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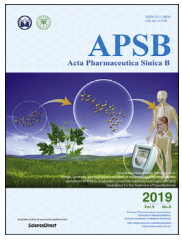

ORIGINAL ARTICLE

Hypericin enhances β-lactam antibiotics activity by inhibiting *sarA* expression in methicillin-resistant *Staphylococcus aureus*

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KEY WORDS

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Abstract Bacteremia is a life-threating syndrome often caused by methicillin-resistant *Staphylococcus aureus* (MRSA). Thus, there is an urgent need to develop novel approaches to successfully treat this infection. Staphylococcal accessory regulator A (SarA), a global virulence regulator, plays a critical role in pathogenesis and β-lactam antibiotic resistance in *Staphylococcus aureus*. Hypericin is believed to act as an antibiotic, antidepressant, antiviral and non-specific kinase inhibitor. In the current study, we investigated the impact of hypericin on β-lactam antibiotics susceptibility and mechanism(s) of its activity. We demonstrated that hypericin significantly decreased the minimum inhibitory concentrations of β-lactam antibiotics (*e.g.*, oxacillin, cefazolin and nafcillin), biofilm formation and fibronectin binding in MRSA strain JE2. In addition, hypericin significantly reduced *sarA* expression, and subsequently decreased *mecA,* and virulence-related regulators (*e.g.*, *agr RNAIII*) and genes (*e.g.*, *fnbA* and *hla*) expression in the studied MRSA strain. Importantly, the *in vitro* synergistic effect of hypericin with β-lactam antibiotic (*e.g.*, oxacillin) translated into *in vivo* therapeutic outcome in a murine MRSA bacteremia model. These findings suggest that hypericin plays an important role in abrogation of β-lactam resistance against

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MRSA through *sarA* inhibition, and may allow us to repurpose the use of β-lactam antibiotics, which are normally ineffective in the treatment of MRSA infections (*e.g.*, oxacillin).

1. Introduction

Staphylococcus aureus (*S. aureus*), a major human pathogen, is capable of causing many infectious diseases (*e.g.*, pneumonia, endocarditis, and bacteremia)[1]. Treatment of methicillin-resistant *S. aureus* (MRSA) infections has become challenging because this pathogen has developed resistance to almost all the standard of care (SOC) antibiotics[2,3]. Therefore, there is an urgent need to develop novel therapeutic strategies against these MRSA life-threatening infections.

SarA, a key global regulator, binds on target promoters to control many virulence factors expression in *S. aureus*[4–7]. As reported from prior studies, laboratory-derived *sarA* mutants exhibited diminished virulence in animal infection models[8,9]. Importantly, we have recently reported that *sarA* mutants become methicillin susceptible with significantly reduced oxacillin resistance *vs.* their respective parental MRSA strains both *in vitro* and in experimental endocarditis model[10]. Therefore, our current studies focus on antimicrobial agents, which could inhibit *sarA* activity, in combination with SOC anti-*S. aureus* β-lactams in order to improve and/or repurpose the treatment efficacy of the antibiotics in MRSA invasive infections.

Hypericin (HYP) is a phenanthropeylene quinine pigment naturally occurring in *Hypericum perforatum* L. (commonly known as St. John's wort). Preclinical and clinical studies demonstrated that it possesses a variety of therapeutic activity (*e.g.*, antidepressant[11], anticancer[12], and antiviral[13]). In addition, its antimicrobial properties have also been reported previously, especially on Gram-positive bacteria (*e.g.*, *S. aureus* and *Listeria monocytogenes*)[14–17]. However, little is known about its mechanism(s) of action.

In the current studies, we investigated the effect of HYP on the susceptibility of β-lactam antibiotics (*e.g.*, oxacillin [OXA], cefazolin [CFZ] and nafcillin [NAF]) and efficacy of HYP in combination with OXA in a murine bacteremia model due to MRSA. We demonstrated that HYP significantly decreased the minimum inhibitory concentrations (MICs) of β-lactam antibiotics, biofilm formation and fibronectin binding in parallel with significantly reduced *sarA*, *agr RNAIII* and virulence related genes expression (*e.g.*, *mecA, fnbA* and *hla*) in MRSA. Importantly, HYP significantly enhanced the efficacy of OXA in an experimental MRSA bacteremia model. These results suggest that the inhibition effect of HYP on *sarA* expression might be responsible for the synergistic effect with OXA both *in vitro* and in the treatment outcome in the MRSA bacteremia model.

