Evaluation of Expression of *NorA* Efflux Pump in Ciprofloxacin Resistant *Staphylococcus aureus* against Hexahydroquinoline Derivative by Real-Time PCR

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Abstract- *Staphylococcus aureus* causes a wide variety of infections worldwide. Methicillin-resistant *S. aureus* is one of most common causes of nosocomial and community acquired infections. The fluoroquinolones are an important class of antibiotics that used to treat infections caused by *S. aureus*. Today, a significant increase in the rate of ciprofloxacin resistance in methicillin-resistant *S. aureus* strains is concerning. The *norA* efflux pump is considered as contributors to antibiotic resistance. Here, we aimed to evaluate the expression of *norA* efflux pump in the presence of hexahydroquinoline derivative in methicillin and ciprofloxacin resistant *S. aureus*. We were determined minimum inhibitory concentration of ciprofloxacin and hexahydroquinoline derivative and their combination by broth microdilution method against ciprofloxacin resistant *S. aureus*. The expression of the *norA* efflux pump gene was evaluated by quantitative Real-time PCR. This study showed that minimum inhibitory concentrations of ciprofloxacin in the presence of hexahydroquinoline derivative in comparison to ciprofloxacin were decreased. Quantitative Real-time PCR identified the increased expression of *norA* efflux pump gene in methicillin and ciprofloxacin resistant *S. aureus* strain. The increased expression of *norA* efflux pump gene may have resulted in the effort of *S. aureus* to survive. The results showed that the hexahydroquinoline derivative enhanced the antibacterial effect of ciprofloxacin against methicillin and ciprofloxacin resistant *S. aureus*. Therefore, the derivatives may be used as inhibitors of antibiotic resistance for combination therapy.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most important cause of both nosocomial and community-acquired infections. *S. aureus* is capable of causing these infections by using various virulence factors (1,2). To date MRSA strains show resistance to many antibacterial agents, and this may cause some limitations in the treatment of patients infected with these bacteria (3). In last decades, there has been increasing concern about bacterial resistance to antibacterial agents because of the counter availability, indiscriminate and inappropriate usage of antimicrobial agents (4,5). The discovery of fluoroquinolone antibiotics, especially ciprofloxacin, made possible the effective treatment of serious diseases caused by *S. aureus* even orally. Unfortunately, soon after the introduction of fluoroquinolone antibiotics for treatment of serious infections caused by *S. aureus*, various strains, especially the methicillin-resistant variety isolates, rapidly became resistant to these antibacterial agents (6,7).

At the present, ciprofloxacin resistant is increasing significantly in MRSA strains (8, 9). Even, in some surveys of nosocomial infections, resistance to ciprofloxacin have been reported to be more than 90

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percent of the *S. aureus* isolates and in some areas, this prevalence has increased to approximately 100 percent (10,11).

There are different mechanisms of antibiotic resistance in *S. aureus*. Among, some mechanisms efflux pumps that extrude antibiotics and decrease the intracellular concentration of the antibiotic are including the important mechanisms involved in the resistance to antibiotics (1,12). These efflux systems can cause resistance to a specific class of antibiotics or to a large number of different antimicrobial agents, which may lead to multidrug resistance (MDR) in bacteria (13).

Recently, many effort has been focused in the usage of inhibitors targeting bacterial resistance in combination therapy (14,15). These inhibitors, which are used in combination therapy, have abrogated resistance mechanism in bacteria and increased the effect of antimicrobial agents (16,17).

Consequently, these compounds are able also to reduce therapeutic doses of antibacterial agents (18,19). When used in combination, these agents are capable of modulating efflux systems and thus restore the activity of antibiotics showing to be promising in overcoming bacterial resistance (20).

Recently, some natural products have been reported which increase antimicrobial activities of different antibiotics such as nitrofurantoin, clindamycin, gentamicin, and the sulfonamides (21,22). Moreover, the effects of new synthetic compounds have been studied on antimicrobial activity of various antibiotics (23,24). Hexahydroquinoline derivative was initially produced by changing the structure of 1, 4-dihydropyridine such as in the above-mentioned synthetic compounds. Lak and colleagues studied the effect of hexahydroquinoline derivatives in clinical strains of *S. aureus* by using the agar disk diffusion method and reported increases in inhibition zone diameter for ciprofloxacin in the presence of these derivatives (25).

