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Isorhamnetin Attenuates *Staphylococcus aureus*-Induced Lung Cell Injury by Inhibiting Alpha-Hemolysin Expression

Lanxiang Jiang[1†], Hongen Li[1†], Laiying Wang[2], Zexin Song[1], Lei Shi[1], Wenhua Li[1], Xuming Deng[1*], and Jianfeng Wang[1*]

[1]*Department of Dermatology, Second Hospital of Jilin University, College of Veterinary Medicine, Jilin University, Changchun 130041, P.R. China*

[2]*The First Affiliated Hospital to Changchun University of Chinese Medicine, Changchun 130021, P.R. China*

Staphylococcus aureus, like other gram-positive pathogens, has evolved a large repertoire of virulence factors as a powerful weapon to subvert the host immune system, among which alpha-hemolysin (Hla), a secreted pore-forming cytotoxin, plays a preeminent role. We observed a concentration-dependent reduction in Hla production by *S. aureus* in the presence of sub-inhibitory concentrations of isorhamnetin, a flavonoid from the fruits of *Hippophae rhamnoides* L., which has little antibacterial activity. We further evaluate the effect of isorhamnetin on the transcription of the Hla-encoding gene *hla* and *RNAIII*, an effector molecule in the *agr* system. Isorhamnetin significantly down-regulated *RNAIII* expression and subsequently inhibited *hla* transcription. In a co-culture of *S. aureus* and lung cells, topical isorhamnetin treatment protected against *S. aureus*-induced cell injury. Isorhamnetin may represent a leading compound for the development of anti-virulence drugs against *S. aureus* infections.

Keywords: *Staphylococcus aureus*, alpha-hemolysin, isorhamnetin, anti-virulence

Introduction

The gram-positive pathogenic bacterium *Staphylococcus aureus* is among the most serious human pathogens encountered in healthcare settings and is a common agent of nosocomial and community-acquired infections, including pneumonia, sepsis, and endocarditis, causing significant morbidity and mortality worldwide [5, 14]. Currently, the treatment of *S. aureus* infections is challenging owing to the bacterium's notable ability to acquire resistance to a wide range of antibiotics [14, 15]. Additionally, the mechanisms employed by traditional antibiotics are primarily variations on a single theme – bacteriostatic or bactericidal effects, which apply strong selective pressure on the target bacteria and, consequently, foster the development of antibiotic resistance [12]. Therefore, the demand to develop new strategies and agents for the war between humans and microorganisms, especially drug-resistant bacteria, is urgent.

