Research Article

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Future Medicinal Chemistry

Influence of subinhibitory concentrations of NH125 on biofilm formation & virulence factors of *Staphylococcus aureus*

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Aim: l-benzyl-3-cetyl-2-methylimidazolium iodide (NH125) can inhibit *Staphylococcus aureus* growth. We investigated the effects of sub-MIC concentrations of NH125 on *S. aureus* biofilm and virulence. **Methodology & results:** Three strains of *S. aureus* were tested. Sub-lethal concentrations of NH125 repressed biofilm formation. At partial sub-MICs, NH125 downregulated the expression of most virulence, while strain-dependent effects were found in the production of α-hemolysin, δ-hemolysin, coagulase and nuclease. In *Galleria mellonella* model, methicillin-resistant *S. aureus* pre-exposed to NH125 demonstrated significantly lower killing ($p = 0.032$ for 1/16 and 1/8 MICs; 0.008 for 1/4 MIC; and 0.001 for 1/2 MIC). **Conclusion:** Sub-MIC concentrations of NH125 inhibited biofilm formation and virulence of *S. aureus*. These findings provide further support for evaluating the clinical efficacy of NH125 in staphylococcal infection.

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Keywords: NH125 • *Staphylococcus aureus* • sub-MIC • virulence factor

Staphylococcus aureus is a major human pathogen that is associated with serious, deep-seated or systemic infections [1–3]. Treatment of staphylococcal infections is complicated by the development of antibiotic resistance. Moreover, the *S. aureus* pathogenicity involves multiple virulence factors, such as toxins, enzymes, adhesions, biofilm and gene-expression regulators [4,5]. These factors enable bacteria to adapt to different living environments and are involved in cell surface adhesion, immune evasion and tissue invasion [6,7].

The action of antimicrobial agents on bacterial virulence factor release, especially at suboptimal concentrations, is one of the pivotal aspects to determine their clinical efficacy [8]. A sub-lethal concentration of tigecycline has been demonstrated to reduce the expression of important virulence factors in *S. aureus*, which may be useful for the treatment of biofilm-mediated infections [7]. Other antibiotics at subinhibitory concentrations, such as tetracycline, erythromycin and quinupristin-dalfopristin, were also found to influence the staphylococcal virulence and biofilm formation [9]. Therefore, the exposure of bacteria to sub-MICs of antimicrobial agents is of great clinical importance as tissue levels of antimicrobial agents vary significantly due to variable blood perfusion of tissues, drug–drug interactions, systemic absorption of topically administered antibiotics, reduction of drug bioavailability or bacterial biofilm development [6].

NH125 (1-Hexadecyl-2-methyl-3-(phenylmethyl)-1H-imidazolium iodide) is an inhibitor of the WalK (a histidine kinase) two-component system (TCS), which is essential in the signal transduction pathway for the cell wall metabolism of notorious pathogens, such as *S. aureus* [10]. This WalK inhibitor has been demonstrated as a bactericidal agent on methicillin-resistant *S. aureus* (MRSA) strains [11–14]. Furthermore, we previously reported that NH125 can kill MRSA persisters, a very robust subpopulation within the *S. aureus* collection of cells that often reside within biofilms or are developed during the biofilm formation process, by inducing membrane perme-

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abilization. Additionally, it can elicit damage to MRSA biofilms [11,15]. Intriguingly, this compound exhibited low toxicity, whereby NH125 did not exhibit significant toxicity to *Caenorhabditis elegans* at 7.5 μg/ml and did not induce lysis of human red cells at 8 μg/ml [11,15].

TCS may directly or indirectly participate in many bacterial processes, including biofilm formation and virulence [16,17], therefore, making this a plausible target for drug development. Some compounds that inhibit WalK were found to have bactericidal effects on biofilm cells of *Staphylococcus epidermidis*, indicating that the inhibitors can serve as potential agents against bacterial biofilms [18]. Here, we investigated whether subinhibitory concentrations of TCS inhibitor NH125 could affect biofilm formation and virulence. Also, we gaged the effect of subinhibitory concentrations of NH125 on virulence using *Galleria mellonella in vivo* model.

Materials & methods

Bacterial strains & antibacterial compound

S. aureus strains MW2 (ATCCBAA-1707), USA300 and Newman were used for this study. The antibacterial compound NH125 was purchased from Sigma-Aldrich (MO, USA) and 5 mg/ml stock solutions of NH125 were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich).

Determination of MICs

MICs of NH125 for *S. aureus* strains were detected in triplicate using the broth microdilution method, according to standard Clinical and Laboratory Standards Institute (CLSI) protocols [19].

Growth curves

S. aureus strains were cultured at 37°C to an optical density (OD_{600}) of 0.1–0.2 in brain heart infusion broth (BHI, Sigma-Aldrich). Three-hundred microliters of the cultures with different subinhibitory concentrations of NH125 (MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC and 1/16 MIC) were placed into the wells of a 96-well plate (Corning, NY, USA) in three replicates for 24 h [20]. Cultures with 1% DMSO (v/v) served as the control. Bacteria were incubated at 37°C with shaking at 200 r.p.m. A SpectraMax M2 multi-mode microplate reader (Molecular Devices, CA, USA) was used to monitor cell growth at designated time points.

Hemolysis assay

S. aureus strains were grown in BHI broth at 37°C (with shaking at 200 r.p.m.) in the presence of graded subinhibitory concentrations of NH125 (1/2, 1/4, 1/8 and 1/16 MIC) to the postexponential growth phase. Culture supernatants were harvested by centrifugation and filtration. Levels of α-hemolysin (using defibrinated rabbit blood, Rockland Immunochemicals, PA, USA) and δ-hemolysin (using human erythrocytes, Rockland Immunochemicals) were measured in culture supernatants according to the method described by Kim *et al.* [11]. A 100 μl volume of 4% blood red cells was mixed with 100 μl of supernatant in a 96-well plate, and incubated for 1 h at 37°C. After being centrifuged at 500 × *g* for 5 min, 100 μl of the clear supernatant was removed to a new microtiter plate, and the OD value was read at 540 nm. Triton-X 100 (0.5%, final concentration) and phosphate-buffered saline (PBS) served as positive and negative controls, respectively. The supernatant of untreated cells (only containing 1% DMSO) was defined as the 100% hemolysis control, and the percent hemolysis was calculated against the control culture. All tests were carried out in triplicate.