Therefore, the study in the field of the efflux pump modulators, since these compounds may increase the antimicrobial effect of antibiotics is important. Accordingly, the evaluation of how hexahydroquinoline derivatives effect the minimum inhibitory concentration (MIC) of ciprofloxacin and expression levels of *norA* efflux pump gene in the presence this derivative against multidrug resistant *S. aureus* is a part of that larger analysis and the impetus to implement this study.

Materials and Methods

Bacterial strain

The study was conducted withciprofloxacin and methicillin resistant clinical isolate of *S. aureus* collected at Shariati University Hospital, Tehran. The antibiotic resistance profile of the isolate was confirmed by the Kirby-Bauer disk-diffusion (MAST Co., UK) method.

Chemicals

The stock solution of hexahydroquinoline derivative (Prepared from Tehran University the Faculty of Pharmacy) was prepared in 100% dimethyl sulphoxide (DMSO), the highest final concentration of DMSO used in the assay (< 1%, v/v) caused no inhibition of bacterial growth. reserpine (final concentration, 20 µg/ml) and ciprofloxacin were purchased from Sigma Chemical Co. USA.

Susceptibility determination

The MICs were determined by the broth microdilution method with interpretation in accordance with CLSI guidelines and carried out in triplicate (26). Briefly, Mueller Hinton broth (Merck Co., Germany) containing two-fold concentration increments of antimicrobial agents were added to 96-well microdilution plates. Test organism suspension equal to a 0.5 McFarland standard was further diluted and added to the plates to achieve a final inoculum of 5×10^5 cfu/ml. The plates were incubated for 18 h at 37 °C in ambient air. For broth microdilution, the MIC was recorded as the lowest dilution showing no growth.

MIC of ciprofloxacin and hexahydroquinoline derivative were determined; then MIC of ciprofloxacin in combination with 1/2 and 1/4 MIC of hexahydroquinoline derivative were separately determined by the broth microdilution method against methicillin and ciprofloxacin resistant *S. aureus* (MCRSA). In this study, the standard ciprofloxacin sensitive *S. aureus* (ATCC8325/4) was used as control.

DNA extraction

DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen Co., Germany) according to the manufacturer, with the modification that 1.5 µl lysostaphin (5 mM) was added to the bacterial suspension. Finally, the purified DNA was used for PCR.

PCR amplification of efflux pump gene

The *S. aureus* strain resistant to methicillin and ciprofloxacin was screened for the presence of *norA* gene by PCR, using primers described in Table 1. PCR reaction was conducted on the final volume of 20 µl using HotStar Taq Master Mix kit (Qiagen Co., Germany) containing 10 µl of 2x HotStar Taq Master Mix, 2 µl of CoralLoad, 1 µl of the DNA template, 2 µl of each primer (20 pmol) and 3 µl of ddH_2O mixed.

DNA amplification was performed in a thermocycler (Eppendorf, Hamburg, Germany) with denaturation at 94 °C for 4 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and an extension at 72 °C for 1 minute plus a final extension at 72 °C for 4 minutes. The amplified products were electrophoresed on 1% gel agarose containing 1x GelRed DNA stain (Biotium, Inc. Co., USA).

Table 1. Primers used in this study

Primer	Sequence (5'-3')	Size (bp)	Ref.
NorA-Fw	TTCACCAAGCCATCAAAAAG	620	27
NorA-Rv	CTTGCCTTTCTCCAGCAATA		
NorA-RT- Fw	ATCGGTTTAGTAATACCAGTCTTGC	112	28
NorA-RT-Rv	GCGATATAATCATTTGAGATAACGC		
Gmk-RT- Fw	TATCAGGACCATCTGGAGTAGG	122	28
Gmk-RT-Rv	CATCAACTTCACCTTCACGC		

RNA extraction

The *S. aureus* strain was grown overnight in Mueller Hinton broth in an incubator shaker at 37 °C and 250 rpm with or without sublethal doses (1/2 and 1/4 MIC) of the hexahydroquinoline derivative (Reserpine was used as a standard).

RNA extraction from *S. aureus* strain was performed using High Pure RNA Isolation kit (Roche Co., Germany) according to the manufacturer's instructions. The RNA was DNase treated using DNase I (Fermentas, Sinagen Co., Iran) to remove remaining genomic DNA and complete removal of contaminating DNA was confirmed by PCR. Reverse transcription was performed with the QuantiTect Reverse Transcription (Qiagen Co., Germany) using 1 µg RNA.