Interference with bacterial virulence and/or cell-to-cell communications is a recent promising strategy, which is an attractive option that is increasingly being characterized [2]. In recent decades, a tremendous effort has been addressed toward understanding how pathogens cause diseases. These efforts clearly indicate that virulence factors evolved as powerful weapons for pathogens and play specific roles at each step of the pathogenesis of infection, facilitating their successful establishment [10, 20]. Thus, the treatment of bacterial infections depends not only on bacterial eradication but also on interfering with the activity of bacterial virulence factors. Furthermore, targeting bacterial virulence factors can expand the repertoire of bacterial targets, preserve the host endogenous microbiome,
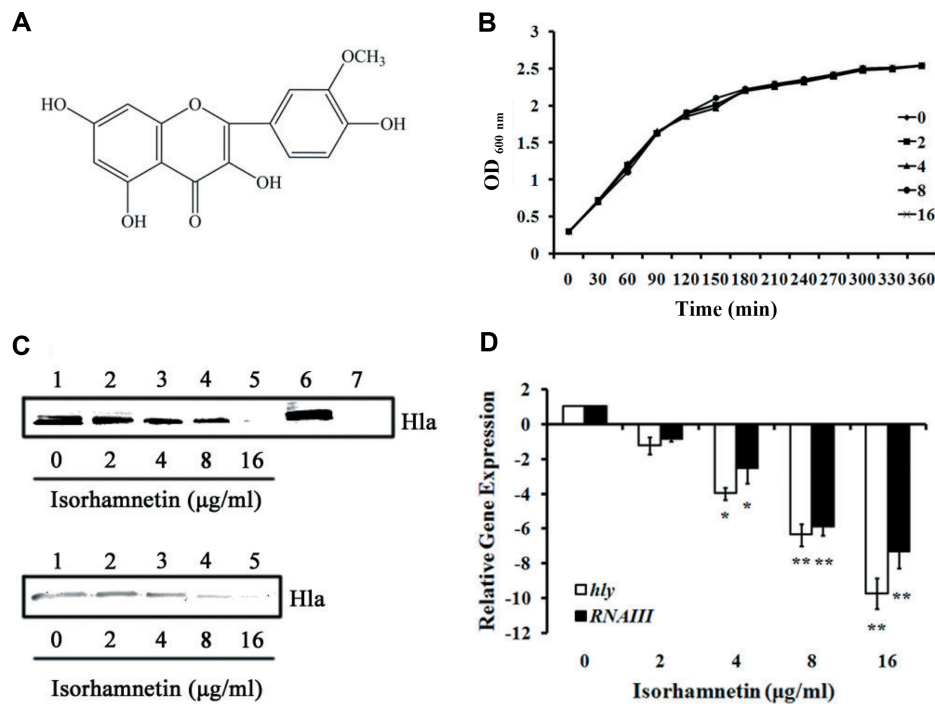
Fig. 1. Isorhamnetin inhibits Hla production in *S. aureus* culture supernatants.
(**A**) Chemical structure of isorhamnetin. (**B**) Growth curves of *S. aureus* strain 8325-4 in the presence of varying concentrations of isorhamnetin. (◆), Isorhamnetin-free; (■), 2 µg/ml; (▲), 4 µg/ml; (●), 8 µg/ml; (×), 16 µg/ml. Curves represent the results of one of three reproducible experiments. (**C**) Western blot analysis of Hla production in the culture supernatants of strains 8325-4 (upper panel) and USA 300 (lower panel) after growth with varying concentrations of isorhamnetin (lanes 1-5). Ten nanograms of purified Hla was used as positive control (lane 6), and strain DU 1090 was used as a negative control (lane 7). (**D**) The influence of different concentrations of isorhamnetin on the transcription of *hla* and *RNA III* in strain 8325-4. Data are expressed as the mean and standard error of three independent experiments. Student's *t*-test was used to determine significant differences. $*p < 0.05$ and $**p < 0.01$, compared with samples without isorhamnetin.

and exert less selective pressure, resulting in decreased resistance [6].

Of the molecular weaponry deployed by *S. aureus*, alpha-hemolysin (Hla), a pore-forming cytotoxin secreted as a water-soluble monomer, is one of the most important virulence factors and causes tissue barrier disruption at host interfaces lined by epithelial or endothelial cells [1]. The role of Hla in the pathogenesis of *S. aureus* is critical, as evident from the fact that mutants lacking Hla display reduced virulence in many animal models of diseases [1, 8, 13, 19]. Some natural compounds identified in our laboratory can attenuate the virulence of *S. aureus* by inhibiting the expression or antagonizing the pore-forming activity of Hla [11, 17, 21]. Thus, this protein is a potential target for the development of anti-virulence agents against *S. aureus* infections. In the present study, among the natural compounds screened using hemolysis assay, we show that isorhamnetin (Fig. 1A), a flavonoid mainly isolated from the fruits of *Hippophae rhamnoides* L., effectively inhibits the

expression of Hla in *S. aureus*, partially by down-regulating the transcription of the *agr* two-component system, and subsequently alleviates the *S. aureus*-induced injury of lung cells.

Materials and Methods

Bacterial Strains, Reagents, and Culture Conditions

All the bacterial strains used in this study (Table 1) were cultured in tryptic soy broth (TSB) (Sparks, MD, USA) at 37°C with varying concentrations of isorhamnetin. Isorhamnetin and oxacillin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Isorhamnetin was dissolved in dimethyl sulfoxide (DMSO ≧99.5%) (Sigma-Aldrich, St. Louis, MO, USA) to make a stock solution, and DMSO was used as a control.

