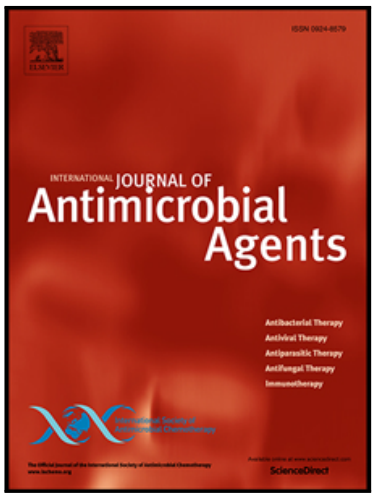

Synergism of sophoraflavanone G with norfloxacin against effluxing antibiotic-resistant Staphylococcus aureus

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Highlights

- Sophoraflavanone G (**SG**), a natural compound isolated from *S. alopecuroides*
- SG exhibits synergetic effect with norfloxacin against SA1199B in vitro and vivo
- NorA Efflux pump inhibition contributes to the synergistic effects of **SG**

Synergism of sophoraflavanone G with norfloxacin against effluxing antibiotic-resistant *Staphylococcus aureus*

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Abstract

Staphylococcus aureus including methicillin resistant variants (MRSA) is still a challeng in the clinical and community settings. The design and discovery of new compounds to deal with resistant bacteria has become one of the most important areas of antiinfective research today. The goal of

this study was to address the problem of MRSA by searching for synergistic antibacterial natural products fromtraditional Chinese herbs that are not substrates for the efflux mechanisms of MRSA strains and that overcome drug-resistance of bacteria by other, as yet, undescribed mechanisms. *In vitro*, synergistic activity was determined using the standard checkerboard method, and mechanistic studies were performed by an ethidium bromide (EtBr) efflux assay. Using *in vivo* experiments, the efficacies of different concentrations of the combinations were compared in a murine model of pyaemia. The natural product sophoraflavanone G (**SG**) showed specific synergistic antibacterial effects both *in vitro* and *in vivo* and may serve as a template for agents with antibiotic-potentiating activity for use against infections caused by *S. aureus*.

Keywords:

Sophoraflavanone G, efflux pump, synergism, *in vitro*, *in vivo*

1 Introduction

Antibiotics have played a vital role in saving the lives of infected individuals and have contributed to major gains in life expectancy over the last century [1]. However, the widespread and inappropriate use of antibiotics has resulted in the worldwide emergence of antimicrobial resistance (AMR) [2]. Currently, there are 15 drug-resistant microorganisms classified as “urgent” or “serious” by the US Center for Disease Control Prevention (CDC), which includes methicillin-resistant *Staphylococcus aureus* (MRSA) [3]. The limited therapeutic options for infections caused by MRSA and effluxing variants may lead to higher mortality rates and costs than those caused by susceptible staphylococci [4]. Researchers are becoming increasingly interested in natural products, which may not be substrates for the mechanisms of drug resistance inbacteria. Some natural products show direct antibacterial activities, whilst others demonstrate synergistic activity [5,6]. Synergy is a widely recognized pharmacological phenomenon and could be utilized to identify new pharmaceuticals [7]. This could offer an alternative strategy to overcome drug-resistance.

Bacteria have four common mechanisms to acquire antibiotic resistance. Among these, multidrug-resistance (MDR)-related effluxis important and has been reported for many organisms [8,9]. Different MDR transporters such as QacA and NorA have been known as resistance mechanisms in *S. aureus* for some time [10,11].

In our current study, an extraction of the Traditional Chinese medicinal herb *Sophora alopecuroides* L. was prepared using alcohol and analysed in broth microtitre plate checkerboard assays in combination with the antibiotics. Tracing the positive synergistic results of the extract

against growth inhibition for the resistant *S. aureus* strains, we gradually focused the synergistically-active fraction by successive chromatographic analyses.