Coagulase titer assay

Bacterial culture supernatants were obtained as described above under 'hemolysis assay'. The supernatants were serially diluted by twofold using BHI broth, and then mixed with iso-volumes (0.5 ml) of BBL lyophilized rabbit plasma (Becton Dickinson, MD, USA) solution. Clotting of the supernatants after incubation at 37°C for 4 h demonstrated the existence of coagulase. The titer was defined as the highest culture supernatant dilution to generate a detectable clot.

Protease activity assay

Proteolytic activity in the supernatants collected as described in the section of hemolysis assay was measured in triplicate [21]. One-hundred microliters of the culture were incubated with 1 ml chromogenic azocasein (1%, Sigma-Aldrich) for 1 h at 37°C. Then, 500 μl of 10% (w/v) trichloroacetic acid (Sigma-Aldrich) was added and incubated for 30 min to cease the reaction. The mixture was centrifuged for 10 min at 10,000 × *g*, and the OD

of the supernatant was measured at 328 nm. The results were reported as the percentage of proteolytic activity in relation to the proteolytic activity of the control.

Nuclease activity assay

Nuclease activity of cultures acquired as depicted above under 'hemolysis assay' was detected by using BBL DNase Test Agar with Methyl Green plate (Becton Dickinson). Twenty microliters of supernatants were added to BBL Blank Test Discs (Becton Dickinson). Then, the discs with bacteria supernatants were placed onto DNase Test Agar plates, and incubated at 37°C for 24 h. BHI broth was used as negative control, and the level of nuclease expression was evaluated by a zone diameter around the disc.

Lipase activity assay

Bacteria culture supernatants were prepared and harvested as described above. Lipase activity was measured in triplicate by detecting hydrolysis of 4-nitrophenyl octanoate in the supernatants [22]. Fifty microliters of culture were incubated with 1.2 ml of 4-nitrophenyl octanoate (Sigma-Aldrich) solution at 37°C for 15 min, and the value of OD was measured at 405 nm immediately. The lipase activity was expressed as a percentage of that of the NH125-free culture control.

Biofilm formation assay

Biofilm formation was detected in triplicate using crystal violet (CV) in a 96-well plate format with a slight modification [23]. Bacterial suspension (OD_{600} = 0.4, 0.5 McFarland standard) was diluted 1:100 in Mueller–Hinton (MH) medium with 2% glucose supplementation (Sigma-Aldrich; MH-G). NH125 dilution was made in MH at graded subinhibitory concentrations (1/2, 1/4, 1/8 and 1/16 MICs). Then, 100 µl of each of bacterial inoculum and the corresponding NH125 dilution were placed into each well of 96-well plates. Inoculated MH-G media with 1% DMSO alone were used as positive control. Plates were cultured statically at 37°C for 48 h. Planktonic bacteria were aspirated and the residual adhesive cells in each well was rinsed three-times with water and stained with 200 µl of 0.3% (w/v) CV (Sigma-Aldrich) for 10 min. The unincorporated CV was washed out and the plates were dried for 3 h at room temperature. The quantity of biofilm in each well was detected by extracting the incorporated dye using 200 µl of 100% ethanol for 30 min and the OD value was determined at 595 nm.

Quantitative reverse transcription polymerase chain reaction

The well-studied *S. aureus* strain MW2 was selected as a representative strain for assessing the effect of subinhibitory concentrations of NH125 on the expression of virulence genes involved in gene expression regulation, surface protein, polysaccharide intercellular adhesion and hemolysin. In brief, an overnight culture was inoculated into BHI medium at an OD_{600} = 0.1. Then, bacteria were incubated at 37°C until the culture reached an OD_{600} of 0.6 and aliquots were subsequently exposed for 3 h to graded subinhibitory concentrations of NH125 [24]. RNA extraction, cDNA synthesis and quantitative reverse transcription (RT)-PCR were carried out as recommended by the manufacturer (Bio-Rad, CA, USA) using the primers listed in Table 1. Cycling conditions were as follows: 95°C for 30 s; 40 cycles at 95°C for 5 s, 55°C for 30 s, and finished with a melt curve analysis from 65 to 95°C. All samples were examined in triplicate, and normalized with respect to the housekeeping gene *gyrA* expression. Relative gene expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method described in [24].

The *G. mellonella–S. aureus* infection model

The inoculum of *S. aureus* MW2 cells was treated with the graded sub-MICs of NH125 according to the method described above under 'Quantitative RT-PCR'. Bacteria were collected by centrifugation at 4°C, rinsed two-times with PBS containing the corresponding sub-MIC of compound, then resuspended in the corresponding PBS-sub-MIC NH125 solution at the density of 7.5×10^9 colony-forming unit/ml, respectively. *Galleriamellonella* larvae were randomly divided into seven groups (ten per group) for the infection experiments. Briefly, four groups of larvae were injected with 10 µl of treated bacteria suspension in PBS containing various sub-MICs of NH125, respectively. The remaining three groups of larvae used as controls were injected with 10 µl sterile PBS, 10 µl of 1 µg/ml of NH125 (1/2 MIC) and 10 µl of untreated bacterial suspension (7.5×10^9 colony-forming unit/ml), respectively. All *G. mellonella* groups were placed at 37°C and mortality was evaluated daily. *Galleria mellonella* experiments were carried out twice on separate occasions.

Table 1. Virulence genes and primers used for quantitative RT-PCR detection.