Susceptibility Testing

The minimum inhibitory concentrations (MICs) of isorhamnetin for *S. aureus* strains were determined in triplicate using the broth

Table 1. Bacterial strains used in this work and their isorhamnetin MICs.

S. aureus strains	Properties	Source	MIC (μg/ml)	
			Oxacillin	Isorhamnetin
ATCC 29212	MSSA, Hla, and β-lactamase producing strain	ATCC	0.25	>1,024
ATCC 10832	MSSA, Wood 46, a natural isolate that produces high levels of Hla	ATCC	0.125	>1,024
USA 300	BAA-1717, the most prevalent CA-MRSA that produces Hla	ATCC	128	>1,024
8325-4	A high-level Hla-producing strain derived from NCTC 8325	Timothy J. Foster	0.125	>1,024
DU 1090	8325-4 defective in Hla, prepared by insertion of a transposon in the *hla* gene	Timothy J. Foster	0.125	>1,024

microdilution procedure as previously described [17]. The MICs were defined as the lowest drug concentration that did not inhibit bacterial growth (OD_{550} < 0.05). Oxacillin was used as a positive control.

Bacterial Growth Kinetics

Overnight cultures (*S. aureus* strains 8325-4 and USA 300) were diluted 1:150 in 15 ml of TSB in a 50 ml flask and grown at 37°C with shaking (200 rpm) with varying concentrations of isorhamnetin for 6 h. Cell growth was monitored in an UV Spectrophotometer at 600 nm against a culture medium blank at 30-min intervals.

Determination of Hemolytic Activity

The hemolytic activity of the culture supernatant was determined as described in our previous study [11]. Briefly, *S. aureus* strains were cultured (37°C, 200 rpm) in TSB supplemented with the indicated concentrations of isorhamnetin and centrifuged at 10,000 ×*g* for 5 min at the post-exponential phase (OD_{600} of 2.5). One hundred microliters of supernatant was mixed with 875 μl of phosphate-buffered saline (PBS; Sigma-Aldrich), and defibrinated rabbit blood was added to achieve a final concentration of 2.5%. The reaction system was incubated at 37°C for 30 min and then centrifuged (1,000 ×*g*, room temperature, 5 min) to pellet the unlysed blood cells. Hemolytic activity of the supernatant was assessed by measuring the optical density at 543 nm. One percent Triton X-100 in TSB was used as a positive control; TSB was used as a negative control.

The influence of isorhamnetin on the hemolysis induced by bacterial culture supernatant was assayed by mixing 100 μl of supernatant with various concentrations of isorhamnetin in 875 μl of pretreated PBS; the mixture was incubated at 37°C for 30 min before the addition of defibrinated sheep erythrocytes and the hemolytic activity of each sample was determined as described above.

SDS-PAGE and Western Blotting

Following boiling with loading buffer for 5 min, supernatants from the above assay were separated on a 12% SDS polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Roche, Basel, Switzerland) with a semi-dry transfer system (Bio-Rad, Munich, Germany). The blot was blocked for 2 h in PBS containing 5% dried skim milk and incubated with anti-Hla primary antibody (Sigma-Aldrich; diluted 1:3,000) and horseradish peroxidase-conjugated anti-rabbit antiserum secondary antibody (Sigma-Aldrich; diluted 1:4,000) in the same buffer. An ECL western blotting kit (GE Healthcare, Buckinghamshire, UK) was used to detect the Hla bands.

