Acta Pharmacol Sin 2007 Nov; 28 (11): 1775–1782

Blackwell Publishing

Full-length article

Inhibition of β-lactamase-mediated oxacillin resistance in *Staphylococcus aureus* by a deoxyribozyme[1]

Zheng HOU[2], Jing-ru MENG[2], Jin-rong ZHAO[3], Ben-quan HU[2], Jie LIU[2], Xiao-jun YAN[3], Min JIA[2], Xiao-xing LUO[2,4]

[2]*Department of Pharmacology and* [3]*Institute of Genetic Diagnosis, The Fourth Military Medical University, Xi'an 710032, China*

Key words

methicillin-resistant *Staphylococcus aureus*; BlaR1; phosphorothioate deoxyribozyme; antibiotic resistance

[1] This work was supported by grants from the National Natural Science Foundation of China (No 30271556) and the Natural Science Foundation of Shaanxi Province (No 2002C2-04).

[4] Correspondence to Prof Xiao-xing LUO.
Phn/Fax 86-29-8477-4591.
E-mail xxluo3@fmmu.edu.cn

Received 2007-01-16
Accepted 2007-04-17

doi: 10.1111/j.1745-7254.2007.00646.x

Abstract

Aim: To investigate the oxacillin susceptibility restoration of methicillin-resistant *Staphylococcus aureus* (MRSA) by targeting the signaling pathway of *blaR1-blaZ* with a DNAzyme. **Methods:** A DNAzyme (named PS-DRz602) targeting *blaR*1 mRNA was designed and synthesized. After DRz602 was introduced into a MRSA strain WHO-2, the colony-forming units of WHO-2 on the Mueller-Hinton agar containing 6 mg/L oxacillin and the minimum inhibitory concentrations of oxacillin were determined. The inhibitory effects of DRz602 on the expressions of antibiotic-resistant gene *blaR1* and its downstream gene *blaZ* were detected by real time RT-PCR. **Results:** PS-DRz602 significantly decreased the transcription of *blaR*1 mRNA and led to the significant reduction of *blaZ* in a concentration-dependent manner. Consequently, the resistance of *S aureus* WHO-2 to the β-lactam antibiotic oxacillin was significantly inhibited. **Conclusion:** Our results indicated that blocking the *blaR1-blaZ* signaling pathway via DNAzyme might provide a viable strategy for inhibiting the resistance of MRSA to β-lactam antibiotics and that BlaR1 might be a potential target for pharmacological agents combating MRSA.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a major cause of nosocomial disease in the world, causing 50% or more of hospital-acquired *S aureus* infections in several countries[1]. In addition, recent reports have indicated that the epidemiology of MRSA may be undergoing a change through the emergence of community-acquired MRSA (CA-MRSA)[2,3]. CA-MRSA is capable of causing infections in otherwise healthy people and may have a serious or even fatal outcome[4,5]. According to the World Health Organization, more than 60% of *S aureus* strains worldwide are now resistant to methicillin. The mortality from severe MRSA infection is reported to be as high as 10%–34%[6,7].

Infections caused by MRSA are not effectively treated by most antibacterial agents and are a major challenge for chemotherapy because these bacteria show resistance to all β-lactam antibiotics. MRSA has traditionally been treated with glycopeptides, such as vancomycin. However, the emergence of vancomycin-intermediate or -resistant *Staphylococci* has spurred renewed efforts in the discovery of new targets in MRSA for novel antibacterial agents with mechanisms radically different from existing compounds[8].

S aureus resist the attack by β-lactam antibiotics in 2 ways: by producing β-lactamase, which inactivates the β-lactam antibiotics, and by expressing new transpeptidases, which are impervious to antibiotic activity. Two genes, *blaZ* encoding β-lactamase and *mecA* encoding the penicillin-binding protein PBP2a, render *S aureus* resistant to antibiotics, respectively[9]. The transcription of these genes is regulated by transmembrane signal sensor/transducer proteins (BlaR1 and MecR1) and their partner repressor proteins (BlaI and MecI). When a β-lactam antibiotic binds to the extracellular sensor domain of BlaR1, a conformational change within BlaR1 leads to autocatalytic activation of the integral-membrane metalloprotease domain. The active protease facing the cytosol specifically cleaves BlaI, directly or indirectly, which subsequently initiates *blaZ* expression. The genes for signal transducers and repressors are contained in a gene operon (*blaR1-blaI*) that is divergently transcribed from its

regulated gene *blaZ*[10]. This system highlights the key role of the methicillin sensor-transducer as the eventual transcriptional regulator of MRSA response[11,12]. Therefore, blocking the sensor/transducer pathway may represent a novel approach to reverse antibiotic resistance in MRSA.