Gene	Function	Sequence of primer†	Size of product (bp)
hla	α-hemolysin	F: 5′-AATGAATCCTGTCGCTAATGCCGC-3′	269
		R: 5′-CTGAAGGCCAGGCTAAACCACTTT-3′	
hld (*RNA III*)	δ-hemolysin/gene expression regulator RNA III	F: 5′-TAATTAAGGAAGGAGTGATTTCAATG-3′	100
		R: 5′-TTTTTAGTGAATTTGTTCACTGTGTC-3′	
agrA	Regulator of gene expression	F: 5′-TGATAATCCTTATGAGGTGCTT-3′	164
		R: 5′-CACTGTGACTCGTAACGAAAA-3′	
arlS	Regulator of gene expression	F: 5′-TGGAATACCAATTCCATGATCT-3′	103
		R: 5′-TGCAATCAAATATGATGTGAAGAA-3′	
srrA	Regulator of gene expression	F: 5′-AGCATGTGTGGGAGGTATGA-3′	118
		R: 5′-CCTCTTGGCCATTACTTGCTT-3′	
saeS	Phosphorylated response regulator	F: 5′-ATCCGAACAACAAGAAAAAACAG-3′	110
		5′-TGATTATACCATCACGTAGTCCTTCA-3′	
yycG	Regulator of gene expression	F: 5′-TACAATCCCTTCATACTAAACTTGTAATTG-3′	198
		R: 5′-GTGCATTTACGGAGCCCTTTTCGTCATATAC-3′	
sasG	Surface protein	F: 5′-GGTTTTCAGGTCCTTTTGGAT-3′	192
		R: 5′-CTGGTGAAGAGCGAGTGAAA-3′	
spa	Surface protein for bacterial aggregation	F: 5′-GCGCAACACGATGAAGCTCAACAA -3′	125
		R: 5′-ACGTTAGCACTTTGGCTTGGATCA-3′	
clfA	Surface protein	F: 5′-CGGTTTTGGACTACTCAGCA-3′	151
		R: 5′- GCTACTGCCGATAAACTA-3′	
fnbA	Surface protein	F: 5′-ACTTGATTTTGTGTAGCCTTTTT-3′	185
		R: 5′-GAAGAAGCACCAAAAGCAGTA-3′	
fnbB	Surface protein	F: 5′-CGTTATTTGTAGTTGTTTGTGTT-3′	118
		R: 5′- TGGAATGGGACAAGAAAAAGAA-3′	
icaA	PIA or PNAG production	F: 5′-AACAGAGGTAAAGCCAACGCACTC-3′	85
		R: 5′-CGATAGTATCTGCATCCAAGCAC-3′	
sigB	Regulator of gene expression	F: 5′-TCAGCGGTTAGTTCATCGCTCACT-3′	156
		R: 5′-GTCCTTTGAACGGAAGTTTGAAGCC-3′	
codY	Regulator of gene expression	F: 5′-AAAGAAGCGCGCGATAAAGCTG-3′	120
		R: 5′-TGCGATTAATAGGCCTTCCGTACC-3′	
sarA	Regulator of gene expression	F: 5′-CCTCGCAACTGATAATCCTTATG-3′	173
		R: 5′-ACGAATTTCACTGCCTAATTTGA-3′	
spoVG	Modulator of gene expression	F: 5′-TGTTCGTTGCAATGCCAAGT-3′	51
		R: 5′-TGTCGCGGAATTCACCATC-3′	
rot	Transcriptional regulator	F: 5′-AAGAGCGTCCTGTTGACGAT-3′	126
		R: 5′-TTTGCATTGCTGTTGCTCTA-3′	
gyrA	DNA gyrase A subunit/internal standard gene	F: 5′-CATTGCCAGATGTTCGTGAC-3′	117
		R: 5′-CACCAACGATACGTGCTGAT-3′	

†The sequence of primers was designed based on the genome sequence of *Staphylococcus aureus* strain MW2 (Accession: BA000033.2).
bp: Base pair; PIA: Polysaccharide intercellular adhesion; PNAG: Polymeric N-acetylglucosamine.

Statistical analysis

Statistical analyses were performed using SPSS 20 software. Data comparisons for the production of hemolysis and exoenzymes, the formation of biofilm and the expression of virulence genes were analyzed by Student's t-test. Data regarding the influence of sub-MICs NH125 on the bacterial growth were made by one-way analysis of variance (ANOVA). Differences in *G. mellonella* survival rates were analyzed using the Kaplan–Meier method and survival curves were determined using log-rank test. A p-value <0.05 was considered significant.

Results & discussion

Influence of NH125 on *S. aureus* growth. The MIC of NH125 against three *S. aureus* strains (MW2, USA300 and Newman) was found to be 2 μg/ml for each of the tested strains. To assess the influence of sub-lethal concentrations of NH125 on the production of bacterial virulence factors, we initially investigated the effect on bacterial growth. As shown in Figure 1, the bacterial growth rates of all three *S. aureus* strains studied were significantly reduced by the addition of 1 μg/ml of NH125 in the media, a concentration that represents 1/2 MIC ($p < 0.001$). Li *et al.* [25] found a similar growth pattern of *S. aureus* treated by chlorogenic acid, which could significantly inhibit the growth at concentration of MIC and 1/2 MIC. In addition, the growth of *S. aureus* was reported to be remarkably attenuated by 1/2 MIC of antimicrobial agents, such as linezolid [8], eugenol [26] and licochalcone A [21]. However, when three *S. aureus* strains grew in the presence of 1/4, 1/8 or 1/16 MIC of NH125, growth was similar to that of the control. Thus, growth was diminished at 1/2 MIC, but was restored at lower concentrations of NH125.

NH125 inhibited the biofilm formation. Since sub-lethal concentrations only had modest effects on the overall growth rate of the bacteria, we sought to explore how the introduction of the compound to the bacteria could affect virulence traits that are employed during the infection process. Kim *et al.* already demonstrated the efficacy of NH125 against persister cells, another part of the sedentary cell population that form a subpopulation harbored within biofilms, where it permeabilized a normally tough membrane [11]. Thus, we speculated that NH125 might be effective against surrounding cells that comprise the biofilm. Our initial investigation focused on the effects to biofilm formation.