Real-Time RT-PCR

S. aureus strain 8325-4 was treated with or without sub-inhibitory concentrations of isorhamnetin, as described in the hemolytic activity assay. Total RNA was extracted from pelleted cells using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The collected RNA was treated with RNase-free DNase I (Qiagen) to remove contaminating DNA. The RNA quantity and purity were determined by measuring the optical density at 260 nm with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The purified RNA was subjected to reverse transcription using the Takara RNA PCR kit (AMV) ver. 3.0 (Takara, Kyoto, Japan), as recommended by the manufacturer. Transcript levels for the *hla* and *RNAIII* genes and the housekeeping gene *16S rRNA* (the gene served as an internal control to normalize the expression levels between samples) were quantified on the 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France) using SYBR Premix Ex Taq (Takara). Cycling conditions consisted of an initial denaturation step at 95°C for 30 sec and 35 cycles at 95°C for 5 sec, 55°C for 30 sec, and 72°C for 20 sec. The primers used for real-time RT-PCR are as follows: *hla* sense: 5'-TTGGTGCAAATGTTTC-3', *hla* antisense: TCACTTTCCAGCCTACT; *RNAIII* sense: 5'-TTCACTGTGTCG ATAATCCA-3', *RNAIII* antisense: 5'-GGAAGGAGTGATTTCAAT GG-3'; *16S rRNA* sense: 5'-GCTGCCCTTTGTATTGTC-3', *16S rRNA* antisense: 5'-AGATGTTGGGGTTAAGTCCC-3'.

Live/Dead and Cytotoxicity Assays

Human alveolar epithelial A549 cells (ATCC CCL 185) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 100 U/ml penicillin/streptomycin at 37°C with 5% CO_2. For experiments, cells were seeded in 96-well plates at 1.0×10^4 cells/well overnight prior to co-culture with 100 μl of staphylococcal suspension (2.5×10^8 CFU/ml) per well in DMEM with isorhamnetin in triplicate wells. After incubation at 37°C for 6 h, cells were fluorescently

Table 2. Hemolytic activity in culture supernatants of *S. aureus* strains treated with increasing concentrations of isorhamnetin.

S. aureus strains	Hemolysis (%) of rabbit erythrocytes by culture supernatant[a]				
	Drug-free	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml
ATCC 29213	100%	78.99 ± 5.67	58.62 ± 4.98**	16.81 ± 2.51**	NO[b]
ATCC 10832	100%	83.99 ± 4.11	65.77 ± 6.35*	33.60 ± 5.19**	8.36 ± 1.02**
USA 300	100%	78.16 ± 8.22	62.84 ± 5.81*	19.49 ± 7.43**	NO[b]
8325-4	100%	86.34 ± 4.38	55.36 ± 6.71**	20.33 ± 3.87**	NO[b]

[a]Hemolytic activity of the drug-free group served as the 100% hemolysis control.
[b]No hemolytic activity was observed.
Data are presented as means with standard error; asterisks indicate a value considered statistically significant (*$p < 0.05$, **$p < 0.01$).

labeled with live/dead (green/red, respectively) reagent (Invitrogen), and the labeled cells were analyzed using a confocal laser-scanning microscope (Nikon, Japan). Cell viability was also assessed using the Cytotoxicity Detection Kit (LDH) (Roche) on a microplate reader (TECAN, Salzburg, Austria) to measure the level of released lactate dehydrogenase (LDH) from A549 cells. The content of secreted Hla in the medium was further assayed by SDS-PAGE and western blotting.

MH-S murine alveolar macrophage cells (ATCC CRL 2019) were maintained in RPMI 1640 medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Invitrogen) and 100 U/ml penicillin/streptomycin at 37°C with 5% CO_2 and the released LDH in the medium was determined as described above.

Statistical Analyses

The data are presented as the mean ± standard deviation of triplicate measurements. Statistical significance was assessed using an independent Student's *t*-test with SPSS 12.0 statistical software (SPSS Inc., Chicago, IL, USA). Values were considered statistically significant when the *p* value was < 0.05.

