Baicalein Suppresses the SOS Response System of *Staphylococcus Aureus* Induced by Ciprofloxacin

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Key Words

Baicalein • SOS response • *Staphylococcus aureus* • Ciprofloxacin

that baicalein may be an SOS-response inhibitor in *S. aureus* through inhibiting ROS formation and ATP production and may be used to prevent excessive mutation induced by antibiotics.

Abstract

Numerous antibiotics can induce an SOS repair system in bacteria that leads to antibiotic-resistant mutation of the bacterium. Therefore, searching for drugs that can prevent the SOS response and thus improve the long-term viability of some antibiotics is important. In this study, we aimed to detect the suppressive effects of baicalein on the SOS system and rifampin-resistant mutation in *Staphylococcus aureus*. We determined the reactive oxygen species (ROS) formation and intracellular ATP level in *S. aureus* with baicalein treatment to investigate the mechanisms involved in its effects on the SOS system. The results showed that baicalein was a potent inhibitor of the expression of the SOS genes RecA, LexA and SACOL1400. The rifampin-resistant mutation rate of *S. aureus* induced by ciprofloxacin was significantly reduced after treatment with baicalein. Treatment with baicalein led to a significant decrease in intracellular reactive oxygen species (ROS) formation and ATP level. Our findings indicate

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Introduction

Staphylococcus aureus infection can be difficult to treat because of multidrug resistance and the organism's remarkable ability to persist in the host. Persistence and the evolution of resistance may be related to several complex regulatory networks such as the SOS response, which is essential in bacterial mutations. SOS system uses the RecA protein, which is stimulated by single-stranded DNA, to inactivate the LexA repressor and induce the response.

In addition to the fluoroquinolones [1-3], other antibiotics, such as rifampin [4], aminoglycosides [5], and β -lactams [5, 6], can induce the SOS response and cause mutations resulting in multidrug resistance. Therefore,

searching for drugs to prevent SOS repair is important to extend the time needed for the pathogenic bacteria to evolve antibiotic resistance and thus improve the long-term viability of some antibiotics. Previous study reported that one mechanism of induced SOS response by quinolones may be their direct inhibition of DNA replication [7]. Another mechanism is low doses of bactericidal antibiotics, such as quinolones, β -lactams and aminoglycosides triggering hydroxyl radical formation by stimulating the Fenton reaction, which damages DNA and thereby induces the SOS response [8, 9]. Although the specific triggers for quinolones and β -lactams remain unknown, mistranslation of envelope proteins and subsequent activation of the stress response is the specific trigger for aminoglycoside-induced hydroxyl radical formation [10]. As well, the activation of RecA in recombination and DNA repair also depends on ATP binding [11, 12].

Therefore, agents that can inhibit the reactive oxygen species (ROS) formation and ATP production should have a suppressive effect on the SOS system and mutation in bacteria. The main component of the Chinese herb *Scutellaria baicalensis* Georgi (Labiateae), baicalein, has well known antimicrobial effects and reverses the antibiotic resistance of *Staphylococcus aureus* [13]. Baicalein is both a radical scavenger [14] and ATP synthase inhibitor in *Escherichia coli* [15]. We hypothesized that these effects also occur in *S. aureus*, and baicalein should be an SOS response inhibitor, which may be of help in reducing the antibiotic mutation of *S. aureus*.

In this study, we aimed to investigate the suppressive effects of baicalein on the SOS system and rifampin-resistant mutation in *S. aureus*. We also determined intracellular ROS formation and ATP production in *S. aureus* with baicalein treatment to investigate the mechanisms involved in the inhibitory effects on the SOS response system.

Materials and Methods

Bacterial strains and reagents

The 2 *S. aureus* isolates used in this study were obtained from the Clinical Microbiology Laboratory of the First Affiliated Hospital of Shantou University, Shantou, China. *S. aureus* ATCC25923 was used as the control strain. Baicalein (purity = 98%) was purchased from Shanghai Usea Biotech Co. Rifampin and ciprofloxacin (purity = 99%) were kindly donated by Prof. Yue Ma (National Institute for the Control of Pharmaceutical and Biological Products, Beijing).

Determination of minimum inhibitory concentration

The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the antibiotics and baicalein. Briefly, a 50- μ l volume of ciprofloxacin (0.06–8 μ g/ml), rifampin (0.004–2 μ g/ml) and baicalein (0.5–256 μ g/ml) was dispensed into each well of microtiter plates, then 50 μ l 10^5 CFU (colony-forming units)/ml bacteria inocula was added to each well. Plates were incubated at 35°C for 24 hr. MICs were read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited. Each experiment was replicated 3 times.