Biofilm formation is extremely important in staphylococcal pathogenesis and it has been associated with seeding chronic infections and establishing recurrent colonization [18]. We found that biofilm formation of the strain MW2 was markedly inhibited by sub-MIC concentrations of NH125 ($p = 0.005$ at 1/2 MIC, 0.011 at 1/4 MIC and 0.009 at 1/8 and 1/16 MICs). Similar reduction was noted when we examined the *S. aureus* strain USA300 ($p < 0.001$ at 1/2 MIC; $p = 0.027$ at 1/4 MIC, 0.016 at 1/8 MIC and 0.031 at 1/16 MICs; Figure 2). In the case of the Newman strain, NH125 reduced the development of biofilm at 1/8 MIC ($p = 0.042$), 1/4 MIC ($p = 0.031$) and 1/2 MIC ($p = 0.032$). Notably, the obvious decrease in biofilm formation, caused by 1/2 MIC of NH125, might partially be attributed to the inhibition on bacterial growth (Figure 1). More generally, inhibition of biofilm formation is ascribed to repression of proteins necessary for the biofilm, such as cell surface adhesions and exopolysaccharide synthases [27,28], or failure to maintain an adequate supportive structure [29]. Therefore, we further investigated the transcription levels of six biofilm-associated genes using RT-PCR. *ClfA* (encoding clumping factor A, ClfA) was significantly downregulated by 3.7-, 2.7- and 2.8-fold ($p = 0.003$ at 1/16 MIC, 0.007 at 1/8 MIC and 0.008 at 1/4 MIC; Figure 3). However, the expression levels of other biofilm-associated genes were increased after exposure to graded sub-MICs NH125. More specifically, *sasG* (encoding surface protein G) and *spa* (encoding protein A) were significantly upregulated in *S. aureus* MW2 cells treated with subinhibitory NH125 concentrations. The transcription levels of *icaA* (encoding intercellular adhesion A) were enhanced by 3.5-fold ($p = 0.001$), 2.2-fold ($p = 0.004$) and 3.0-fold ($p = 0.001$), when exposed to 1/16, 1/8 and 1/4 MIC of NH125, respectively. The expression levels of *fnbA* (encoding fibronectin binding protein A) were highly induced at the lower sub-MICs of NH125, while this compound had no prominent influence on *fnbB* (fibronectin binding protein B) expression. As described above, only *clfA* among the investigated genes was significantly repressed by all suboptimal concentrations of NH125, indicating that its downregulation could be a major factor to attenuate biofilm formation. Notably, ClfA is a fibrinogen-binding protein anchored to the *S. aureus* cell wall, and also plays an important role in binding of *S. aureus* in adhesion to both polyethylene and polyvinyl surfaces [30,31]. Accordingly, we reason that NH125 may interfere with cell wall metabolism and biofilm formation by at least partial inhibiting *clfA* expression so as to exert its antibiofilm effects.

Effects of NH125 on hemolysin and hemolysin gene. In *S. aureus*, TCS is involved in cell viability, biofilm formation and virulence [17]. Hemolysin, one of the main staphylococcal virulence factors, is a pore-forming protein with cytolytic, hemolytic and toxic characteristics that disintegrates erythrocytes and other mammalian cells [32,33]. We evaluated the effects of subinhibitory concentrations of NH125 on the production of α-hemolysin and δ-hemolysin by MRSA strains MW2, USA300 and Newman. The production of hemolysin by *S. aureus* MW2 cells was reduced by sub-MICs of NH125 in a dose-dependent manner (Table 2). Specifically, NH125 significantly reduced α- and δ-hemolysin within the MW2 strain, when exposed to all the sub-MIC concentrations. On the contrary, NH125 significantly increased δ-hemolysin in the USA300 strain at 1/4 MIC ($p = 0.027$) and 1/8 MIC ($p = 0.035$), while the α-hemolysin production kept stable at all tested concentrations of NH125. For the Newman

