nosocomial patients, mostly causes infections of respiratory tract, burns, surgical site and bloodstream. The elderly patients are one group of the main risk individuals (3). MRSA infections often become the fatal pathogeny among patients with multiple organ malfunctions or tumors. Although vancomycin is still one of the most effective anti-MRSA agent, it has a lot of adverse effects such as hypotension, phlebitis, nephrotoxicity, ototoxicity, hypersensitivity reactions, red man syndrome, neutropenia, chills, fever and interstitial nephritis (4). The emergence of decreased vancomycin susceptibility and even resistant strains in MRSA has exhibited a significant clinical problem as well (5). Therefore, new treatment strategies to cope with MRSA infections and to alleviate selection pressure of MRSA on the current anti-MRSA agents are inevitably needed.

Salvianolate (SAL) is a prescribed medicine derived from Danshen's principal active constituent Salvianolic acid B (6). It contains mainly Magnesium salvianolic acid B (≥85%, Fig. 1) and other phenolic acids (≤15%) (7, 8). Danshen is originated from the well-known Chinese herb *Salvia miltiorrhiza* Bunge (Labiatae) which has the function of promoting blood circulation (9, 10). Both SAL and Danshen has been widely used in the treatment of cardiovascular and cerebrovascular diseases (6, 10). A recent report demonstrated that Salvianolic acid B could protect against acetaminophen hepatotoxicity (11). However, no detailed evaluation of

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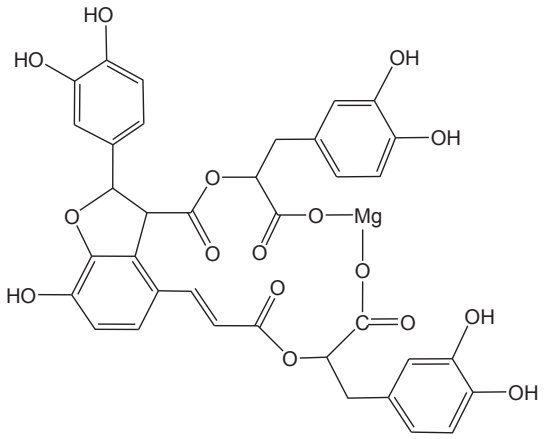

Fig. 1. The schematic diagram of magnesium salvianolic acid B.

SAL on methicillin-resistant *S. aureus* (MRSA) has been reported. Therefore, we herein present the report which deals with *in vitro* antibacterial and synergistic effects of SAL on conventional antibacterial agents against clinical isolates of MRSA.

2. Materials and methods

2.1. Bacterial strains and culture media

MRSA strains (ten isolates with SCCmec III genotype) were isolated and identified from the clinical sample of patients in Kunming General Hospital as previous reports (12–14). ATCC 25923 was used as the control strain. Standard Mueller–Hinton agar and broth (MHA and MHB, Tianhe Microbial Agents Co., Hangzhou, China) were used as bacterial culture media.

2.2. Antibacterial agents

SAL and ten antibacterial agents represented different conventional types were purchased from the manufacturers, *i.e.* Salvianolate (SAL (contains ≥85% magnesium salvianolic acid B), Shanghai Greenvalley Pharmaceutical Co., Ltd, Shanghai, China), and Ampicillin (AMP, North China Pharmaceutical Co., Ltd, Shijiazhuang, China), Ceftazidime (CAZ, Baiyunshan Tianxin Pharmaceutical Co., Ltd, Guangzhou, China), Cefazolin (CFZ, Harbin Pharmaceutical Co., Ltd, Harbin, China), Clindamycin (CLI, Lifeshine Pharmaceutical Co., Ltd, Beijing, China), Cefoperazone-sulbactam, Erythromycin, Fosfomycin and Piperacillin-tazobactam (CPS, ERY, FOS and PTZ, Hunan Central South Kelun Pharmaceutical Co., Ltd, Changsha, China). Amikacin (AMK, Jiangsu Wuzhong Pharmaceutical Group Co., Ltd.); Levofloxacin (LEV, Yangzhijiang Pharmaceutical Co., Ltd, Taizhou, China). Vancomycin (VAN) (Eli Lilly Japan K. K., Seishin Laboratories) was used as the positive control agent. Cefoxitin disks were purchased from Tiantan biological products Co., Ltd (Beijing, China).