2. Materials and methods

2.1. Bacterial strains and growth medium

JE2 strain, a plasmid-cured derivative of LAC MRSA USA300, was obtained from the National Institutes of Health Network on Antimicrobial Resistance in *S. aureus* (NARSA)[18]. A *sarA* deletion in MRSA strain JE2 was achieved by transducing *sarA::*kan mutation from ALC2543[19]. JE2 *ΔmecA* is a transposon mutant with insertion in *S. aureus* USA 300_0032 and obtained from the Nebraska Transposon Mutant Library (NTML, Omaha, NE, USA)[10]. JE2 *ΔsarA/pmecA* is a *sarA* mutant strain complemented with pALC6185, which carries the entire *mecA* locus[10]. pALC 6185 is a plasmid pEPSA5 containing a 2-kb DNA fragment containing the *mecA* coding region[20]. The study strains were stored at −80 °C until thawed for use. Bacteria were routinely grown in tryptic soy broth (TSB) or TSB agar plates otherwise unless specified. All bacterial culture media were purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

2.2. Determination of MICs

The MICs of HYP (Meilun Biotech, Dalian, China) and β-lactam antibiotics, including OXA, CFZ, NAF, and other SOC antibiotics on the study MRSA strains, were determined by a standard broth microdilution method[21] as recommended by the Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA). All the assays were conducted at least three times on different days and the most consistent results were presented.

2.3. Checkerboard assay

Checkerboard assays were employed to determine *in vitro* interactions of HYP and β-lactam antibiotics on the study MRSA strain JE2 according to the CLSI guidelines[22]. In brief, a final inoculum of 5×10^5 colony-forming unit/mL (CFU/mL) of MRSA cells was added into 96-well plates containing two-fold diluted HYP and β-lactam antibiotics in cation-adjusted Mueller Hinton (CAMH) broth (+2% NaCl for OXA). After incubation at 37 °C for 24 h, the combinational activity of HYP with β-lactam antibiotic was analyzed by FIC index[21]. The FIC was interpreted as follows: synergism, FIC $\leq$ 0.5; antagonism, FIC $>$ 4.0; and indifferent when 0.5 $<$ FIC $\leq$ 4[23].

2.4. In vitro time-kill curves

Time-killing experiments were performed using CAMH broth (+2% NaCl for OXA) with an initial inoculum of ~5×10^5 CFU/mL of MRSA cells in the presence of sub-MICs of β-lactam antibiotics (*e.g.*, 1/4 × MIC of OXA, CEF, and NAF) or HYP (at 1/16 × MIC or 1/8 × MIC) alone and in combination[21]. The concentrations of HYP and antibiotics were chosen based on the checkerboard assay results. Viability counts were performed at 0, 2, 4, 6, 8, 12 and 24 h of incubation at 37 °C. Synergistic effect was defined as a $\geq$2lg decrease of CFU/mL in combination *vs.* the most active drug alone at 24 h of incubation[24].

2.5. Biofilm formation

Biofilm formation of the study strains was performed as previously described[25,26]. Briefly, MRSA cells from fresh culture plates were washed and adjusted to a density of 0.5 McFarland standard and diluted 1:10 into brain heart infusion broth supplemented with 0.5% glucose. This suspension was transferred to 96-well tissue culture plates with study compound alone and in combination (1/16 × MIC or 1/8 × MIC of HYP; 1/256 × MIC of OXA, CEF, and NAF) exposure and incubated for 18 h at 37 °C. The specific concentrations of OXA and HYP were chosen based on the impact of the antibiotic alone on the biofilm formation (See Supporting Information Tables S1 and S2). After incubation, the wells were washed, air dried, and stained with safranin (0.1% in distilled water). The adhering dye was dissolved in 30% acetic acid, and absorption was measured at OD_{490nm} to quantify biofilm formation[5,26].

2.6. Adherence to fibronectin

Fibronectin adherence assay was performed as previously described[5]. Briefly, 6-well tissue culture plates were coated with purified human fibronectin (50 mg/L, Sigma Chemicals, St. Louis, USA) for overnight at 4 °C and then treated with 3% bovine serum albumin (Sigma Chemicals) for 3 h at 37 °C to prevent nonspecific adhesion. Overnight cultured MRSA cells with/without HYP (1/16 × MIC or 1/8 × MIC), OXA (1/256 × MIC) alone and in combination were adjusted to $OD_{600\ nm} = 1.0$ ($\sim 10^9$ CFU/mL) and subsequently diluted 1:100 into fresh TSB with the same exposures of the compounds as for the overnight culture and incubated at 37 °C to $OD_{600\ nm} \approx 0.5$. Then the MRSA cells ($\sim 10^3$ CFU/well) were added into the plates and incubated for 1 h. After 1 h incubation, plates were washed with PBS, TSB agar was added into each well, and incubated overnight at 37 °C. Adherence to fibronectin was expressed as the percentage (±standard deviation [SD]) of the initial inoculum bound as previously described[27].

2.7. Transcription analyses by quantitative real-time PCR (qRT-PCR)

Exponential phase of MRSA cells with/without HYP and/or OXA exposure as descripted in Section 2.6 above were used for the isolation of total RNA by using a RNeasy kit (Qiagen, Los Angeles, CA, USA) as described previously[10]. Briefly, 2 μg of DNase treated RNA was transcribed into complementary DNA. The amplification of *sarA, agr RNAIII, mecA, fnbA, hla* and *gyrB* were performed using primers as described previously (see Table 1)[7,28,29]. qRT-PCR was performed using an ABI Prism 7000 instrument (Applied Biosystems, Los Angeles, CA, USA) and SYBR green PCR master kit (Applied Biosystems). *gyrB* was used as a control to normalize for transcript quantification. Relative quantification was calculated by the $\Delta\Delta C_T$ method.