We finally isolated the dihydroflavonoid sophoraflavanone G (**SG**), which has previously been reported to have anti-MRSA activity [12]. We studied the inhibition effects of SG on five different types of MRSA strains from different genetic background (SA1199B, XU212, RN4220, EMRSA-15 and EMRSA-16) combined with different antibiotics, but it only showed synergistic effects with norfloxacin against strain SA1199B. The *S. aureus* strain SA1199B, which exhibits norfloxacin resistance through increased expression of the NorA-efflux protein [13]. Using strain SA1199B, we evaluated the synergistic effects of **SG** combined with norfloxacin *in vitro* using the broth microdilution method [14] and *in vivo* in a murine model of pyaemia [15]. The mechanism of synergy was then studied using an efflux inhibition assay with EtBr as efflux substrate.

2 Materials and methods

2.1 Bacterial strain and antimicrobial agents

The *S. aureus* strain SA1199B used for both *in vitro* and *in vivo* studies, which overexpresses the *norA* gene encoding the NorA efflux protein, was the generous gift of Professor Glenn W. Kaatz [16]. The antibiotic norfloxacin was purchased from Dalian Meilun Biology Technology Co. Ltd. (China). Sophoraflavanone G, the NMR data for which ispresented in Supplementary Table S1 and the structure is shown in Fig.1, was isolated and purified from the extract of *Sophora alopecuroides* L., with a purity of 99% observed using an HPLC-UV detector (Supplementary Fig. S1).

2.2 *In vitro* synergy tests

Broth microdilution antimicrobial checkerboard assays were performed to examine the presence of a synergistic interaction [17]. **SG** was tested in combination with norfloxacin against SA1199B at concentration ranges of 0.125-8 and 0.125-64 mg/L, respectively. All of the treatments were tested in duplicate wells. The effect of the combination of **SG** with norfloxacin was evaluated by calculating the fractional inhibitory concentration index (FICI) according to theformula:

$$\text{FICI} = \frac{\text{MIC (antibiotic combined with compound)}}{\text{MIC (antibiotic alone)}} + \frac{\text{MIC (compound combined with antibiotic)}}{\text{MIC (compound alone)}}$$

"Synergistic" effects were defined as when the FICI was less than or equal to 0.5; an"indifference" effect was defined as when the FICI was greater than 0.5 and less than or equal to 4.0; and an "antagonistic" effect was observed when the FICI was greater than 4.0 [18].

2.3 EtBr efflux assay

Considering that the test strain SA1199B overexpresses the NorA efflux pump, we postulated that the synergistic effect results from inhibition of the NorA efflux pump, for which norfloxacin is a substrate. To determine the mechanism of the interaction between **SG** and norfloxacin, an efflux assay was performed as previously described [19] (See Supplementary Material).

2.4 *In vivo* synergy tests

An *in vivo* infection assay was performed according to a previously reported method with some modifications [20]. Six groups of ten female ICR mice (each weighing 18-22 g; Shanghai Sippr-BK Laboratory Animal Co.Ltd.) were used in the study (See Supplementary Material).

3 Results

SG showed significant synergistic effects when combined with norfloxacin against strain SA1199B, resulting in a 16-fold reduction in the MIC of norfloxacin against SA1199B, from 32 to 2 mg/L. The minimum inhibitory concentration (MIC) of SG and norfloxacin were 4 and 32 mg/L respectively. The FICI value of the combination was 0.188. The results suggest that **SG** has notable synergistic activity towards the drug-resistant strain SA1199B.

Whilst **SG** showed no inhibitory effects alone at the concentrations tested, it had a strong 'efflux pump inhibitory' effects against SA1199B. Within the first half hour of the assay, **SG** showed competitive efflux pump inhibitory effects compared with the positive control CCCP, whereas this effect was weaker compared with that observed for CCCP over the subsequent half hour (Fig. 2).

To examine the *in vivo* synergistic effects between **SG** and norfloxacin, mice were administered (i.v.) with 10^6 CFU/mL of SA1199B and monitored for 7 consecutive days. As shown in Fig. 3, the mice in the vehicle group died within 12 h after infection, whereas six mice in the group treated with **SG** (180 mg/kg) alone group died within 12 h, and all died within 24 h. In the group treated with norfloxacin (180 mg/kg) alone, a weak effect was observed on the survival rate of the infected mice, where six mice died within 7 days. The three groups of mice with the same dose of **SG** (100 mg/kg) and different doses of norfloxacin (60, 90 and 120 mg/kg; the synergism groups) had survival rates of 40, 50 and 70% respectively. These data showed thatat a dose of 100 mg/kg, **SG** synergistically increased the antibacterial activity of norfloxacin *in vivo* in a dose-dependent manner (Fig. 3).