2.3. Susceptibility of SAL and antibacterial agents alone against MRSA

Susceptibility of each drug used alone against MRSA was determined by standardized broth microdilution techniques with inoculums of 5×10^5 CFU/mL according to CLSI guidelines and incubated at 35 °C for 24 h (15, 16). The testing solution was prepared by dissolving SAL or the agents in sterile saline. Serial dilutions were made with the corresponding culture broth. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) representing a drug's antibacterial activity against MRSA isolates. All experiments were performed in triplicate.

2.4. Synergy testing of SAL in combination with the antibacterial agents against MRSA

Potential anti-MRSA synergy of SAL in combination with the ten antibacterial agents against MRSA was measured by fractional inhibitory concentration (FIC) indices (FICI) with chequerboard method and by time-killing curves as previously reported (17,18). The FIC of the combination was calculated through dividing the MIC of the SAL/antibacterial agent combined by the MIC of SAL or the antibacterial agent alone, and the FICI was obtained by adding the FIC of SAL and that of an antibacterial agent. The FICI results were interpreted as follows: FICI ≤ 0.5, synergy; 0.5 < FICI ≤ 1, additivity; and 1 < FICI ≤ 2, indifference (or no effect) and FICI >2, antagonism (17). In the killing curves, synergy was defined as ≥2 $\log_{10}$ CFU/mL increase in killing at 24 h with the combination in comparison with the killing by the most active single drug (△Log_{10} CFU/mL at 24 h, *i.e.* △LC_{24}). Additivity was defined as a 1–2 $\log_{10}$ CFU/mL increase in kill with the combination in comparison with the most active single agent. Indifference was defined as ±1 $\log_{10}$ CFU/mL killing or growth. Combinations that resulted in >1 $\log_{10}$ CFU/mL bacterial growth in comparison with the least active single agent were considered to represent antagonism (18). All experiments were performed in triplicate.

3. Results

3.1. Effects of SAL used alone against MRSA

The MICs (mg/L) of Salvianolate (SAL, Fig. 1) and the ten clinical antibacterial agents AMP, AMK, CAZ, CFZ, CLI, CPS, ERY, FOS, LEV and PTZ used alone against 10 clinical MRSA isolates of SCCmec III type are shown in Table 1. SAL showed MIC_{50} and MIC_{90} of both 256 mg/L (n = 10). Meanwhile, SAL used alone showed similar activity against standard Gram-positive *S. aureus* (methicillin-susceptible *S. aureus*, MSSA) by MIC/MBC at 128/256 mg/L.

3.2. Anti-MRSA potentiation of the agents by SAL

The combined anti-MRSA MICs of SAL with the ten antibacterial agents are shown in Table 2. The potentiation effects could be

Table 1
MICs of SAL and the ten antibacterial agents against MRSA strains (mg/L, n = 10).

Agent[a]	Range	MIC_{50}	MIC_{90}	MIC interpretive criteria[b]		
				S	I	R
AMP	16–128	64	64	≤0.25	–	≥0.5
CAZ	256–512	512	512	≤8	16	≥32
CFZ	64–512	256	512	≤8	16	≥32
CLI	128–1024	256	512	≤0.5	1–2	≥4
CPS	256–1024	256	1024	–	–	–
PTZ	32–256	128	256	≤8/4	–	≥16/4
ERY	256–4096	2048	2048	≤0.5	1–4	≥8
AMK	16–32	32	32	≤16	32	≥64
FOS	16–256	64	256	–	–	–
LEV	7.8–125	31.25	62.5	≤1	2	≥4
SAL	128–256	256	256	–	–	–
VAN	1	1	1	≤4	8–16	≥32

[a] SAL: Salvianolate; AMP: Ampicillin; CAZ: Ceftazidime; CFZ: Cefazolin; CPS: Cefoperazone-sulbactam; PTZ: Piperacillin-tazobactam; AMK: Amikacin; CLI: Clindamycin; ERY: Erythromycin; FOS: Fosfomycin; LEV: Levofloxacin; VAN: Vancomycin.
[b] MIC Interpretive Criteria (2012 CLSI M100-S22); "S": susceptible; "I": intermediate; "R": resistant.