Results

Isorhamnetin Has No Influence on *S. aureus* Growth

The MIC values of isorhamnetin for *S. aureus* strains tested in this study (Table 1) were all >1,024 µg/ml. Furthermore, the growth of *S. aureus* 8325-4 was not visibly affected by the addition of increasing sub-inhibitory concentrations of isorhamnetin (Fig. 1B). Although the different bacterial strains vary in their growth kinetics, isorhamnetin did not influence the growth of USA 300 (data not shown). Therefore, isorhamnetin has little antibacterial activity and,

at levels of 2 to 16 µg/ml, has no influence on *S. aureus* growth.

Isorhamnetin Inhibits Hla Production in Culture Supernatants

Hemolysis assays were performed to evaluate the effect of isorhamnetin on the biological relevance of the inhibition of Hla in culture supernatants. Isorhamnetin, at the concentrations that do not affect bacterial growth, dose-dependently repressed the hemolytic activity of bacterial culture supernatants from all tested *S. aureus* strains (Table 2). The exposure to 8 µg/ml of isorhamnetin resulted in a significant reduction (83.19%, 66.40%, 80.51%, and 79.67% for ATCC 29213, ATCC 10832, USA 300, and 8325-4, respectively) for all tested strains relative to their drug-free controls. Remarkably, no hemolytic activity was observed in the culture supernatants co-cultured with 16 µg/ml of isorhamnetin for ATCC 29213, USA 300, and 8325-4. In the present study, isorhamnetin, as an agent, did not cause lysis of rabbit erythrocytes (data not shown) or inhibit the hemolysis induced by bacterial culture supernatant, even at concentrations up to 32 µg/ml (Table 3). Thus, we hypothesized that the decreased hemolytic activity is due to the inhibition of Hla production by isorhamnetin in the culture supernatants.

Western blotting was performed to directly evaluate this hypothesis. As expected, the level of Hla protein in the supernatants was also concentration-dependently decreased (Fig. 1C). Consistent with the hemolysis assay, visibly reduced Hla production was observed in culture supernatants in the presence of 8 µg/ml of isorhamnetin, and almost no

Table 3. Hemolytic activity of culture supernatants treated with various concentrations of isorhamnetin.

Hemolysis (%) of rabbit erythrocytes by culture supernatant[a]					
Drug-free	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml
100%	98.66 ± 5.67	99.37 ± 4.98	100.85 ± 6.51	95.85 ± 4.14	97.95 ± 4.59

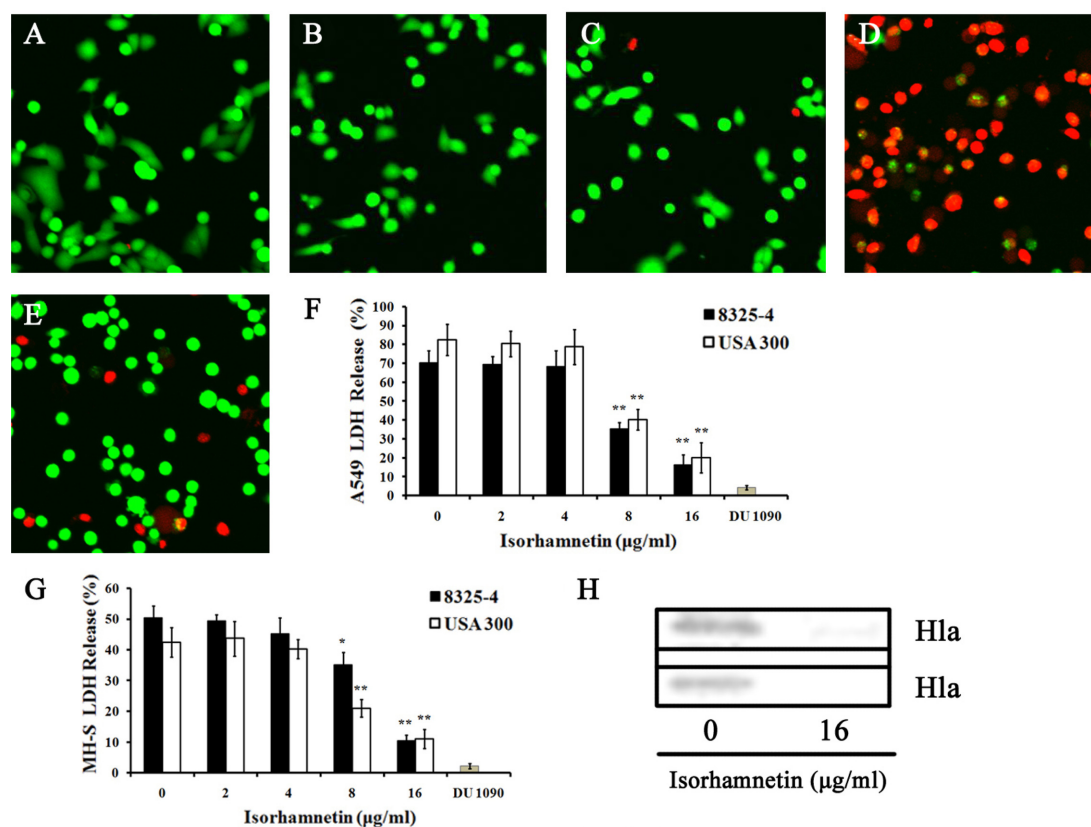[a]Hemolytic activity of the drug-free group served as the 100% hemolysis control.