Quantitative real-time RT-PCR

Each strain was inoculated into Luria-Bertani (LB) broth and incubated for 18 hr. Cultures were diluted 1:100 and incubated in LB containing 0, 2, 4, 8 μ g/ml baicalein with or without 1/4 MIC ciprofloxacin. After 24-hr culture at 35 °C, 2 ml culture was centrifuged and total RNA was extracted by use of a Bacterial RNeasy Mini kit (Dongsheng Biotech, China). This procedure was repeated 3 independent times to generate 3 samples for each strain.

First-strand cDNAs were synthesized from 2 μ g of total RNA in a 100- μ L reaction volume by use of the First-Strand cDNA Synthesis Kit (GeneCopoeia, USA). Primers for genes involved in the SOS system (*recA*, *lexA* and *SACOL 1400*) and 16s RNA were as described [16]. Real-time PCR involved use of an iCycler (Bio-Rad), with an All-in-One™ miRNA quantitative RT-PCR (qRT-PCR) Detection Kit (GeneCopoeia) at an initial denaturing step of 95°C for 3 min, then 40 cycles of 95°C for 10 s and 55°C for 30 s. Expression of 16S rRNA was a normalization control. The fold change was calculated using the $2^{-\Delta\Delta C_t}$ method presented as the fold change in expression of post-treatment groups relative to the control group (no drug treatment).

Mutation rate with baicalein treatment

Mutation rate was determined by a rifampin-based selection method. Briefly, overnight cultures of 2 clinical isolates and ATCC25923 were diluted 1:100 into 10 ml LB in the presence of 0, 2, 4 μ g/ml baicalein with or without 1/4 MIC ciprofloxacin for 24 hr at 35 °C and 250 rpm. A sample of 100 μ l was diluted and plated on antibiotic-free LB agar for total viable count determination. The remaining culture was centrifuged for 10 min at 2,500 $\times$ g. The pellet was resuspended in 500 μ l LB broth and seeded on LB agar plates containing 4 μ g/ml rifampin (Sigma, St. Louis, MO). The number of rifampin-resistant colonies was detected after 48-hr incubation. The colony counts from 10 replicates were used for determining mutation by MSS maximum-likelihood method [17]. Mutation rates were calculated by use of Fluctuation AnaLysis CalculatOR (<http://www.mitochondria.org/protocols/FALCOR.html>) [18].

ROS detection by fluorescence microplate reader

ATCC25923 was cultured overnight in 25 ml LB medium in 250-ml flasks and incubated at 35°C and 300 rpm, then 1/4 MIC ciprofloxacin, 4 μ g/ml baicalein or ciprofloxacin plus 4 μ g/ml baicalein was added at an early exponential phase (OD_{600} =0.3). Each culture was then continuously incubated

under the same condition until $OD_{600}=1.0$. A 1-ml sample was collected, washed once with phosphate buffered saline (PBS; pH 7.0), then incubated with the fluorescent reporter dye DCFH-DA (Beyotime, China) at a final concentration of 10 mM for 20 min at 35°C before measurement. The samples were centrifuged and washed with PBS 3 times. The precipitate was then suspended with 1 ml PBS and 100 μ l of each sample was added to each well of a black microplate. A SpectraMax M2 microplate reader (Molecular Devices) in fluorescence mode (excitation 488 nm, emission 524 nm) was used to detect the cellular DCF fluorescence. Each experiment was repeated 3 times.

Bioluminescence assay for intracellular ATP determination

An overnight culture of ATCC25923 was diluted 1:100 into LB medium and incubated at 35°C and 300 rpm. An amount of 1/4 MIC ciprofloxacin, 4 μ g/ml baicalein or ciprofloxacin plus 4 μ g/ml baicalein was added to each culture at early exponential phase ($OD_{600}=0.3$) and incubated at 35°C and 300 rpm for 3 hr. Each sample was centrifuged and washed by water twice. The intracellular ATP concentration was analyzed by use of the Enliten ATP Detection Kit (Promega, USA). A standard curve was generated from known concentrations of ATP and used to calculate the concentration of ATP in each sample. Luminescence increased linearly with the negative log of the ATP concentration in the samples over the range of concentrations measured. Data were normalized to total protein concentration as determined by the BCA protein assay Kit (Beyotime, China). The ATP levels were standardized by the ATP content with control group (no drug treatment).

Statistical analysis

Differences among groups were tested by one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Determination of MIC

The MIC of the 2 clinical isolates and ATCC25923 are in Table 1. Each strain was susceptible to both ciprofloxacin and rifampin. Baicalein had a weak antibacterial effect, with MIC ranging from 32 to 64 μ g/ml against *S. aureus*.