Quantitative Real-Time PCR

Q-RT-PCR was performed in triplicate using a Power SYBR Green PCR Master Mix (Applied Biosystems Co., UK) on a StepOne ABI Real-time PCR equipment (Applied Biosystems, Foster City, CA). cDNA was used as a template in 20 µl reactions including 10 µl Power SYBR® Green PCR Master Mix (Applied Biosystems) and 2 pmol of each primer. The qPCR cycling was performed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 1 min at 60 °C and finally a melting stage to determine the unspecific PCR product or possible primer dimers. Triplets of a negative control were included in all qPCR runs, and *gmk* gene (guanylate kinase) was used as an endogenous control. The primers used in this study are described in Table 1. The relative expression of *norA* efflux pump gene was determined using $\Delta\Delta C_T$ method.

Results

Susceptibility determination

The MIC values of ciprofloxacin against methicillin and ciprofloxacin resistant *S. aureus* and ATCC8325/4 strains were 32 and 0.03125 µg/ml, respectively. Moreover, the MIC values of 60 and 30 µg/ml determined for hexahydroquinoline derivative against methicillin and ciprofloxacin resistant *S. aureus* and ATCC8325/4 strains, respectively.

Effect of hexahydroquinoline derivative on MICs of ciprofloxacin

MIC of ciprofloxacin in combination with 1/2 and 1/4 MIC of hexahydroquinoline derivative were separately determined by the broth microdilution method against MCRSA and ATCC8325/4. The MIC values of ciprofloxacin against MCRSA in combination with 1/2 and 1/4 MIC of hexahydroquinoline derivative decreased to four and two-fold, respectively (29). A reduction of the MICs to at least a quarter of their original values in the presence of the hexahydroquinoline derivative was considered as indicative of the presence of efflux activity. In studies on ATCC8325/4 strain, significant discrepancy in the MIC of ciprofloxacin in the presence of hexahydroquinoline derivative was not found in this strain.

Screening of norA efflux pump gene

In this research, gene that coded for the norA efflux pump in MCRSA strain were screened by PCR, using the primers described in Table 1.

Quantitative Real-Time PCR of norA

The relative expression of the *norA* efflux pump gene in MCRSA strain in the presence of hexahydroquinoline derivative and reserpine (as standard) evaluated byqRT-PCR analysis, which showed that *norA* in the presence of both compounds is overexpressed (Figure 1).

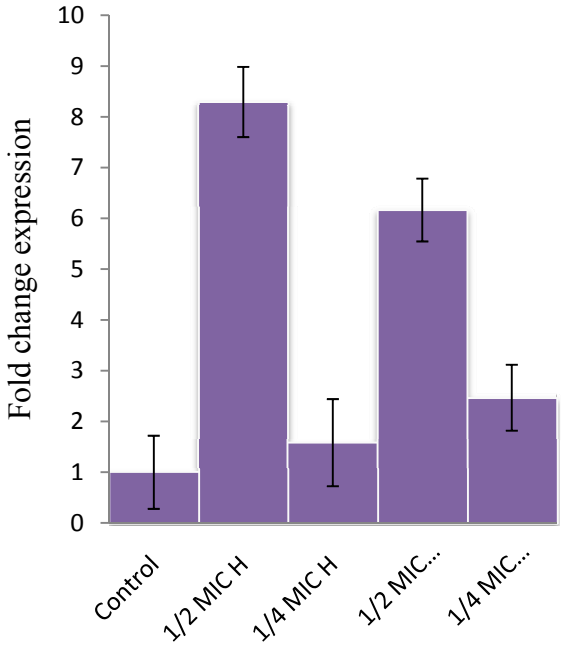

Figure 1. Relative expression of the norA efflux pump gene in MCRSA strain in presence and absence of hexahydroquinoline derivative. MCRSA: Methicillin and ciprofloxacin resistant Staphylococcus aureus; Control: Methicillin and ciprofloxacin resistant S. aureus untreated by hexahydroquinoline derivative; H: Hexahydroquinoline derivative; Reserpine: In this study used as the standard

Discussion

Emergence and spread of antibiotic resistance in bacteria have led to the substantial efforts on the discovery of new antibiotics and modulators of antibiotic resistance. There are different mechanisms of antibiotic resistance in *S. aureus*. One of the most important of those mechanisms is the efflux pumps, which extrude antibiotics and decrease the intracellular concentration of the antibiotic (1,12).