Fig. 2. Isorhamnetin alleviates *S. aureus*-induced injury of lung cell via reducing Hla production.
Human A549 cells were imaged using confocal laser scanning microscopy 6 h after co-culture with strain 8325-4. Live cells and dead cells were stained with green fluorophore and red fluorophore, respectively. Cells were left uninfected (**A**), treated with 16 μg/ml isorhamnetin (**B**), or infected with strain DU 1090 (**C**); A549 cells were co-cultured with strain 8325-4 without (**D**) or with (**E**) isorhamnetin (16 μg/ml). LDH release by A549 cells (**F**) or MH-S cells (**G**) co-cultured with strain 8325-4 or USA 300 in the presence of the indicated concentrations of isorhamnetin. (**H**) The level of Hla in the medium of co-culture system of *S. aureus* strain 8325-4 and A549 cells (upper panel) or MH-S cells (lower panel) was evaluated by western blotting in the presence or absence of isorhamnetin (16 μg/ml). Data shown in panel (**F**) and (**G**) represent means of at least three independent experiments ± standard deviations. *$p < 0.05$ and **$p < 0.01$.

immunoreactive protein was found in the samples treated with 16 μg/ml of isorhamnetin (Fig. 1C).

Real-time RT-PCR was further employed to assess the effect of isorhamnetin concentrations from 2 to 16 μg/ml on *hla* transcription. The transcription of *RNAIII*, the effector molecule of the *agr* system, was also determined because more than 100 virulence factors, including Hla, are positively regulated by the *agr* two-component system [16]. Treatment with sub-inhibitory concentrations of isorhamnetin led to inhibition, also in a dose-dependent manner, of the transcriptional levels for both the *hla* and *RNAIII* genes (Fig. 1D). At 16 μg/ml, isorhamnetin remarkably reduced *hla* and *RNAIII* transcript levels (10.74-fold reduction and 8.35-fold reduction for *hla* and *RNAII*, respectively) compared with those without the drug (Fig. 1D).

Isorhamnetin Alleviates the Injury of Lung Cell Caused by *S. aureus* via Reducing Hla Expression

The critical role of Hla in *S. aureus*-induced human alveolar epithelial A549 cell injury coupled with the inhibition of Hla production in culture supernatants induced by isorhamnetin prompted us to investigate the potential protective effect of isorhamnetin on the injury of lung cell in the co-culture system. As expected, the cytotoxicity depended on Hla, as the strain deficient in Hla production (DU 1090) failed to caused cytotoxicity (Fig. 2C), but the wild-type strain (8325-4) caused significant cell injury, indicated by red fluorescent dead cells (Fig. 2D). No such injury was observed in uninfected cells (Fig. 2A) or cells treated with isorhamnetin (Fig. 2B). Treatment with 16 μg/ml isorhamnetin provided robust protection against *S. aureus*-