2.8. Determination the impact of HYP on SarA-mecA binding by a gel shift assay

Gel shift assay was performed to determine if SarA regulates *mecA* expression by directly binding to the *mecA* promoter, as *sarA* mutants exhibited increased OXA susceptibility *vs*. their respective isogenic MRSA parental strain[10]. Purified SarA protein was kindly provided by Dr. Ambrose Cheung at Dartmouth Medical School (Hanover, New Hampshire, USA)[30]. A 200 bp fragment encompassing the *mecA* promoter was generated by PCR amplification of JE2 DNA using the primer as listed in Table 1[31]. Then, the *mecA* promoter DNA was incubated at room temperature for 20 min with various amounts of purified SarA protein (*e.g*., 0.3, 0.6 and 1.2 mg/L) in binding buffer[30]. To determination the impact of HYP on Sar-*mecA* binding, the *mecA* promoter DNA and 1.2 mg/L purified SarA protein were incubated at room temperature for 20 min with various concentrations of HYP (*e.g*., 4 and 8 mg/L) in binding buffer. The reaction mixtures were analyzed in a 6% Tris-Glycine gel (Novex, San Diego, CA, USA). The band shifts were stained by SYBR® Green Electrophoretic Mobility-Shift Assay (EMSA) Kit (Panomics, Fermont, CA, USA) and detected by exposing to $UV_{302\ nm}$ following the manufacturer's instructions.

2.9. Cell cytotoxicity assay

Cell cytotoxicity was tested by the cell counting kit-8 (CCK-8) assay, as we previously reported[32,33]. Briefly, human embryonic kidney 293 (HEK-293) cells were plated in a 96-well plate. After overnight incubation, different concentrations of HYP were added. After 24 h of incubation, the cells were treated with CCK-8 assay reagent, and OD at 450 nm was measured.

Table 1 Primers used in this study.

Primer	Sequence	Purpose
sarA	Forward: 5′-TCTTGTTAATGCACAACAACGTAA-3′ Reverse: 5′-TGTTTGCTTCAGTGATTCGTTT-3′	RT-PCR
fnbA	Forward: 5′-CGACACAACCTCAAGACAATAGCGG-3′ Reverse: 5′-CGTGGCTTACTTTCTGATGCCGTTC-3′	RT-PCR
mecA	Forward: 5′-TCCAGATTACAACTTCACCAGG-3′ Reverse: 5′-CCACTTCATATCTTGTAACG-3′	RT-PCR
RNAIII	Forward: 5′-AATTAGCAAGTGAGTAACATTTGCTAGT-3′ Reverse: 5′-GATGTTGTTTACGATAGCTTACATGC-3′	RT-PCR
hla	Forward: 5′-ACAATTTTAGAGAGCCCAACTGAT-3′ Reverse: 5′-TCCCCAATTTTGATTCACCAT-3′	RT-PCR
gyrB	Forward: 5′-CGCAGGCGATTTTACCATTA-3′ Reverse: 5′-GCTTTCGCTAGATCAAAGTCG-3′	RT-PCR
mecA promoter	Forward: 5′-ATATCGTGAGCAATGAAC TG-3′ Reverse: 5′-TATATACCAAACCCGACAAC-3′	Gel shift

2.10. Murine bacteremia model due to MRSA strain

To further define the effect of HYP on OXA susceptibility against MRSA *in vivo*, a well-characterized murine bacteremia model was used[34]. All the animal studies complied with the ARRIVE guidelines[35] and the Institutional Animal Care and Use Committee of Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center (Los Angeles, CA, USA) approved the animal study protocol. CD1 male mice (~6 weeks) were infected by using exponential phase of MRSA JE2 cells *via* tail vein (10^8 CFU/mouse). At 24 h post-infection, animals were randomized into one of the following groups: control (without treatment); HYP alone at 5 mg/kg, iv, once daily; OXA alone at 100 mg/kg, im, three times a day (tid); or HYP-OXA combination at the doses listed above. The HYP dose was selected according to previously reported regimen in murine model[36]. The treatment strategy of OXA encompassed dose-regimens used in prior studies in murine sepsis model[37]. Treatment lasted for 3 days. The control animals were euthanized at 24 h post-infection in order to determine the MRSA density in target tissues (*e.g.*, blood, spleen and kidney) at the beginning of treatment. Antibiotic treated animals were sacrificed at 24 h after the last treatment dose. At sacrifice, the target organs (*e.g.*, blood, kidney and spleen) were removed and quantitatively cultured. The mean lg(CFU/g) of tissue or lg(CFU/mL) of blood (±SD) was calculated for each group for statistical comparisons.