In recent years, catalytic nucleic acids were composed entirely of DNA, which have been generated by *in vitro* selection strategies. These molecules ideally combine the catalytic activity of ribozymes with the stability of oligodeoxynucleotides. The typical deoxyribozyme or DNAzyme, known as the "10–23" model, is capable of cleaving a specific phosphodiester linkage between an unpaired purine (A, G) and a paired pyrimidine (C, U) under simulated physiological conditions. DNAzymes have considerable advantages over ribozymes in that they are easier to synthesize and less sensitive to chemical and enzymatic degradation than RNA-based reagents. They also exhibit greater catalytic efficiency than conventional hairpin and hammerhead ribozymes[13,14], so it is considered as an easily "drugable" tool to knock down target genes. In this study, we explored the use of a synthetic deoxyribozyme targeting *blaR*1 transcripts as a potential tool in inhibiting the expression of *blaR*1, blocking the signal pathway of *blaR1-blaI-blaZ* and thereafter leading to the reduction of BlaZ expression. The PS-DRz602 (anti-*blaR1* phosphorothioate deoxyribozyme) was found to reduce β-lactamase mRNA expression concomitantly and led to the restoration of the susceptibility of MRSA to oxacillin.

Materials and methods

Bacterial strain and electrocompetent *S aureus* preparation The strain of MRSA, WHO-2, obtained from Chinese National Center for Surveillance of Antimicrobial Resistance (Beijing, China) was used in the study. WHO-2 exhibited a moderate level of resistance to oxacillin (minimum inhibitory concentration [MIC]=32 μg/mL), in which the *mecR1-mecA*[15] and *blaR1-blaZ*[16] genes were detected by PCR. A methicillin-susceptible *S aureus* strain, ATCC (American Type Culture Collection) 29213, was used as a positive control.

Competent *S aureus* was prepared according to a previously published paper[17]. Briefly, 2 mL of overnight-cultured WHO-2 was transferred to 200 mL broth medium and incubated at 37 ºC with moderate agitation until the optical density $(OD)_{600}$ reached 0.55–0.65. The cells were centrifuged at 2817×*g* for 10 min at 4 ºC and the supernatant was removed. The pellet was washed by resuspending in ice-cold deionized water in the same volume of culture medium and the suspension was centrifuged. The supernatant was carefully removed and the pellet was washed a second time using the same procedure. The pellet was then washed 4 times with 40, 10, 2, and 1 mL of 10% cold glycerol, respectively, with the procedure mentioned above. Finally, the cell pellet was resuspended in 1 mL of cold 10% glycerol and 50 μL aliquots and stored at -80 ºC.

Modified DNAzyme The sequence of most active DNAzyme in this study was PS-DRz602: 5'-GCTTGA-GTTGAGGGCTAGCTACAACGACGCAGTA-3', which is complementary to the sequence of nt 1378–1359 of *blaR1* in *S aureus*; the internucleoside linkages in 2 arms of DNAzyme were phosphorothioated to increase nuclease resistance (modified bases are underlined). PS-DRz341 (control mismatched sequence) had been randomly aligned with the same number of bases as 5'-CAGTATGCATGCACGCTTG TAA-CCGTAAGTACGC-3', in which the underlined bases were also phosphorothioated. PS-DRz602 and PS-DRz341 were synthesized by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China).

DNAzyme delivery Different concentrations of phosphorothioated PS-DRz602 (5, 10, and 15 mg/L) were introduced into the *S aureus* strain WHO-2 by the Electroporator (JY2000-1B electroporation apparatus, Ningbo Scientz Biotechnology, Ningbo, China) at conditions of 25 μf, 900 V, 200 Ω, and time constant 3.6–4.2 ms. Briefly, the cells of bacteria and PS-DRz602 were mixed and transferred into prechilled cuvettes. After the pulses were applied, the cuvettes were removed and 1 mL of SOC (Super Optimal Catabolite) medium (0.5% yeast extract, 2% tryptone, 10 mmol/L NaCl, 2.5 mmol/L KCl, 10 mmol/L $MgCl_2$, 20 mmol/L $MgSO_4$, and 20 mmol/L glucose [pH 7.0]) was added immediately. The cells were allowed to recover by incubating for 1 h at 37 ºC with shaking. Transformation efficiencies should be approximately 9×10^8 transformants/μg of DNAzyme.

Bacterial susceptibility assay The growth determination of the cells receiving different concentrations of PS-DRz602 by electroporation was carried out as follows: the cells were diluted 10^6 times and 50 μL dilution was then spread onto the Mueller-Hinton agar that contained 6 mg/L oxacillin; the plates were incubated for 48 h at 35 ºC. The number of colonies were counted for plates with >10 and <500 colonies, and the total colony-forming unit (CFU) per sample was determined by correcting the colony count for the dilution.