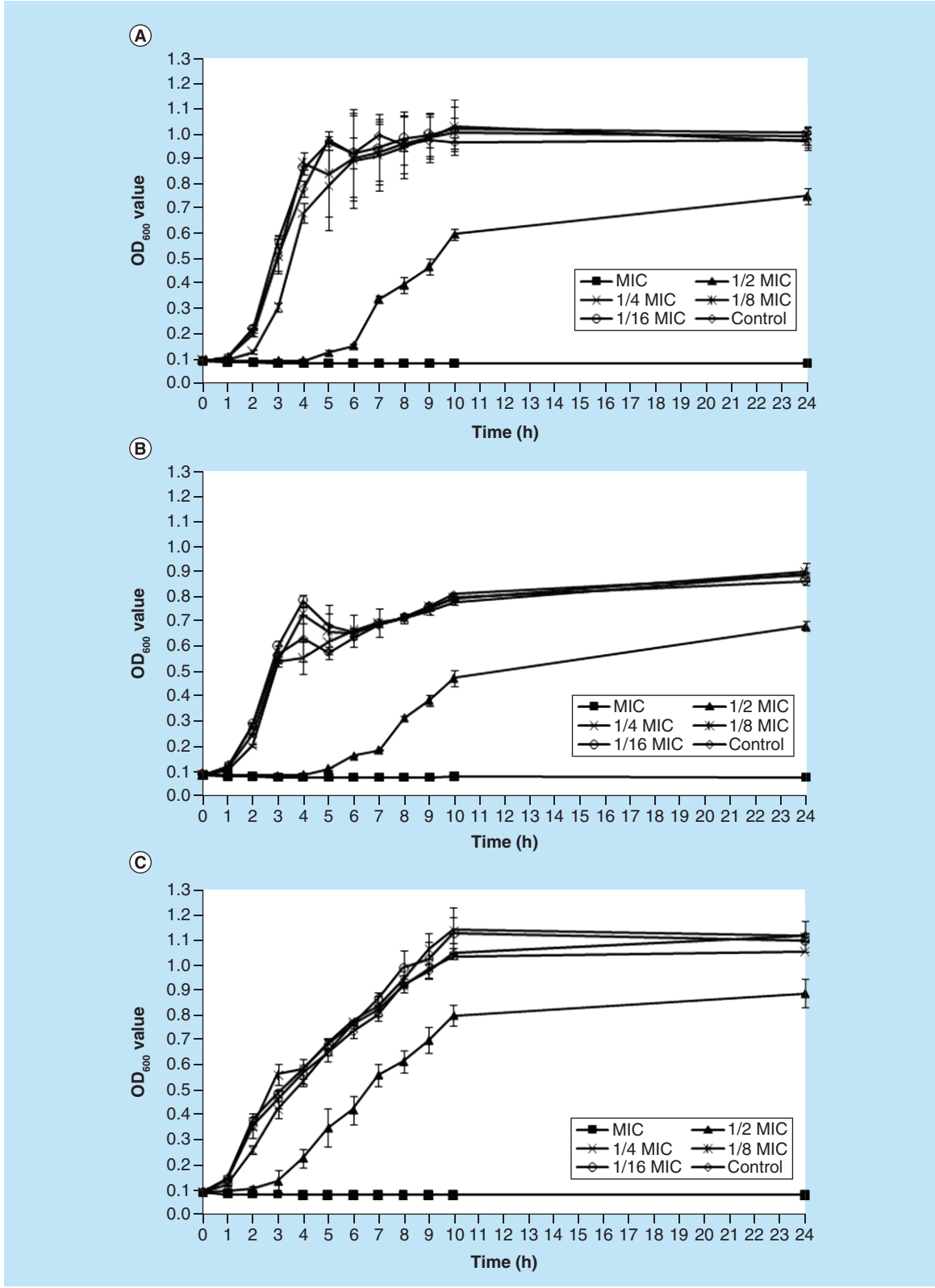

Figure 1. Growth curves of *Staphylococcus aureus* strains in the presence of graded subinhibitory concentrations of NH125. These curves represent the mean values of three repeat testing results. Error bars represent standard deviation. **(A)** Strain MW2; **(B)** strain USA300; **(C)** strain Newman; control strain treated by 1% DMSO.
OD: Optical density.

Table 2. Hemolytic and enzymatic activities of *Staphylococcus aureus* culture supernatants dealt with graded subinhibitory concentrations of NH125.

Toxin/enzyme	Strain MW2					Strain USA300					Strain Newman				
	1/2 MIC	1/4 MIC	1/8 MIC	1/16 MIC	Control	1/2 MIC	1/4 MIC	1/8 MIC	1/16 MIC	Control	1/2 MIC	1/4 MIC	1/8 MIC	1/16 MIC	Control
α-hemolysin[†]	48.8 ± 0.3[#]	55.9 ± 18.4[¶]	72.3 ± 7.3[¶]	64.4 ± 19.8[¶]	100	94.4 ± 7.8	98.9 ± 2.1	104.7 ± 3.9	106.2 ± 1.8	100	98.8 ± 3.2	115.9 ± 4.1[#]	102.3 ± 3.2	101.5 ± 3.7	100
δ-hemolysin[†]	22.4 ± 5.6[#]	39.2 ± 5.7[#]	50.7 ± 12.5[#]	146.4 ± 32.5	100	120.7 ± 19.5	156.6 ± 27.8[¶]	145.2 ± 24.0[¶]	103.2 ± 55.3	100	121.1 ± 9.1	130.7 ± 18.9	293.1 ± 28.1[#]	263.7 ± 18.0[#]	100
Coagulase titer[‡]	1:1	1:1	1:1	1:2	1:4	1:16	1:16	1: 32	1: 32	1:256	1:512	1:2048	1: 512	1: 512	1:256
Proteolytic activity[†]	96.2 ± 4.7	97.7 ± 6.2	98.0 ± 5.8	95.6 ± 5.8	100	97.8 ± 3.6	98.2 ± 5.8	99.7 ± 4.6	97.3 ± 3.2	100	102.0 ± 3.2	99.5 ± 4.9	101.5 ± 4.3	101.0 ± 3.3	100
Nuclease activity[§]	10.0 ± 0.9	10.0 ± 0.5	12.0 ± 0.5[#]	12.0 ± 0.9[#]	9.0	10.0 ± 0.5	10.0 ± 0.5	10.0 ± 0.9	12.0 ± 0.5[#]	9.0	11.0 ± 0.5	11.0 ± 0.9	11.0 ± 0.5	11.0 ± 0.5	10.0
Lipase activity[†]	81.9 ± 9.1	88.5 ± 4.5	86.4 ± 4.2	77.2 ± 13.6	100	93.3 ± 5.5	102.2 ± 5.3	99.7 ± 3.1	114.4 ± 12.0	100	102.4 ± 0.3	97.6 ± 3.4	101.0 ± 6.2	100.8 ± 2.5	100

[†]The data were expressed as percentage. The compound-free culture supernatants were used as the 100% control. Values were the means ± standard deviation of findings of three repeat experiments.
[‡]The titer is the highest dilution of sample causing coagulation.
[§]The data indicated the zone size (mm).
[¶]$p < 0.05$.
[#]$p < 0.01$, compared with the results of the corresponding control. Control strain treated with 1% DMSO.