2.11. Statistic analyses

All *in vitro* assays were conducted with at least two biological replicates in triplicate. Two-tailed Student's *t*-test was used to analyze the *in vitro* data and *S. aureus* counts in the target tissues in the murine bacteremia model between different groups. Data were expressed as Mean (±SD). P values < 0.05 were considered significant. No adjustment was made for all the P values reported in this study.

3. Results

3.1. MICs and FIC index

As expected, the MRSA strain JE2 was resistant to OXA, CFZ and NAF with MICs ranging 32−256 mg/L (Table 2). The MIC of HYP against the JE2 strain was 64 mg/L (Table 2). A synergistic activity was observed in the combination of HYP with the study β-lactam antibiotics against the JE2 stain with FIC index values ranged 0.10−0.19 (Table 2). In addition, the effect of HYP in combination with other SOC antibiotics against the study MRSA strain was tested. Except amoxicillin, ciprofloxacin, and streptomycin, all the antibiotics showed a reduction in MIC values when used in combination with 1/8 × MIC of HYP (See Supporting Information Table S3).

Table 2 MICs of HYP, OXA, NAF and CFZ, and FIC index of the combination of HYP with the study β-lactam antibiotics against MRSA strain JE2.

MICs (mg/L)				FIC index		
				HYP + β-lactam		
HYP	OXA	CFZ	NAF	OXA	CFZ	NAF
64	256	128	32	0.10	0.19	0.19

Table 3 MICs and FIC index of OXA in the presence of HYP against MRSA JE2 wild-type, its isogenic *sarA*, *mecA* mutants and the *sarA* mutant complemented with *mecA* strains.

Strain	MIC (mg/L)			FIC index
	HYP	OXA	OXA+1/8 × MIC HYP	
JE2 wild-type	64	256	8	0.16
ΔsarA in JE2	32	4	2	0.56
ΔmecA in JE2	64	0.5	0.25	0.63
ΔsarA/pmecA in JE2	64	128	64	0.56

In addition, as we reported recently[10], the *sarA* and *mecA* mutants in the JE2 background had significantly decreased OXA MICs, and the *sarA* mutant with plasmid *mecA* complemented strain returned the OXA MIC value to near its parental JE2 level (Table 3). In the current study, we found that the MICs of HYP on the JE2 strain set were similar with MICs ranging 32−64 mg/L. Importantly, addition of 1/8 × MIC HYP significantly reduced the MIC of OXA on the JE2 parental strain (decreased OXA MICs

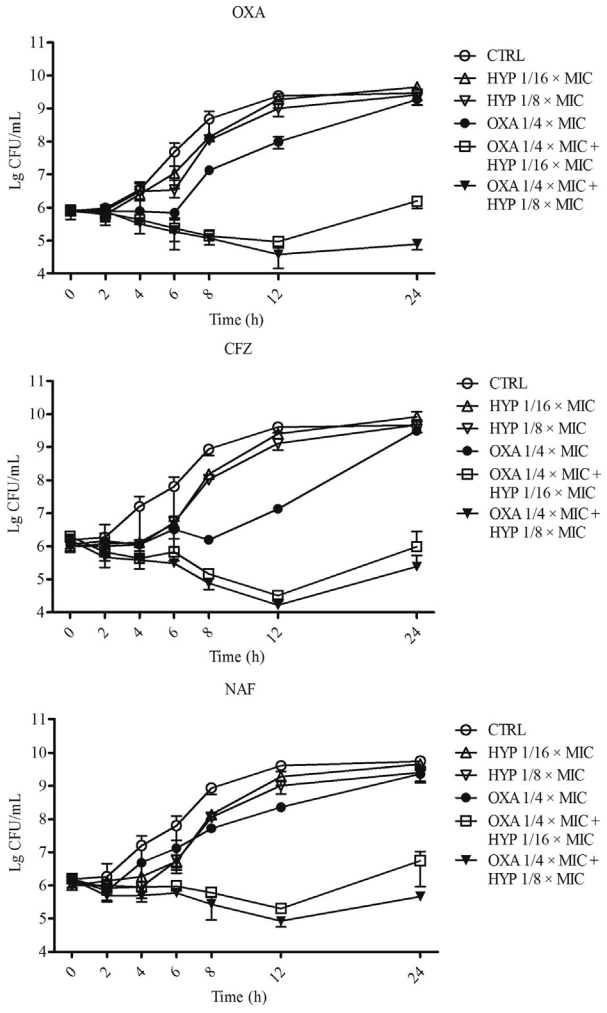

Figure 1 *In vitro* time−killing curves of HYP, OXA, CFZ or NAF alone; and the combination of HYP with OXA, CFZ or NAF against MRSA JE2 strain. The data are the mean ± SD of MRSA counts in each group of at least two biological replicates.