Effect of baicalein on SOS response in S. aureus

To test the effect of baicalein on SOS response in *S. aureus*, we detected the transcription of genes involved in the SOS system, including RecA, LexA and SACOL1400, which encodes LexA-regulated DNA polymerase [16]. Real-time RT-PCR revealed that treatment with 1/4 MIC ciprofloxacin significantly upregulated RecA, LexA and SACOL1400. Treatment with 2 and 4 μ g/ml baicalein significantly ameliorated the expression of these genes induced by ciprofloxacin (Fig.

Fig. 1. RT-PCR analysis of change in level of SOS genes. Data are mean $\pm$ SD. (A) Fold change of RecA expression of post-treatment *S. aureus* relative to the level of control group (no drug treatment); (B) Fold change of LexA expression post-treatment group relative to the level of control group (no drug treatment); (C) Fold change of SACOL1400 expression post-treatment group relative to the level of control group (no drug treatment). 1: control group (no drug treatment); 2: 2 μ g/ml baicalein; 3: 4 μ g/ml baicalein; 4: 1/4 minimum inhibitory concentration (MIC) ciprofloxacin; 5: 1/4 MIC ciprofloxacin plus 2 μ g/ml baicalein; 6: 1/4 MIC ciprofloxacin plus 4 μ g/ml baicalein.

Fig. 2. Change in rifampin-resistance mutation rates after treatment (data are mean $\pm$ SD). 1: control group (no drug treatment); 2: group treated with 2 μ g/ml baicalein ; 3: group treated with 4 μ g/ml baicalein; 4: group treated with 1/4 MIC ciprofloxacin 5: group treated with 1/4 MIC ciprofloxacin plus 2 μ g/ml baicalein; 6: group treated with 1/4 MIC ciprofloxacin plus 4 μ g/ml baicalein.

1). Increasing the concentration of baicalein resulted in a stronger inhibitory effect.

Baicalein reduced the mutation rate to rifampin

We next examined whether baicalein reduced the ciprofloxacin-induced mutability. As shown in Fig. 2, the rifampin-resistant mutation rate was higher by more than eight-fold with 1/4 MIC ciprofloxacin than with no treatment ($p < 0.05$; Fig. 2). Treatment with 4 μ g/ml baicalein and ciprofloxacin significantly reduced the mutation rate to 1.7-fold the untreated level ($p < 0.05$).

Effect of baicalein on the formation of ROS

As shown in Fig. 3, the fluorescence intensity for detecting ROS in *S. aureus* was higher by more than 20% with ciprofloxacin treatment than with no treatment, so ciprofloxacin induced cellular ROS formation. The ROS formation was lower by more than 40% with ciprofloxacin plus baicalein than with ciprofloxacin alone ($p < 0.05$). As well, the fluorescence intensity was decreased significantly with baicalein alone ($p < 0.05$), which suggests that baicalein acted as a strong ROS scavenger.

Effect of baicalein on intracellular ATP production

The intracellular ATP level in *S. aureus* was higher by more than 45% with 4 μ g/ml ciprofloxacin than with no treatment ($p < 0.05$; Fig. 4). Baicalein alone significantly decreased the intracellular ATP production ($p < 0.05$). The intracellular ATP level was significantly lower with ciprofloxacin and baicalein combined than with ciprofloxacin alone ($p < 0.05$).

Discussion

The SOS response is an inducible DNA repair system that allows bacteria to survive sudden increases in DNA damage. Various antibiotics can induce the SOS response and thereby launch error-prone DNA repair, which results in increased antibiotic-resistant mutation.

Fig. 3. Fluorescence intensity of reactive oxygen species in *Staphylococcus aureus* after treatments (data are mean $\pm$ SD). Doses are 4 μ g/ml baicalein and 1/4 MIC ciprofloxacin.

Fig. 4. Intracellular ATP levels in *Staphylococcus aureus* after treatments as in Fig. 3 (data are mean $\pm$ SD).

The SOS response can be induced by quinolones because of their direct inhibition on DNA replication. Low doses of bactericidal antibiotics, such as quinolones, β -lactams

	MIC (μg/ml)		
	Baicalein	Ciprofloxacin	Rifampin
SA1	64	0.5	0.015
SA2	32	1	0.03
ATCC25923	32	0.5	0.008

Table 1. Antimicrobial susceptibility of *Staphylococcus aureus* used in this study. MIC, minimum inhibitory concentration; SA1, *Staphylococcus aureus* isolate 1; SA2, *Staphylococcus aureus* isolate 2

and aminoglycosides, can also trigger hydroxyl radical formation by stimulating the Fenton reaction, which damages DNA and thereby induces the SOS response [8, 9] and mutation frequency.