The used of inhibitors of bacterial resistance are a possibility for the treatment of the patient who has an infection caused by antibiotic-resistant bacteria. Using such inhibitors may provide re-treatment of patients by ineffective antibiotics in clinics and could prevent the emergence of new MDR strains (20).

To date, with the importance of the efflux pump in antibiotic resistance especially in multidrug resistant *S. aureus*, researchers have performed a number of studies in this field. For instance, in a study conducted by Kumar *et al*., novel structural analogues of piperine were evaluated as inhibitors of antibiotic extrude pumps, and it was shown that some of these compounds were capable of inhibiting *norA* pump in *S. aureus* (30). In another study, activity of synthetic materials Phenylpiperidine selective serotonin reuptake inhibitors (PSSRI) was investigated in *S. aureus*. The results indicated that compound 3-aryl piperidine increased significantly activity of antibiotics in *S. aureus* possessing the *norA* and *mepA* efflux pumps (31). Moreover, in a study conducted by Couto *et al*., overexpression of the *norA* efflux pump gene was reported in the presence of inhibitors (27).

The present study showed that the antimicrobial effect of ciprofloxacin against methicillin and ciprofloxacin resistant *S. aureus* strain increased in the presence of hexahydroquinoline derivative. This study also showed that expression of *norA* efflux pump gene increased while the hexahydroquinoline derivative was present in vitro. When the *S. aureus* encountered the hexahydroquinoline derivative in two concentrations, the expression of *norA* efflux pump increased markedly.

This increase may have resulted in the noticeable effort of bacteria for survival and growth in vitro. The increased expression of the efflux pump and intracellular concentrations reduction of hexahydroquinoline derivative in bacterial cells may lead to the survival of bacteria in the presence of non-lethal concentration of this derivative. Combination of hexahydroquinoline derivative and ciprofloxacin may cause changing antibiotic resistance in *S. aureus*. With these results, perhaps this compound may be used as inhibitors of antibiotics in combination therapy and reducing the effective therapeutic doses of antimicrobial agents.

In conclusion, the hexahydroquinoline derivative decreased the MIC of ciprofloxacin and increased expression of *norA* efflux pump gene, thus it is suggesting a study on the evaluation of direct effect of hexahydroquinoline derivative on *norA* efflux pump. Further evaluation of the toxicity of this derivative in animal models should also be considered in future studies.