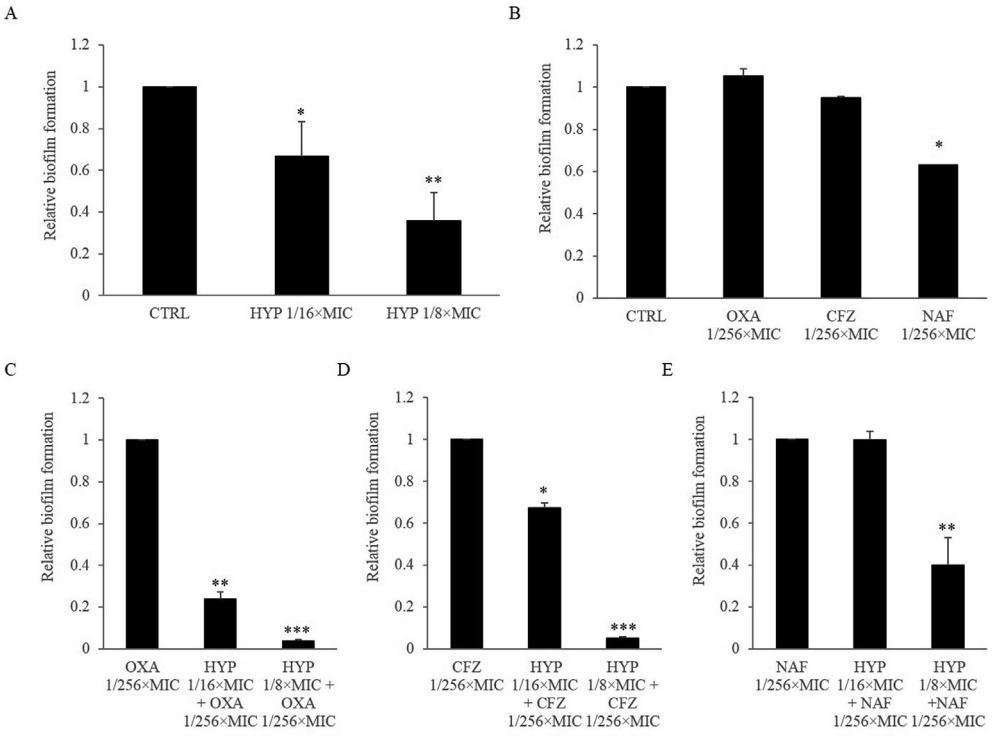

Figure 2 The effect of HYP (penal A), β-lactam alone (penal B), and the combination of HYP with OXA (penal C), CFZ (penal D), or NAF (penal E) on biofilm formation in MRSA. Control or β-lactam alone groups were set up as 1. Relative biofilm formation levels were represented as mean ± SD of at least two biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Penals A and B *vs.* control; Penal C, D and E *vs.* β-lactam alone.

from 256 mg/L to 8 mg/L), and showed synergistic effect with FIC index of 0.16 (Table 3). However, this synergistic effect was not observed on the mutant strains (Table 3; FIC index >0.5). These data indicate that the synergistic effect of HYP with OXA might be due to its impact on *sarA*, then subsequence on *mecA*.

3.2. Time−killing curves

The time−killing profiles of HYP, OXA, CFZ, and NAF alone, as well as in combination against the MRSA strain JE2 are presented in Fig. 1. Control cultures without antibiotic exposure increased ~4lg(CFU/mL) over the 24 h of incubation. HYP and β-lactam antibiotics alone at sub-MIC concentrations showed minor inhibition effect on the growth of the bacteria during 12 h of incubation, while had similar bacterial growths *vs.* the control groups at 24 h of incubation. Importantly, the combinations of HYP with OXA, CFZ, or NAF demonstrated synergistic effect against the MRSA strain JE2 with ~2lg(CFU/mL) killing compared to their respective most active single drug exposure during 12 h incubation. More importantly, the combinations resulted >2lg(CFU/mL) reduction *vs.* HYP and β-lactams alone at 24 h time point (Fig. 1).

3.3. The effect of HYP, β-lactam antibiotics alone and in combination on biofilm formation

HYP alone at sub-MIC levels resulted significantly less biofilm formation than control group in a dose-dependent manner in MRSA strain JE2 (Fig. 2A). In addition, sub-MIC of NAF had significantly effect on the reduction of biofilm formation as compared to the control group, while OXA and CFZ alone had similar biofilm formation *vs.* the control (Fig. 2B). Of note, the combination of HYP with the study β-lactams at sub-MIC levels significantly reduced biofilm formation in a dose-dependent manner as compared to β-lactams alone (Fig. 2C−E for OXA, CFZ and NAF, respectively), except the combination of HYP at 1/16 × MIC with NAF at 1/256 × MIC (Fig. 2E).

