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Short Communication

Isovalerylshikonin, a new resistance-modifying agent from *Arnebia euchroma*, supresses antimicrobial resistance of drug-resistant *Staphylococcus aureus*

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ABSTRACT

Antimicrobial resistance is the greatest threat to the treatment of bacterial infectious diseases. The development of resistance-modifying agents (RMAs) represents a promising strategy to mitigate the spread of bacterial antimicrobial resistance. In this study, a natural product, isovalerylshikonin (IVS), was isolated from *Arnebia euchroma*, a traditional Chinese medicine herb, that exhibited marginal antibacterial activity against drug-resistant *Staphylococcus aureus* RN4220, with a minimum inhibitory concentration (MIC) of 16 mg/L. In addition, a synergistic effect between IVS and streptomycin (STM) was detected by the microdilution antimicrobial chequerboard assay, with a reduction in the MIC of STM by up to 16-fold against strain RN4220. A bacterial ethidium bromide efflux assay and reverse transcription PCR were performed to investigate the synergistic mechanism. IVS significantly inhibited bacterial efflux and expression of *msrA* mRNA in vitro. A murine peritonitis/sepsis model was employed to test the in vivo synergistic activity of IVS and STM. IVS synergistically decreased bacterial counts with STM in peritoneal, spleen and liver tissue and increased mouse survival with STM in 7 days. The acute toxicity of IVS was tested and the 50% lethal dose (LD_{50}) of IVS with a single exposure was 2.584 g/kg in mice. Overall, IVS, a low-toxicity RMA, exhibited synergistic antibacterial activities in vitro and in vivo against drug-resistant *S. aureus*. The effects were mediated by suppression of *msrA* mRNA expression and reduced bacterial efflux. In addition, these data support that IVS is a potential RMA against microbial resistance caused by the MsrA efflux pump.

1. Introduction

Extensive and unrestricted use of antibiotics has imposed a selective pressure on bacteria, resulting in the development of antimicrobial resistance [1]. Antimicrobial resistance is considered the greatest threat to the treatment of bacterial diseases by the World Health Organization (WHO) [2]. Among these problematic bacteria, drug-resistant *Staphylococcus aureus* are responsible for a large number of hospital-acquired infections in many countries [3,4]. The major mechanisms of bacterial resistance to antibacterial drugs include drug inactivation, target modification, and alteration of accessibility to the target through drug efflux and decreased uptake. Drug efflux is an important and widely investigated mechanism of antimicrobial resistance [2,5].

An important potential strategy to overcome the resistance problem is the discovery and development of new drugs that are capable of partly or completely inhibiting bacterial resistance mechanisms, which have been termed resistance-modifying agents (RMAs) [2,6]. Plants are an important potential source of RMAs, providing hundreds of metabolites with differing structures.

In China, the root of *Arnebia euchroma* (Royle) Johnst. is a traditional medicinal herb that has been used since ancient times to treat skin eruption, small pox, jaundice, burns, eczema and constipation [7]. Pharmacological studies have demonstrated that extracts of *A. euchroma*, specifically naphthoquinones, have antibacterial activity [8]. In this study, a naphthoquinone, isovalerylshikonin (IVS), was isolated from *A. euchroma* and the in vitro and in vivo synergistic effects with streptomycin (STM) against drug-resistant *S. aureus* as well as the mechanisms involved in the action and acute toxicity of this compound were investigated.

2. Materials and methods

2.1. Chemicals and materials

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Biosharp (Hefei, China). STM and

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vancomycin were purchased from Dalian Meilun Biological Technology Co., Ltd. (Dalian, China). Water was purified using a Milli-Q water purification system (Millipore, Milford, MA). IVS was isolated from *A. euchroma* by chromatographic methods (Supplementary material).

Staphylococcus aureus RN4220 overexpresses the *msrA* gene encoding the MsrA multidrug efflux pump. This strain was provided by Simon Gibbons (UCL School of Pharmacy, London, UK). Male BALB/c mice (6–8 weeks old) were obtained from Lingchang Biotechnology Co., Ltd. (Shanghai, China). Pathogen-free BALB/c mice were bred in the animal facility of the School of Pharmacy at Fudan University (Shanghai, China) and were supplied with food and water ad libitum.

2.2. Antibacterial minimum inhibitory concentration (MIC) assay and synergy testing

MICs were determined following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [9]. *Staphylococcus aureus* RN4220 was used in a Mueller–Hinton broth microdilution antimicrobial chequerboard assay [10] to examine the presence of a favourable interaction, as described in the Supplementary material.