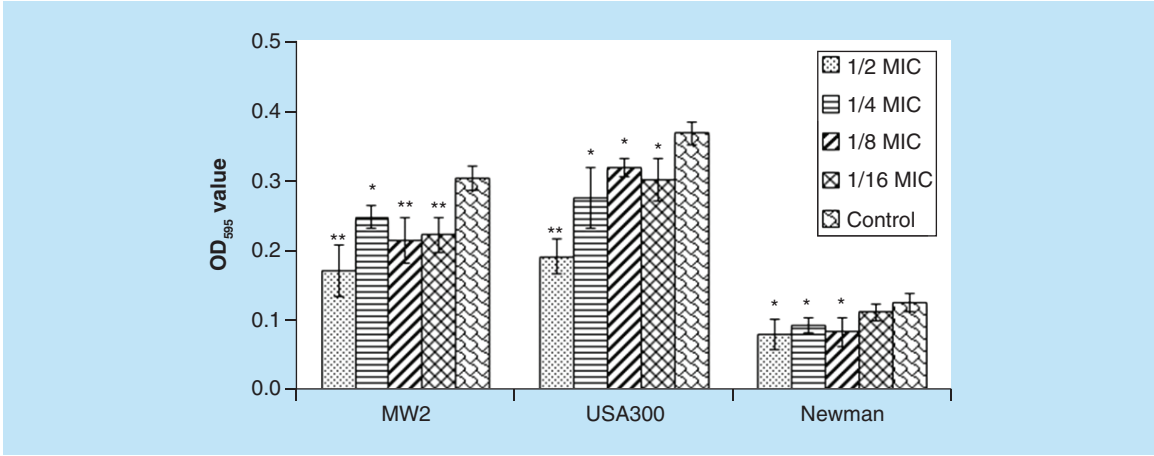

Figure 2. Influence of graded subinhibitory concentrations of NH125 on the biofilms formation of *Staphylococcus aureus* MW2, USA300 and Newman. The data are presented as mean ± standard deviation (three independent experiments). Control strain treated by 1% DMSO.
*$p < 0.05$ and **$p < 0.01$, compared with the results of the corresponding control.
OD: Optical density.

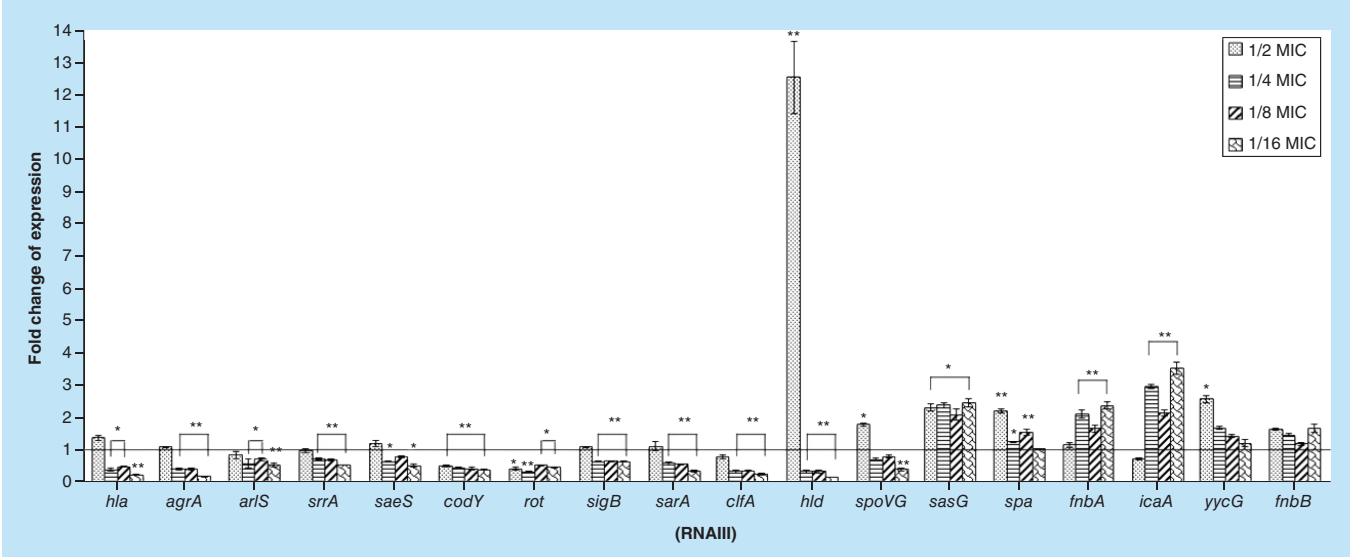

Figure 3. Influence of graded subinhibitory concentrations of NH125 on the virulence genes expression of *Staphylococcus aureus* MW2. The bar graph is a mean of three independent experiments. Error bars represent standard standard deviation. Control strain treated by 1% DMSO.
*$p < 0.05$ and **$p < 0.01$, compared with the results of the corresponding control.

strain, NH125 promoted the δ-hemolytic activities at 1/8 MIC ($p = 0.001$) and 1/16 ($p < 0.001$) MIC, and the α-hemolytic activity only at 1/4 MIC ($p = 0.004$; Table 2). Taken together, these results demonstrated that sub-MICs of NH125 affected the production of hemolysins by *S. aureus* in a strain-dependent manner.

To determine whether the reduced α-hemolysin and δ-hemolysin production in the presence of various subinhibitory concentrations of NH125 was due to the diminished transcription of *hla* (α-hemolysin) and *hld* (δ-hemolysin, encoded by *RNA III*), we performed real-time RT-PCR to determine the relative expression levels of the investigated genes in *S. aureus* MW2 after treatment with NH125. We found that the transcription of *hla* was downregulated by 4.2-fold ($p = 0.003$), 2.0-fold ($p = 0.035$) and 2.5-fold ($p = 0.016$) at 1/16, 1/8 and 1/4 MIC of NH125, respectively, where the transcription of *hld* was inhibited by 5.8-fold ($p < 0.001$), 2.9-fold ($p = 0.002$) and 2.8-fold ($p = 0.003$) (Figure 3). Notably, treatment with NH125 led to a significant increase by 12.6-fold in

the expression of *hld*. We speculated that NH125 might decrease the hemolysins by controlling other regulatory elements, such as global regulators, which were investigated in the series of experiments detailed below.