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References

1. Costa SS, Falcao C, Viveiros M, et al. Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of Staphylococcus aureus. BMC Microbial 2011;11(1):241.
2. Havaei SA, Moghadam SO, Pourmand MR, et al. Prevalence of genes encoding bi-component leukocidins among clinical isolates of Methicillin-Resistant Staphylococcus aureus. Iran J Publ Health 2010;39(1):8-14.
3. Kurlenda J, Grinholc M. Alternative therapies in Staphylococcus aureus diseases. Acta biochim Pol 2012;59(2):171-84.
4. Neuhauser MM, Weinstein RA, Rydman R, et al. Antibiotic resistance among gram-negative bacilli in US intensive care units: implications for fluoroquinolone use. JAMA 2003;289(7):885-8.
5. Sahm DF, Critchley IA, Kelly LJ, et al. Evaluation of current activities of fluoroquinolones against gram-negative bacilli using centralized in vitro testing and electronic surveillance. Antimicrob Agents Chemother 2001;45(1):267-74.
6. Harnett N, Brown S, Krishnan C. Emergence of quinolone resistance among clinical isolates of methicillin-resistant Staphylococcus aureus in Ontario, Canada. Antimicrob Agents Chemother 1991;35(9):1911-3.
7. Mesak LR, Davies J. Phenotypic changes in ciprofloxacin-resistant Staphylococcus aureus. Res Microbiol 2009;160(10):785-91.
8. Acar JF, Goldstein FW. Trends in bacterial resistance to fluoroquinolones. Clin Infect Dis 1997;24(1):S67-73.
9. Schmitz FJ, Fluit AC, Brisse S, et al. Molecular epidemiology of quinolone resistance and comparative in vitro activities of new quinolones against European Staphylococcus aureus isolates. FEMS Immunol Med Mic 1999;26(3-4):281-7.
10. Drlica K, Malik M. Fluoroquinolones: action and resistance. Curr Top Med Chem 2003;3(3):249-82.
11. Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis 2005;41(Suppl 2):S120-6.
12. Savjani JK, Gajjar AK, Savjani KT. Mechanisms of resistance: useful tool to design antibacterial agents for drug-resistant bacteria. Mini-Rev Med Chem 2009;9(2):194-205.
13. Marquez B. Bacterial efflux systems and efflux pumps inhibitors. Biochimie 2005;87(12):1137-47.
14. Braga LC, Leite AA, Xavier KG, et al. Synergic interaction between pomegranate extract and antibiotics against Staphylococcus aureus. Can J Microbiol 2005;51(7):541-7.
15. Schito GC. The importance of the development of antibiotic resistance in Staphylococcus aureus. Clin Microbiol Infect 2006;12(Suppl 1):3-8.
16. Wright GD. Bacterial resistance to antibiotics: enzymatic degradation and modification. Adv Drug Deliver Rev 2005;57(10):1451-70.
17. Chan BCL, Ip M, Lau C, et al. Synergistic effects of baicalein with ciprofloxacin against NorA over-expressed methicillin-resistant Staphylococcus aureus (MRSA) and inhibition of MRSA pyruvate kinase. J Ethnopharmacol 2011;137(1):767-73.
18. Gibbons S. Plants as a source of bacterial resistance modulators and anti-infective agents. Phytochem Rev 2005;4(1):63-78.
19. Shin S, Pyun MS. Anti-Candida effects of estragole in combination with ketoconazole or amphotericin B. Phytother Res 2004;18(10):827-30.
20. Stavri M, Piddock LJV, Gibbons S. Bacterial efflux pump inhibitors from natural sources. J Antimicrob Chemoth 2007;59(6):1247-60.
21. Shahverdi AR, Rafii F, Tavassoli F, et al. Piperitone from Mentha longifolia var. chorodictya Rech F. reduces the nitrofurantoin resistance of strains of enterobacteriaceae. Phytother Res 2004;18(11):911-14.
22. Rafii F, Shahverdi AR. Comparison of essential oils from

three plants for enhancement of antimicrobial activity of nitrofurantoin against enterobacteria. Chemotherapy 2007;53(1):21-5.

23. Shahverdi AR, Monsef-Esfahani HR, Tavasoli F, et al. Trans-cinnamaldehyde from Cinnamomum zeylanicum bark essential oil reduces the clindamycin resistance of Clostridium difficile in vitro. J Food Sci 2007;72(1):55-8.
24. Laue H, Weiss L, Bernardi A, et al. In vitro activity of the novel diaminopyrimidine, iclaprim, in combination with folate inhibitors and other antimicrobials with different mechanisms of action. J Antimicrob Chemoth 2007;60(6):1391-4.
25. Lak P, Amini M, Safavi M, et al. Enhancement of the antibacterial activity of ciprofloxacin against Staphylococcus aureus by 3-alkyl esters and 3-aryl esters of hexahydroquinoline derivatives. Arznei-Forschung 2008;58(9):464-8.
26. Wayne PA. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 21th Inform Suppl 2011;31(1):M100-S21.
27. Couto I, Costa SS, Viveiros M, et al. Efflux-mediated response of Staphylococcus aureus exposed to ethidium bromide. J Antimicrob Chemoth 2008;62(3):504-13.
28. Ding Y, Onodera Y, Lee JC, et al. NorB, an efflux pump in Staphylococcus aureus strain MW2, contributes to bacterial fitness in abscesses. J Bacteriol 2008;190(21):7123-9.
29. Yousefi M, Pourmand MR, Shahverdi AR, et al. Minimum inhibitory concentration of ciprofloxacin in combination with hexahydroquinoline derivatives against Staphylococcus aureus. Tehran Univ Med J 2012;70(9):525-30.
30. Kumar A, Khan IA, Koul S, et al. Novel structural analogues of piperine as inhibitors of the NorA efflux pump of Staphylococcus aureus. J Antimicrob Chemoth 2008;61(6):1270-6.
31. German N, Kaatz GW, Kerns RJ. Synthesis and evaluation of PSSRI-based inhibitors of Staphylococcus aureus multidrug efflux pumps. Bioorg Med Chem Lett 2008;18(4):1368-73.