4 Discussion

Sophoraflavanone G (**SG**), isolated and purified from the traditional Chinese medicine *Sophora alopecuroides*, showed a significant synergistic effect together with the classical antibiotic

norfloxacin at a low concentration (1 mg/mL) against the fluoroquinolone-resistant strain SA1199B *in vitro*. The MIC of norfloxacin combined with **SG** was sixteen times less than that of norfloxacin alone, which resulted in an FICI value of 0.188, less than the synergy evaluation threshold of 0.5.

Considering that SA1199B overexpresses the NorA efflux protein, we postulated that the synergistic mechanism of the interaction between **SG** and norfloxacin was efflux pump inhibition. The results of the EtBr efflux assayssupported this postulation. The natural product **SG** showed a similar efflux pump inhibitory activity on SA1199B compared to the positive control (CCCP), and they both showed a significant difference compared with the vehicle in the EtBr efflux assay (Fig. 2).

Whilst **SG** has been previously described and there are some reports ofits antibacterial effects, there are no reports describing its synergistic activities on drug-resistant strains *in vivo*. As we were able to observe an *in vitro* synergistic effect with norfloxacin, we performed an *in vivo* test in mice and observed a positive effect. A synergistic effect between **SG** (100 mg/kg, p.o.) and norfloxacin against SA1199B infection was observed, which increased the survival of mice at 7 days after infectionina dose-dependent manner (Fig. 3). The same survival rate was observed for mice treated with norfloxacin alone (180 mg/kg) and those treated with a combination of **SG** (100 mg/kg, ineffective dose alone) and norfloxacin (60 mg/kg), yielding the same effect on mouse survival with a dosage of norfloxacin that was decreased by 2/3 compared to the treatment with norfloxacin alone. The survival rate observed usinga combination of **SG** (100 mg/kg, ineffective dose alone) and norfloxacin (120 mg/kg) was much higher than that observed for norfloxacin alone (180 mg/kg). But the statistical analysis result between synergism group (SG 100 mg/kg + norfloxacin

120 mg/kg) and norfloxacin alone group (180 mg/kg) showed no statistical difference (p<0.17) and this maybe due to individual difference or too few samples used for statistical analysis.

5 Conclusion

The results of this study show that sophoraflavanone G exhibits synergistic antibacterial effects against SA1199B by inhibiting the NorA efflux pump in this norfloxacin-resistant strain. Recently, norfloxacin has been shown to exhibit a series of serious side-effects and toxicity during its clinical use [20]. However, it is an inexpensive and useful antibiotic against many types of bacteria. The synergy with a non-toxic compound may reduce the dose of norfloxacin required for its antibacterial efficacy, thereby avoiding some side-effects. Furthermore, by improving the solubility and pharmacokinetic parameters as well asenhancing the synergistic effects, a compound such as **SG** may be a potential drug-lead for treating infections by effluxing bacteria.

Declarations

Funding: This work was supported by the NSFC grant (21672041) of China.

Competing Interests: None to declare.

Ethical Approval: All animal experimental procedures were conducted according to the university guidelines and were approved by the Institutional Animal Careand Use Committee (IACUC), School of Pharmacy, Fudan University with an ethical approval document no. 201603-TY-MQ-01(Shanghai, China).

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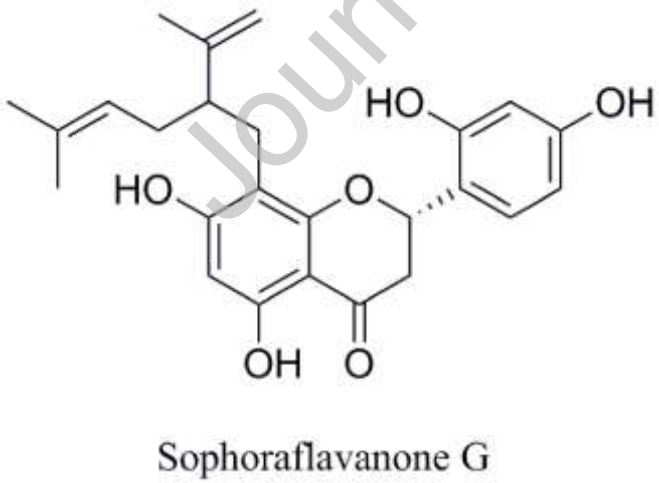
Legends of Figures