NH125 reduced coagulase production. Previous studies demonstrated that the subinhibitory concentrations of compounds with little or no influence on cell growth, could substantially affect the expression of bacterial virulence factors, such as hemolysin, coagulase, protease, nuclease and lipase [24–26,34]. Thus, we also studied the coagulase production and found that the graded subinhibitory concentrations of NH125 altered the production of coagulase in a strain- and dose- dependent manner (Table 2). More specifically, the coagulase titer in *S. aureus* strain MW2 was reduced by two- and foufolds at 1/8 and 1/16 MIC, whereas the increasing subinhibitory concentrations of NH125 led to a decrease of coagulase production in *S. aureus* USA300. As for the *S. aureus* strain Newman, however, the coagulase activities treated with NH125 at 1/2, 1/4, 1/8 and 1/16 MIC were two-, eight-, two- and twofold greater than that of the control, respectively. Interestingly, a strain-dependent difference in the production of coagulase has been previously reported, and Blevins *et al.* explained that this strain-dependent behavior might be associated with the mutations or deletions of some important regulatory elements [35].

Influence of NH125 on activities of protease, nuclease and lipase. Protease is another crucial virulence factor, which is correlated with the biofilm development in *S. aureus* [36]. Thus, we detected the activities of *S. aureus* extracellular enzymes, such as protease, lipase and nuclease, which are the critical virulence factors. As shown on Table 2, NH125 did not significantly affect the total activity of protease in all tested strains. Accordingly, we surmise that the decreased biofilm formation is not related to the protease-mediated destabilization of the biofilm.

In addition, nuclease and lipase are important for bacterial evasion of host response [37]. Our finding showed that sub-MICs of NH125 had no striking influence on the total activity of lipase by the strains studied. The nuclease activity in *S. aureus* strain Newman was quite stable after treated with grade sub-MIC of NH125, while the increased nuclease activities were observed in *S. aureus* MW2 at 1/8 MIC ($p = 0.002$) and 1/16 MIC ($p = 0.007$), and USA300 at 1/16 MIC ($p = 0.007$). This change may involve TCS-associated genes and global regulators, which control the expression of many virulence factors [7,25].

Effects of NH125 on TCS-associated gene and global regulator expression. The subinhibitory effects of NH125 are not just restricted to biofilm formation. The compound has more broad scope effects resulting in the altered transcription of other factors that culminate to affect virulence. subinhibitory concentrations of antibiotics may disturb the expression of modulatory determinants in *S. aureus* [34,38]. As depicted in Figure 3, following exposure NH125 at concentrations ranging from 1/16 to 1/4 MIC, the transcription levels of TCS-associated genes *agrA* (accessory gene regulator A), *arlS* and *srrA* (staphylococcal respiratory response A) in *S. aureus* MW2 were suppressed by 5.1-, 2.5- and 2.4-fold ($p < 0.001$ for all three concentrations), 1.8-fold ($p = 0.003$), 1.4-fold ($p = 0.013$) and 1.7-fold ($p = 0.029$), and 1.8-fold ($p = 0.001$), 1.4-fold ($p = 0.004$) and 1.4-fold ($p = 0.007$), respectively. The levels of expression of *saeS* (encoding *S. aureus* exoprotein expression S) were decreased by 1.5-fold ($p = 0.042$) and 1.9-fold ($p = 0.014$) at 1/4 and 1/16 MIC of NH125, respectively, whereas *yycG* (also called WalK) showed upregulation by 2.6-folds ($p = 0.014$) following the addition of 1/2 MIC NH125.

Among the tested global gene regulators, the expression levels of *codY* and *rot* (repressor of toxin) were significantly suppressed by all sub-MICs of NH125 ($p < 0.05$; Figure 3). The levels of transcription of *sigB* (alternative sigma factor B) and *sarA* (staphylococcus accessory regulator A) were remarkably decreased by the addition of all subinhibitory concentrations of NH125 but 1/2 MIC, respectively. The expression of *spoVG* (the stage V sporulation protein G) gene was also impacted by NH125 in a specific manner (promoted by 1.8-fold [$p = 0.015$] at 1/2 MIC, but reduced by 2.4-fold [$p = 0.005$] at 1/16 MIC).

Overall, 11 TCS genes and global regulatory genes, which are associated with the regulation of the expression of genes involving bacteria cell density sensing, interconnecting metabolism and virulence, showed dramatic alteration of expression in response to NH125 [14,22,39–45]. The transcription of the genes *agrA*, *saeS*, *arlS*, *srrA*, *sarA*, *sigB*, *codY* and *rot* was markedly decreased at different subinhibitory concentrations, while NH125 at different sub-MICs had the opposite impact on the expression of *spoVG* and *hld* (*RNAIII*). The expression of *agrA* and *sarA* was obviously downregulated at lower concentrations of NH125 and this finding is in accordance with reports that *S. aureus* strains expressing *agr*/*sarA* at low levels demonstrate increased capacity to form biofilm [46–48]. Also, it should be noted that NH125, as a YycG (WalK) repressor, strikingly enhanced the expression level of *yycG* at 1/2 MIC, indicating that NH125 inhibits the kinase activity of WalK and regulates kinase expression. Apparently, NH125 may also indirectly modulate the expression of *walK* by affecting the regulatory factors and NH125 is a nonspecific inhibitor that forms aggregates that bind to multiple proteins [49]. In this context, our finding that NH125 affected the expression of many virulence factors and their regulators may further provide evidence to support that the