2.3. Growth kinetics, ethidium bromide efflux assay, total RNA extraction and real-time PCR

Growth kinetics of strain RN4220 were determined as previously described [11] with some modifications. IVS was evaluated in a bacterial efflux assay as described previously [12]. Total RNA was isolated from bacteria using TRIzol reagent (Life Technologies, Rockville, MD) according to the manufacturer's instructions. The reverse transcription step was carried out using a RevertAid^{TM} First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) to synthesise cDNA. PCR analysis was performed using an ABI 7300 real-time fluorescent quantitative PCR system (Applied Biosystems, Foster City, CA). Detailed methods are provided in the Supplementary material.

2.4. In vivo infection and acute toxicity studies

In vivo infection and acute toxicity studies were performed according to previously published methods, with some modifications [13,14] (Supplementary material).

2.5. Statistical analysis

Data are shown as the mean ± standard deviation of three separate experiments. Significant differences were established by one-way analysis of variance (ANOVA) using PASW Statistics v.18 (SPSS Inc., Chicago, IL). The Kaplan–Meier method was used to compare differences in mortality rates between groups. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Antibacterial minimum inhibitory concentration assay and synergistic effects

IVS was isolated from *A. euchroma* with 98.7% purity (Supplementary Fig. S1) and showed marginal antibacterial activity against the drug-resistant test strain *S. aureus* RN4220 with an MIC of 16 mg/L (Table 1; Supplementary Fig. S2). As a positive control, the antibiotic vancomycin inhibited the growth of strain RN4220 with an MIC of 2 mg/L.

Strain RN4220 was resistant to the antibiotic STM (MIC = 256 mg/L). However, IVS exhibited a synergistic effect with STM against strain RN4220, with a fractional inhibitory concentration index (FICI) of 0.19, reducing the MIC of STM.

Table 1
Minimum inhibitory concentrations (MICs), fractional inhibition concentration (FIC) and fractional inhibition concentration index (FICI) of isovalerylshikonin (IVS) from *Arnebia euchroma* and streptomycin against drug-resistant *Staphylococcus aureus* RN4220.

Agent	MIC (mg/L)		FIC	FICI[a]
	Alone	Combination		
Isovalerylshikonin	16	2	0.125	0.19
Streptomycin	256	16	0.063	
Vancomycin	2	–	–	–

[a] A synergistic effect was defined when the FICI was ≤0.5.

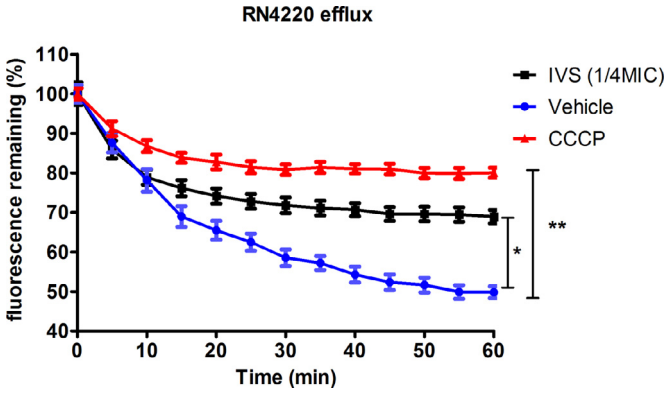

Fig. 1. Inhibitory effect of isovalerylshikonin (IVS) on bacterial efflux. Bacteria were treated with IVS (4 mg/L, 0.25 × MIC), the known efflux inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (100 μmol/L) or vehicle. Results are the mean ± standard deviation of three independent experiments. * $P < 0.05$; ** $P < 0.01$. MIC, minimum inhibitory concentration.

3.2. Growth kinetics, ethidium bromide efflux assay and effect of isovalerylshikonin on induction of msrA at the transcriptional level

As shown in Supplementary Fig. S3, the growth curves of bacteria treated with the vehicle or with 0.25 × MIC of IVS almost overlapped. The growth of strain RN4220 treated with 0.25 × MIC of STM was almost completely halted within 12 h and grew quickly thereafter. Compared with STM alone, strain RN4220 treated with STM (0.25 × MIC) combined with IVS (0.25 × MIC) grew at a slower rate. Thus, a synergistic effect between STM and IVS against strain RN4220 was observed.