3.4. The effect of HYP, OXA alone and in combination on fibronectin binding

As shown in Fig. 3, in the presence of sub-MICs of HYP, the MRSA strain JE2 showed significantly decreased capability of fibronectin binding in a dose-dependent manner *vs.* the control group. Interestingly, sub-MIC of OXA exposure showed

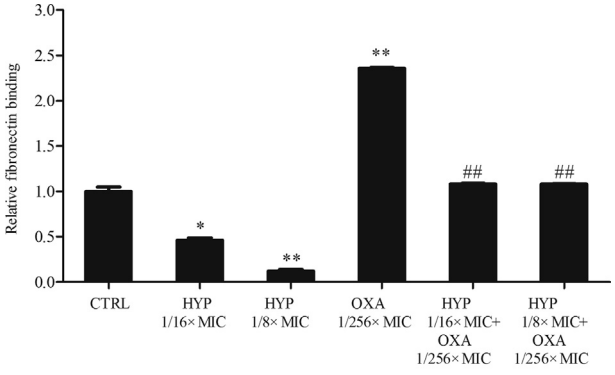

Figure 3 The effect of HYP or OXA alone and in combination on fibronectin binding in MRSA strain JE2. Control group was set up as 1. Relative fibronectin binding levels were represented as mean ± SD of at least two biological replicates. $^{*}P < 0.01$, $^{**}P < 0.001$ *vs.* control; $^{\#\#}P < 0.001$ *vs.* OXA alone.

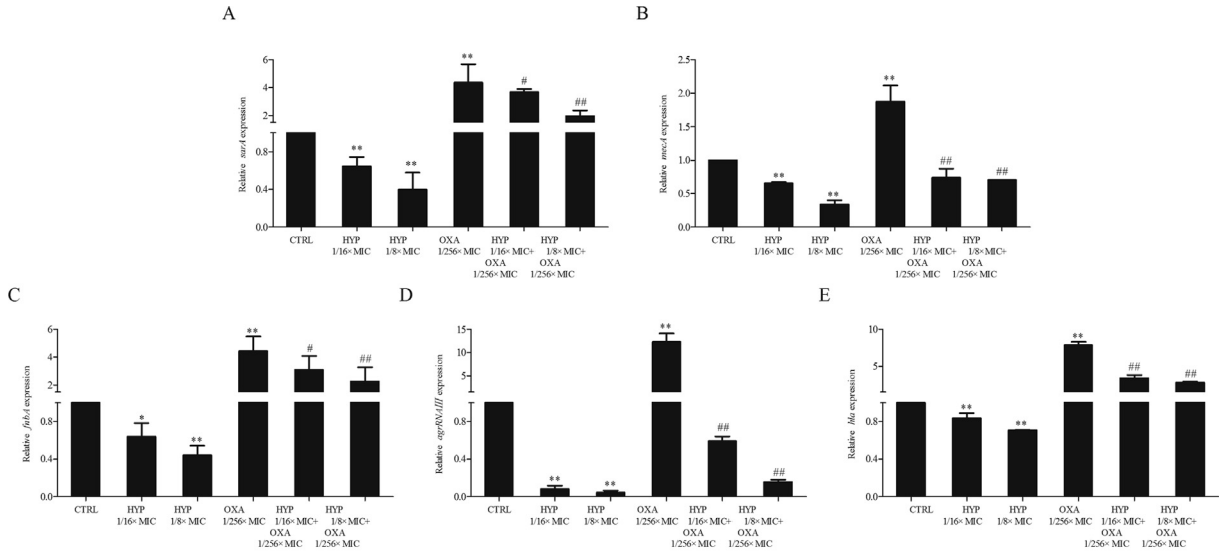

Figure 4 The effect of HYP or OXA alone; and in combinations on *sarA* (Penal A), *mecA* (Penal B), *fnbA* (Penal C), *agrRNAIII* (Penal D) and *hla* (Penal E) expressions in MRSA. Control group was set up as 1. Relative transcript levels of *sarA, mecA, fnbA, agrRNAIII,* and *hla* were represented as mean ± SD of at least two biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. OXA alone.

significantly increased binding to fibronectin as compared with the control group (Fig. 3). Of importance, the combination of HYP with OXA significantly decreased the fibronectin binding ability of the JE2 strain *vs*. OXA alone exposure (Fig. 3).

3.5. *The effects of HYP, OXA alone and in combination on sarA, mecA and virulence related genes expression*

It is known that *mecA,* encoding penicillin binding protein 2a (PBP2a), mediates the resistance to β-lactam antibiotics in MRSA[35]. In addition, we have recently demonstrated the important role of the global regulator, *sarA*, in β-lactam antibiotics resistance in MRSA[10]. In the current study, we found that HYP at sub-MIC levels significantly decreased *sarA* and *mecA* expressions as compared to their respective control groups (Fig. 4A and B for *sarA* and *mecA* expression, respectively). Consistent with our previous findings[10,38], sub-MICs of OXA exposure significantly induced *sarA* and *mecA* expressions *vs*. their respective controls (Fig. 4A and B for *sarA* and *mecA* expression, respectively). Interestingly, the combination of HYP and OXA significantly reduced the expression of *sarA* and *mecA* as compared with OXA alone (Fig. 4A and B for *sarA* and *mecA* expression, respectively). More importantly, HYP also significantly decreased *agr RNAIII*, which is a well-known *sarA* downstream key regulator and other virulence related genes (*e.g.*, *fnbA* and *hla*) expression as compared to their respective control groups. Although sub-MIC of OXA exposure significantly induced these genes expression, the combination groups significantly reduced these genes expression as compared with OXA alone (Fig. 4 D and E).