induced cell injury, indicated by a dramatic reduction in dead cells and limited transformations to the cellular morphology of the live cells observed in the co-culture system in the presence of isorhamnetin (Fig. 2E). The level of released LDH in the medium was further examined to quantitatively measure the protective effect, and, as expected, it was concentration-dependent for both A549 cells (Fig. 2F) and MH-S cells (Fig. 2G). The level of secreted Hla in the co-culture system was further evaluated to verify whether such protection was attributed to the inhibition of Hla production caused by isorhamnetin. Consistent with earlier results, the production of Hla in the medium was also significantly decreased in the presence of 16 μg/ml isorhamnetin (Fig. 2H). Therefore, isorhamnetin confers effective protection against lung cell injury caused by *S. aureus* in co-culture via reducing Hla production (Fig. 2).

Discussion

S. aureus is a common colonizer of the host mucosal layers and can also cause many life-threatening infections with long hospital stays and high costs [7]. The ability of this pathogen to develop resistance to antibiotics introduced in clinical application makes its treatment especially difficult [14, 15]. Currently, clinical treatment of most bacterial infections is facing this crisis, which necessitates the development of new therapeutic strategies or drugs. Although both are resistant to methicillin and other β-lactam antibiotics, community-associated MRSA is much more likely to cause aggressive disease than hospital-associated MRSA owing to the presence of numerous virulence factors that are used as molecular weapons, leading to cell lysis and tissue injury [3]. Thus, disarming bacterial virulence may represent an alternative treatment option that does not apply selective pressure and lead to further resistance development.

On this basis, we screened natural compounds for effective inhibitors of Hla, a critical virulence factor in the pathogenicity of *S. aureus*. We found that isorhamnetin, an abundant flavonol aglycone in herbal medicinal plants, significantly inhibited the hemolytic activity of bacterial culture supernatants with little antibacterial activity (MICs >1,024 μg/ml). Western blotting and real-time RT-PCR indicated that this inhibition was due to a reduction of Hla production in supernatants via the inhibition of the transcription of *hla*, the gene encoding Hla, and *RNAIII*, a key feature of *agr* function. Finally, isorhamnetin conferred a remarkable degree of protection against *S. aureus*-induced injury in our co-culture system. Such inhibition would

apply less evolutionary pressure on *S. aureus* for the development of resistance.

The *agr* two-component system, a quorum sensing system that modulates most secreted virulence factors and surface proteins in *S. aureus*, is a global regulator responsible for the virulence of this pathogen [16]. More than 100 protein-coding genes, including *hla*, are transcriptionally controlled by this regulatory system [16]. Based on the results of real-time RT-PCR showing isorhamnetin-induced transcription inhibition of *RNAIII*, we infer that the production of *agr*-dependent virulence-related exoproteins is also regulated by this compound. The important role of the *agr* locus in *S. aureus* virulence has been well established in a variety of animal models of infection [4], as *agr*-defective *S. aureus* showed considerable attenuation of virulence. Thus, isorhamnetin may represent a candidate for the development of anti-virulence treatments against *S. aureus* infections by interfering with the production of virulence factors.

Recently, evidence has emerged indicating that treatment of infections with an anti-virulence agent facilitates the host immune system and effectively eradicates pathogens, with minimal impact on resistance [16, 18]. When anti-virulence agents are insufficient to clear an infection, they can still be used in combination with established or novel antimicrobials in a synergistic manner, reducing the usage of both agents [12]. Additionally, Ohlsen *et al.* [9] showed that exposure to sub-inhibitory concentrations of β-lactams led to a strong induction of *hla* expression in *S. aureus*, which may result in an unfavorable impact on the outcome of an infection. With respect to this challenge, combinations of isorhamnetin and commonly used antibiotics might provide improved clinical performance and extend the clinical lifetime of these drugs.

Acknowledgments

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