Ethidium bromide was quickly depleted to <50% by vehicle-treated bacteria from 1–60 min, followed by a slower reduction, and the final level of fluorescence was ca. 30% (Fig. 1). IVS inhibited bacterial efflux against strain RN4220 ($P < 0.05$), although this inhibition was weaker than in the positive control carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).

Expression of *msrA* mRNA in bacteria treated with vehicle was set as the reference level of induction (1-fold). Strain RN4220 was incubated with STM (32 mg/L, 0.0125 × MIC) resulting in substantial expression of *msrA* mRNA, which was decreased significantly when the bacteria were incubated with STM and IVS ($P < 0.01$). However, the level of mRNA expression in bacteria treated with STM combined with IVS was higher than in those treated with the vehicle (Supplementary Fig. S4).

3.3. In vivo infection and acute toxicity studies

The 7-day survival curve of mice infected with strain RN4220 was recorded. Vehicle group mice died within 24 h of infection, as did those treated with IVS (40 mg/kg) alone or with STM

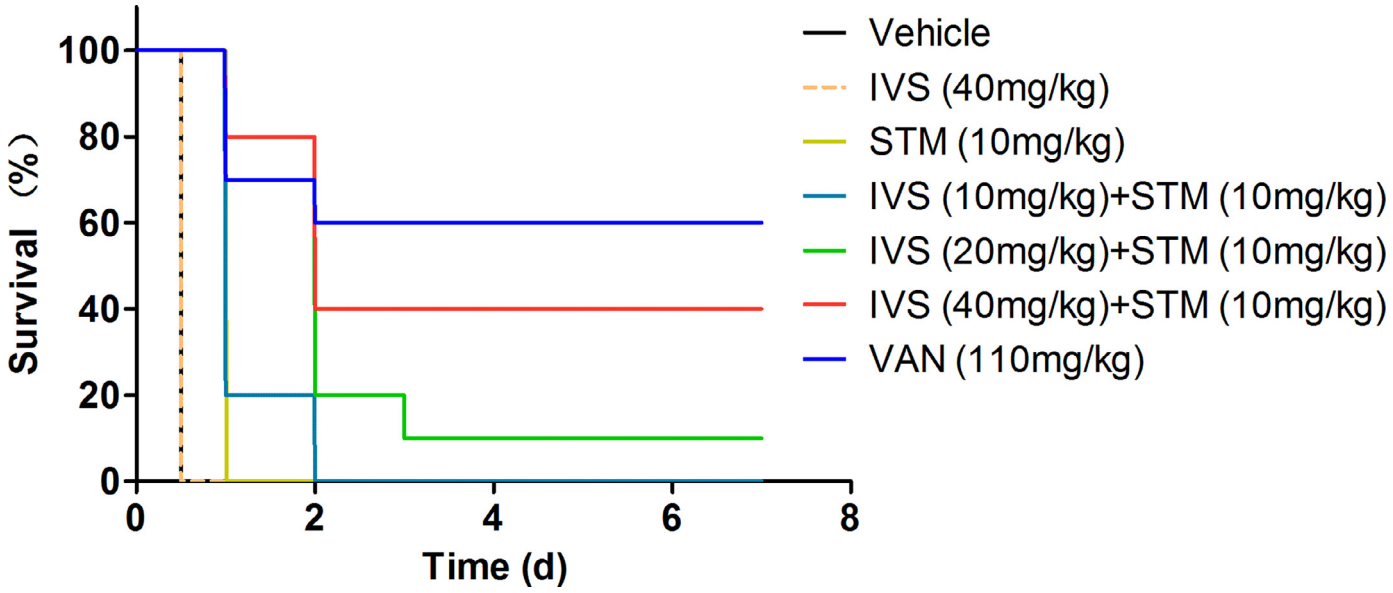

Fig. 2. Synergistic protective effect on survival of the combination of isovalerylshikonin (IVS) and streptomycin (STM) in mice infected with drug-resistant *Staphylococcus aureus* RN4220. Mice were administered phosphate-buffered saline (vehicle control, p.o.), IVS (40 mg/kg, p.o.), STM (10 mg/kg, i.m.), vancomycin (VAN) (110 mg/kg, i.h.), or IVS (10, 20 or 40 mg/kg, p.o.) in combination with STM (10 mg/kg, i.m.) every 12 h during the first 24 h. Mouse survival was monitored at 12-h intervals for 7 days. The result is representative of two independent experiments. p.o., oral; i.m., intramuscular; i.h., hypodermic injection.