3.6. *The impact of HYP on the SarA-mecA binding*

A direct binding of SarA to the *mecA* promoter fragment was observed in a SarA protein concentration dependent manner (Fig. 5). For instance, a nearly complete shift occurred in the presence of 1.2 μg of SarA protein. Of importance, sub-MICs of HYP substantially reduced the SarA−*mecA* binding (Fig. 5). These results indicate that SarA positively controls *mecA* expression through its direct binding to *mecA* promoter, and HYP decreases the binding ability of SarA to *mecA* promoter.

3.7. *The cytotoxic effect of HYP on HEK-293*

The cytotoxic effect of HYP on HEK-293 cells was investigated by using CCK-8 assay. The results showed that HEK-293 cells were well tolerated to HYP at concentrations tested (Supporting Information Fig. S1).

3.8. *Therapeutic efficacy of HYP, OXA alone and in combination in the murine bacteremia model*

HYP monotherapy showed no significant decreased in MRSA densities in kidney and spleen *vs*. the untreated controls, while blood samples from mice treated with HYP had significantly lower MRSA densities than those from the control animals (Fig. 6). Treatment of OXA alone resulted significant reduced MRSA density in all target tissues as compared with the control groups (Fig. 6). Of great interest, the combination of HYP and

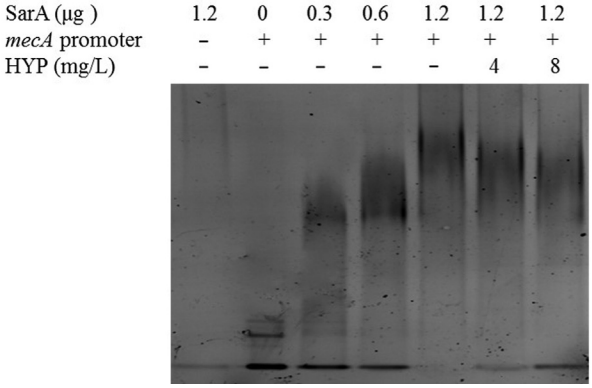

Figure 5 The effect of HYP on SarA binding ability to the *mecA* promoter.

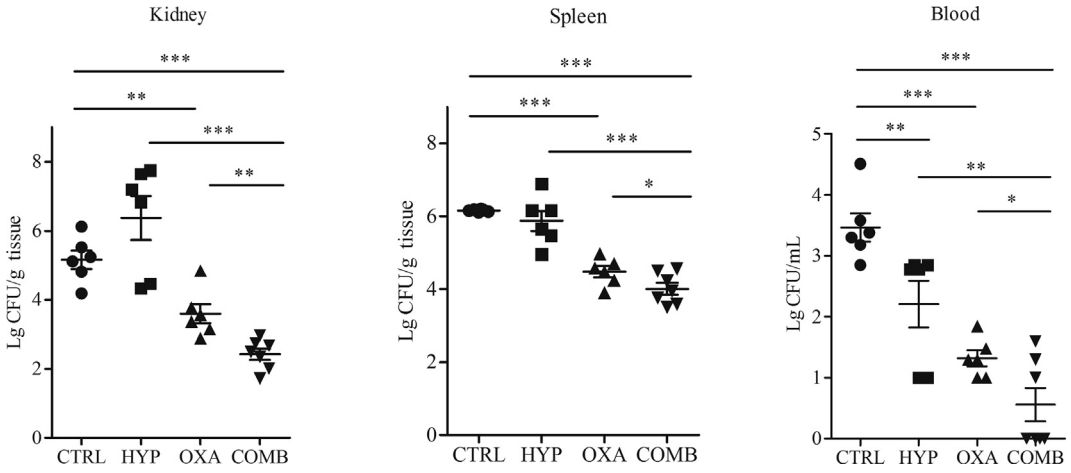

Figure 6 Therapeutic efficacy of HYP and OXA alone, and in combination in a mouse bacteremia model due to MRSA strain JE2. Each dot represents MRSA density in target tissues in the bacteremia model in one mouse ($n = 6$). Horizontal black bars indicate mean ± SD MRSA density in the target tissues. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

OXA exhibited significantly greater efficacy in reducing MRSA densities in all target tissues *vs*. all other three groups (Fig. 6).

4. Discussion

MRSA is a major cause of invasive infections (*e.g*., bacteremia) with unacceptable high morbidity and mortality since its emergency in 1960s[39]. Vancomycin and daptomycin have been considered as the first line of antibiotics against MRSA infections. However, many MRSA strains have developed resistance to these antibiotics[40,41]. Thus, an alternative treatment approach against MRSA infections is urgently needed. In our previous studies, the importance of *sarA* in β-lactams resistance was reported[10]. For instance, we demonstrated that *sarA* mutant strains became more susceptible to OXA as compared to their respective parental MRSA strains both *in vitro* and in an experimental endocarditis model. Hence, we speculated that *sarA* could be an optimal potential target to anti-MRSA.