Sophoraflavanone G

Figure 1. Chemicaltructure of **SG** isolated from *Sophora alopecuroides.*

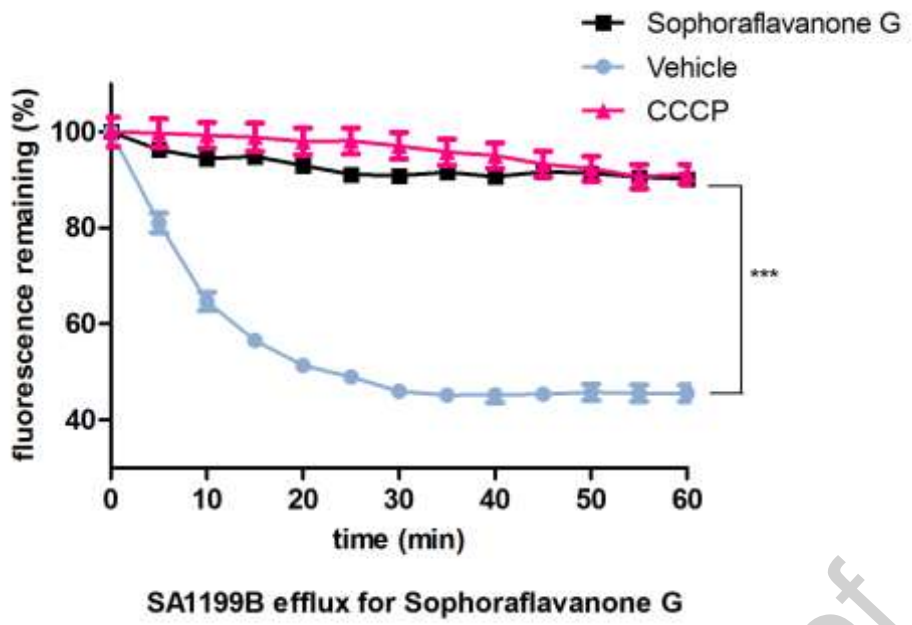


Figure 2. EtBr efflux inhibitory effects of **SG** against *S. aureus* SA1199B.

SA1199B was treated with **SG** (1 mg/L, 1/4 MIC), the known efflux inhibitor CCCP (100 μmol/L) orvehicle.The data are expressed as the means ± S.D. *** $p<0.001$

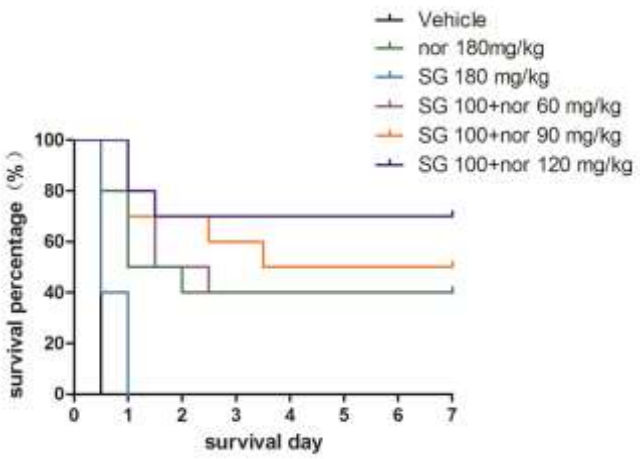


Figure 3. Survival curves of combinations of **SG** and norfloxacin for SA1199B-infected mice.

Mice were administered PBS (vehicle), **SG** (180 mg/kg) alone, norfloxacin (180 mg/kg) alone, or combinations of **SG** (100 mg/kg) and norfloxacin (60, 90 and 120 mg/kg). The survival of all groups was monitored at 12-h intervals for 7 consecutive days.