(10 mg/kg) alone (Fig. 2). Most mice administered a combination of STM (10 mg/kg) and IVS (10 mg/kg) died within 24 h of infection, with a survival percentage of 20%, and the rest died within the following 24 h. Eight of the combination group mice treated with the combination of STM (10 mg/kg) and IVS (20 mg/kg) died within 2 days (48 h) of being infected by strain RN4220, such that the final 7-day survival percentage of this group was 10%. Combination group mice administered STM (10 mg/kg) and a high dose of IVS (40 mg/kg) died slowly within 24 h of infection, and six died within 3 days of infection, with a final 7-day survival percentage of 40%. Mice in the positive control group injected with vancomycin (110 mg/kg) had a 60% survival rate at 7 days of infection with strain RN4220. IVS significantly suppressed bacterial levels in infected mice (Supplementary Fig. S6), increasing the in vivo antibacterial activity of STM. The acute toxicity of IVS in mice was measured and it was found to have a 50% lethal dose (LD_{50}) of 2.584 g/kg, indicating that IVS is a low-toxicity compound (Supplementary Fig. S8).

4. Discussion and conclusion

4.1. Discussion

Expression of efflux pumps that extrude antibiotics from bacterial cells is one of the most important mechanisms of microbial resistance to antibiotics. The MsrA efflux pump is an important resistance mechanism in methicillin-resistant *S. aureus* (MRSA); in one study the prevalence of *msrA* in clinical *S. aureus* isolates was 6.9% [15]. The current study focused on the investigation of a new RMA against the MsrA efflux pump. *Staphylococcus aureus* RN4220, which has been engineered to overexpress the *msrA* gene encoding the MsrA efflux pump, was used in this study to measure the antimicrobial resistance effect of IVS as well as the synergistic effect of IVS and STM. The MsrA efflux pump confers resistance to macrolides and, in some cases, to lincosamides and type B streptogramins [16]. Strain RN4220 is resistant to STM with an MIC of 256 mg/L. Currently STM is not used much globally. However, development of RMAs represents a promising approach to recycling this antimicrobial agent as well as other well established antibiotics that are no longer in wide use [17].

Other compounds extracted from many different plants have been previously investigated for their antimicrobial activities. It has been reported that carnosic acid showed an eight-fold reduction in the MICs of antibiotics against *S. aureus* RN4220 expressing MsrA [18]. Two isopimarane diterpenes isolated from an extract of *Lycopus europaeus* showed a two-fold potentiation of antibiotics against strain *S. aureus* RN4220 possessing MsrA [19]. However, many of these data are preliminary, having been derived from in vitro antimicrobial assays or potentiation assays. Efflux studies, or any other studies to establish the mechanism of action, have not usually been performed. IVS isolated from the Chinese herb *A. euchroma* as a pure chemical entity had a synergistic effect with STM against drug-resistant *S. aureus* RN4220. Limiting the access of antimicrobials to their target through drug efflux is one of the most important and widely investigated antibacterial resistance mechanisms. The current studies indicate that IVS may exhibit its synergistic effect against drug-resistant *S. aureus* by suppressing the efflux pump of the bacteria. IVS significantly decreased the expression of *msrA* at the mRNA level as well as overexpression of MsrA induced by the antibiotic.

Investigation of efficacy and safety in animal models is important in drug development. The synergistic effect between IVS and STM against drug-resistant *S. aureus* infection was shown in vivo in a mouse model. The acute toxicity of IVS with a single exposure in mice was measured with an LD_{50} of 2.584 g/kg in present study (Supplementary material).

In this study, we focused on synergy between IVS and STM. However, IVS could have a broader application in offering synergy with other antibacterials that are affected by the MsrA efflux pump. However, first these observations need to be confirmed with other bacterial strains.

4.2. Conclusion

This study focused on the discovery of a new RMA against the MsrA efflux pump. IVS isolated from *A. euchroma* was shown to be an effective RMA against microbial resistance caused by the MsrA

efflux pump, exhibiting in vitro and in vivo synergistic activities against *S. aureus* RN4220. The effects were mediated by suppression of *msrA* expression at the mRNA level and by a reduction of bacterial efflux. In addition, IVS is a low toxicity agent with an LD_{50} of 2.584 g/kg in mice and may serve as a potential agent for therapeutic use in infections.

Funding

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Competing interests

None declared.

Ethical approval

All procedures were conducted in accordance with the university guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC), School of Pharmacy, Fudan University (Shanghai, China) [ethical approval document no. 201603-TY-MQ-01].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.08.021.

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