In the current investigation, we observed that HYP had anti-MRSA activities with MIC of 64 mg/L. Of major importance, a synergistic effect of HYP with β-lactams (*e.g*., OXA, CFZ and NAF) was observed against MRSA strain JE2. In addition, the combination resulted ∼2lg(CFU/mL) reduction during 12 h incubation as compared with HYP or β-lactams alone group. However, regrowth was observed at 24 h incubation.

It is well known that *mecA* plays a key role in β-lactam resistance (*e.g*., OXA) in MRSA[42,43]. In addition, we recently demonstrated that *sarA* regulates β-lactam antibiotic resistance in MRSA both *in vitro* and in an experimental endocarditis model at least in part through its effect on *mecA* expression[10]. In this study, we observed that HYP exposure significantly decreased *sarA* and *mecA* expression. Although OXA alone significantly induced *sarA* and *mecA* expression, which was consistent with others previous reported[10,38,44], the addition of HYP significantly reduced *sarA* and *mecA* expression *vs*. OXA alone. To further investigate whether the synergistic effect of HYP and OXA was mediated by *sarA* and/or *mecA,* isogenic *sarA*, *mecA* and *sarA/pmecA* mutant strains in the JE2 background were used. Our results demonstrated that there were no synergistic effects between HYP and OXA against these mutant strains. Moreover, HYP exhibited inhibition effect on SarA−*mecA* promoter binding. Taken together, these data indicate that the synergistic effect at least in part was due to the impact of HYP on the inhibition of *sarA* activity, then subsequent on *mecA* expression.

Biofilm formation plays an important role in the pathogenesis of staphylococci infections[45]. Numerous studies have demonstrated that *sarA* is an important positive regulator on biofilm formation[5,46–48]. The mechanism partly due to *sarA* negatively regulate the activity of protease and nuclease[5], while positively control Bip[49] and PIA/PNAG production[50]. Consistent with the previous studies[10] , we found that JE2 *sarA* mutant strain formed significantly less biofilm than its isogenic parental strain. Of importance, HYP, as a *sarA* inhibitor, showed significantly reduced biofilm formation in a concentration-dependent manner in JE2 strain. These data were consistent with other studies showing that HYP and SarA inhibitor had anti-biofilm activity against *S. aureus* strains[6,15]. However, the specific mechanism of HYP against MRSA biofilm formation still need further studies.

SarA is an important transcriptional regulator that interacts with other regulators (*e.g*., *agr*) and controls many virulence genes (*e.g*., *mecA, fnbA* and *hla*) in *S. aureus*[51,52]. As reported from prior studies, SarA binds to *fnbA* promoter fragments, positively regulates FnBPs production and increases capacity of MRSA strains binding to fibronectin[5,53]. HYP, as a *sarA* inhibitor, significantly decreased *fnbA* expression and fibronectin binding capacity, which were consistent with previous results related to *sarA* mutants and other SarA inhibitor[5,7,54]. Of importance, the combination of HYP and OXA showed significantly decreased *fnbA* expression and fibronectin binding capacity *vs*. OXA alone. In addition, Sub-MIC of HYP exposure significantly decreased *agr RNAIII* and its downstream *hla* genes expression[6,7], while sub-MIC of OXA exposure significantly induced these genes expression[55,56]. Of importance, the combination of HYP and OXA showed significantly decreased these genes expression *vs*. OXA alone. Taken together, our data indicate that HYP, as a *sarA* inhibitor, significantly decreased *agr RNAIII* and its downstream virulence genes expression.

The most important finding in the current study was that the *in vitro* results were well translated into the *in vivo* treatment outcomes in the murine bacteremia model due to the study MRSA

strain. Similar MRSA densities in kidney and spleen were observed in the HYP monotherapy group *vs*. the untreated controls. However, OXA treatment alone had therapeutic efficacy with significantly reduced MRSA counts in all the target tissues as compared with the control group. These results were in agreement with previous studies using similar OXA treatment regimen and bacteremia model[37,38]. Of important, the combination of HYP with OXA exhibited significantly lower MRSA densities in the target tissues *vs*. OXA monotherapy. These combinational therapeutic effects were thought to occur by inhibition of *sarA*, which subsequently reduces *mecA* expression and OXA resistance. These data underscored the significance of HYP, as a *sarA* inhibitor, in the use with β-lactam antibiotic (*e.g*., OXA) for the treatment of bacteremia due to MRSA.

5. Conclusions

We demonstrated that HYP had inhibition effect on *sarA* expression, and subsequently downregulated the expression of *mecA,* and increased β-lactams susceptibility in MRSA. In addition, HYP, as a *sarA* inhibitor, significantly reduced biofilm formation, fibronectin binding and virulence-related gene expression. Notably, combination therapy regiments of HYP and OXA significantly enhanced *in vivo* efficacy of OXA in a murine bacteremia model due to MRSA. This combinational approach may present a novel treatment strategy against infections caused by MRSA strains by using anti-methicillin susceptible *S. aureus* (MSSA) β-lactam antibiotics.

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Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2019.05.002.

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