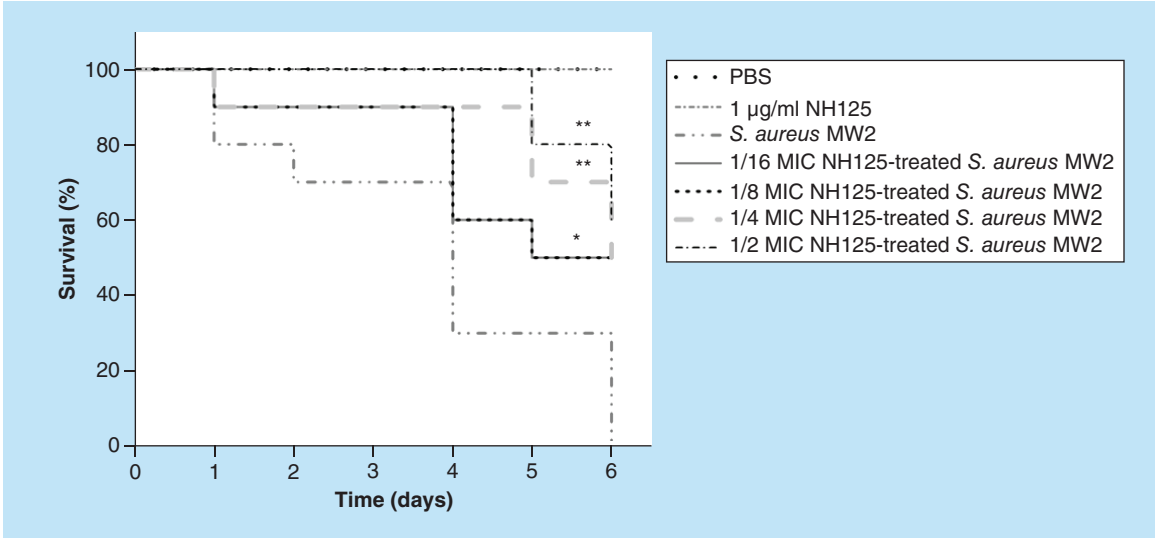

Figure 4. Survival curves for *Galleria mellonella* larvae inoculated with sub-MIC NH125-treated *Staphylococcus aureus MW2*. Phosphate-buffered saline and 1 μg/ml (1/2 MIC) of NH125 were injected alone as the control groups. n = 10/group and the experiments were done in duplicate.
*$p < 0.05$ and **$p < 0.01$, were considered as with a significant difference, compared with untreated bacteria-infected larvae.
PBS: Phosphate-buffered saline; *S. aureus*: *Staphylococcus aureus*.

mechanism might involve multiple regulators or regulatory pathways. Future studies should focus on exploring further the impact of this compound on regulatory pathways that alter the expression of virulence genes.

In vivo efficacy of NH125 against *S. aureus* virulence. The effects of subinhibitory NH125 on virulence traits were ultimately evaluated using the *in vivo* infection model *G. mellonella*, a host that has been successfully utilized to assess the virulence of bacterial pathogens [50,51]. To investigate the capacity of NH125 against virulence factors *in vivo*, the *G. mellonella* infection model were adopted to evaluate the effect of subinhibitory concentration of NH125 on *S. aureus* virulence. Herein, each larvae was infected with *S. aureus* MW2 cells that were pretreated with graded sub-lethal NH125. As shown in Figure 4, NH125 at the concentrations of 1/2 MIC ($p = 0.001$) and 1/4 MIC ($p = 0.008$) could remarkably prolong the survival of the infected larvae. Compared with the group infected by untreated bacteria, the survival rates on day 6 were increased by 50% when infected larvae were injected with 1/16 and 1/8 MICs of NH125 ($p = 0.032$). Therefore, sequential exposure of *S. aureus* MW2 to sub-MIC of NH125 could attenuate its virulence, corresponding to our conclusion *in vitro*. The elevated survival rate of infected *Galleria* further confirms the excellent performance of sub-lethal NH125, which provides important sight into combating the *S. aureus*.

Conclusion

NH125 could significantly reduce the formation of MRSA biofilms, and this was at least partially due to the reduction of *clfA* expression. In the presence of graded sub-MICs of NH125, a strain-dependent and concentration-dependent effect was found in the productions of the ubiquitous virulence factors, including α-hemolysin, δ-hemolysin, coagulase and nuclease. Accordingly, some virulence-related genes: *agrA*, *saeS*, *arlS*, *srrA*, *sarA*, *sigB* and *codY* were found to be repressed by the subinhibitory NH125, whereas the transcription levels of *spa*, *icaA*, *fnbA*, *sasG* and *yycG* were increased. Further, the subinhibitory concentrations of NH125 could reduce the virulence of *S. aureus* MW2 *in vivo*. Strains of *S. aureus* secrete a wide list of enzymes and cytotoxins, including hemolysins, nucleases, proteases, lipases and collagenase, while some strains produce one or more additional exoproteins like enterotoxins. Screening for additional virulence factors is beyond the scope of this work. However, future studies should evaluate the effect of NH125 (or other similar compounds) to other virulence factors.

Future perspective

Staphylococcus aureus forms biofilms and produces a wide variety of virulence factors, such as hemolysins, nucleases, proteases and lipases, which contribute to its ability to colonize and cause disease. Moreover, biofilm formation and the expression of these virulence factors hinder our ability to combat staphylococcal infections. Accordingly, the effect of antimicrobial agents on these factors has become the major focus in the study of antimicrobial agents. Herein, we studied the effects of sub-MICs NH125 on the biofilm formation and the ubiquitous and important virulence factors of *S. aureus*, such as hemolysins, nucleases, proteases, lipases and collagenase. Our results demonstrated that subinhibitory concentrations of NH125 not only inhibited the biofilm formation of *S. aureus*, but also attenuated the virulence *in vitro* and *in vivo*. Future works should evaluate the effect of NH125 (or other similar compounds) to other virulence factors and focus on exploring further the impact of this compound on regulatory pathways to explain why sub-lethal NH125 could alter the expression of certain virulence genes. Additionally, investigation with murine models should be conducted to determine whether NH125 has the therapeutic effects for the treatment of biofilm-mediated infections.

Summary points

- NH125, a WalK two-component system inhibitor, could inhibit biofilm formation of *Staphylococcus aureus*.
- In the presence of graded sub-MICs of NH125, a strain-dependent effect was found in the productions of α-hemolysin, δ-hemolysin, coagulase and nuclease.
- Sub-MICs of NH125 had no effect on the total activity of protease and lipase.
- At sub-MICs, NH125 repressed the expression of most tested virulence genes, including part of two-component system and global regulatory regulators.
- Using the alternative model host *Galleria mellonella*, we confirmed that NH125 reduces the virulence of *S. aureus* at sub-MICs *in vivo*.

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