Therefore, searching for drugs that can prevent SOS response and thus improve the long-term viability of some antibiotics is important. In the present study, we aimed to detect the suppressive effects of baicalein on the SOS system and rifampin-resistant mutation in *S. aureus*, particularly ROS formation and intracellular ATP level, to investigate the mechanisms involved in inhibitory effects on the SOS system.

Previous study has reported that ciprofloxacin, a quinolone antibiotic, could induce the SOS system via a RecA-LexA dependent pathway in *S. aureus*. SACOL1400, which encodes the error-prone DNA polymerase, is also involved in the RecA-LexA-mediated SOS system of *S. aureus* [16]. We first investigated the inhibitory effect of baicalein on the ciprofloxacin-induced SOS response in *S. aureus*. As expected, low-dose of ciprofloxacin (1/4 MIC) significantly induced the expression of the SOS genes RecA, LexA and SACOL1400, and increased the mutation rate of rifampin resistance. Baicalein strongly inhibited the transcription of RecA, LexA and SACOL1400 in *S. aureus* treated with or without ciprofloxacin. This suppressive effect on the SOS system may result in the reduced rifampin-resistant mutation rate.

Baicalein is a direct radical scavenger and also inhibits the formation of endogenous hydroxyl radicals produced through the Fenton reaction by forming stable and inert complexes with iron [14]. To determine whether baicalein reduced ROS formation in *S. aureus*, we detected ROS production affected by baicalein. Baicalein significantly reduced the ROS formation in *S. aureus* induced by ciprofloxacin, which might explain in part the suppressive effect of baicalein on the SOS response

induced by antibiotics.

Baicalein is a polyphenol compound belong to the flavonoids group. Many flavonoids including baicalein are potent inhibitors of ATP synthase from *E. coli* [15]. This inhibitory effect of baicalein on ATP synthase may be one of the important mechanisms involved in the antibacterial activity of baicalein. The proposed mode of this action is binding of polyphenol at the polyphenol binding pocket of ATP synthase and blocking the rotation of the γ subunit [15]. Because the sequence of ATP synthase is highly conserved across species, this suppressive effect of baicalein may also occur in *S. aureus*, thus resulting in a direct decrease in intracellular ATP production. Therefore, we sought to determine the intracellular ATP production affected by baicalein. Ciprofloxacin increased the ATP production in *S. aureus*. This result may be explained by upregulated NADH dehydrogenase I induced by fluroquinolone, which was previously shown in *E. coli* [19], thereby leading to increased ATP generation. We found that baicalein inhibited the intracellular ATP production in *S. aureus*. Previous studies have reported that ATP binding is essential for storage structure formation and ability to interact with other RecA proteins in a filament [11, 12]. RecA must first bind ATP to adopt a high affinity conformation for single-stranded DNA [20, 21] and interacts with the LexA repressor protein to initiate the SOS response. Therefore, another factor related to the inhibitory SOS response may be baicalein's inhibition of intracellular ATP production. Further study is required to investigate the inhibition of ATP synthase of *S. aureus* by baicalein.

Although radical scavengers such as thiourea and iron chelator dipyrindyl weaken ampicillin-mediated killing by reducing the hydroxyl radical formation [19], we found that baicalein and ciprofloxacin had no antagonistic effects against *S. aureus* concerning cell viability in this study (data not shown). Conversely, β lactams, the antibiotics that can also induce the production of ROS, and baicalein have a synergic effect against methicillin-resistant *S. aureus* [13], which suggests that baicalein significantly increases the antibacterial effect of β lactams despite the unclear synergic mechanism. The toxicity of baicalein is negligible when administered orally [22]. Therefore, baicalein could be used as a safe antimutagen combined with antibiotics to induce the SOS response to prevent the excess antibiotic resistance of *S. aureus* without sacrificing the antibacterial effects of antibiotics. However, further *in vivo* study of baicalein's inhibitory effect on antibiotic resistance mutation is needed to

determine whether it can be used as an antimutagen *in vivo*.

In conclusion, this study indicated that baicalein can inhibit the SOS response and reduce the rifampin resistance mutation rate of *S. aureus* induced by ciprofloxacin. Baicalein may contribute to the suppression of SOS response by its inhibitory effects on ROS formation and intracellular ATP production. Therefore, baicalein might be used as an antimutagen to prevent excess

antibiotic resistance of *S. aureus*.

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