The MIC of oxacillin for PS-DRz602-treated MRSA and MSSA (methicillin sensitive *S aureus*) were determined by the 2-fold microdilution method with the Mueller-Hinton broth supplemented with oxacillin in the range of 0.25–128 μg/mL. 100 μL of 5×10^5 CFU/mL test bacteria was added to 96-well microtitre plates and grown at 35 °C for 20 h. 10 μL of

1% triphenyl tetrazolium chloride (TTC), a colorimetric indicator, was added to each well of the microtitre plates and incubated for 1.5 h at 35 °C. The TTC-based MIC was determined as being the lowest concentration of oxacillin that showed no red color changes and indicated the complete growth inhibition.

RNA extraction The culture of the *S aureus* strain WHO-2 was centrifuged at 1957×*g* for 10 min at 4 °C and the supernatant was decanted. The cell pellet was suspended in 100 μL lysis solution (50 mmol/L Tris-HCl [pH 7.5], 5 mmol/L EDTA [pH 8], and 50 mmol/L NaCl) with 300 μg lysozyme (Sigma-Aldrich, St Louis, MO, USA) and 5 μg lysostaphin (Sigma-Aldrich, USA) for 30 min at room temperature. The total RNA was extracted from the bacterial lysis with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacture's instructions, and the RNA samples were treated with DNase I to remove any genomic DNA contamination.

Reverse transcription reaction The cDNA of *blaR1* and *blaZ* was synthesized, respectively, by reverse transcription (RT) from 1 μg of each RNA sample using SuperScriptIII reverse transcriptase (Invitrogen, USA). The 14 μL mixture (1 μg RNA, 0.1 μg random primer, 4 μL of 2.5 mmol/L dNTP mix, and sterile water) was heated at 65 °C for 5 min and incubated on ice for at least 1 min. 1 μL of 0.1 mol/L DTT (dithio-threitol), 4 μL of 5× RT buffer, 20 U RNase inhibitor, and 100 U SuperScriptIII reverse transcriptase was added to the mixture in a final volume of 20 μL. The RT conditions were 5 min at 25 °C, 45 min at 50 °C, and 15 min at 70 °C.

Real-time PCR detection The nucleotide sequences for the various primers are listed in Table 1. All the primers were synthesized commercially (Shanghai Sangon Biological Engineering Technology and Services, China).

The PCR was run in a DNA Engine Opticon (MJ Research, Waltham, MA, USA) with SYBR Green I. The PCR reagents consisted of: 12.5 μL SYBR Premix Ex Taq (DRR041S, TaKaRa, Otsu, Shiga, Japan), 0.5 μL of 50×ROX reference dye (DRR041S, TaKaRa, Japan), 0.75 μL of each primer (10 μmol/L), and 1 μL of sample cDNA in a final volume of 25 μL. Each plate included its own negative controls: no template controls (where all the reaction reagents except for cDNA were used). The thermal cycling conditions were: an initial denaturation step at 95 °C for 5 min, then 50 cycles at 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s. The melting curves of the PCR products were acquired by the stepwise increase of the temperature from 60 to 90 °C (temperature transition 0.5 °C/s). The standard deviation of the fluorescence values recorded from cycles 3 to 15 was multiplied by 10 to define the cycle threshold line. The specificity of the amplified products was verified by analysis of the dissociation curves as well as by ethidium bromide-stained 1% agarose gels.

The DNA Engine Opticon system detects and plots the increase of each PCR product in fluorescence versus the PCR cycle number to produce a continuous measurement of PCR amplification. To provide the precise quantification of the initial target in each PCR reaction, the amplification plot was examined at a point during the early log phase of product accumulation. This was accomplished by assigning a fluorescence threshold above the background and determining the time-point at which each sample's amplification plot reached the threshold (defined as the threshold cycle number or C_t). Differences in the threshold cycle number were used to quantify the relative amount of PCR target contained within each tube.

Construction of standard curves for *blaR1*, *blaZ*, and *16SrRNA* genes The cDNA of control group was 10 fold series diluted and the analysis was performed as follows: For each sample, a difference in C_t values (ΔC_t) was calculated for target genes (*blaR1/blaZ*) by taking the mean C_t of duplicate tubes and subtracting the mean C_t of the duplicate tubes for the reference RNA (*16SrRNA*) measured on an aliquot from the same RT reaction. $\Delta C_t = C_{t\ (\text{target gene})} - C_{t\ (16SrRNA)}$.

Comparative calculation and determination of the relative expression levels of *blaR1* and *blaZ* in the differently treated groups The relative expression of *blaR*1 or *blaZ* mRNA was calculated using the comparative C_t method.

Table 1. Oligonucleotide primers used for PCR.

Gene	Primers	Primer sequence (5'–3')	Location (bp)	Size (bp)
blaR1	Forward	acaatgaagtagaagccgatagat	719–742	489
	Reverse	gtcggtcaagtccaaaca	1207–1190	
blaZ	Forward	agagatttgcctatgcttca	311–330	461
	Reverse	agtatctccgcttttattattt	771–750	
16SrRNA	Forward	gttattagggaagaacatatgtg	446–468	750
	Reverse	ccaccttcctccggtttgtcacc	1195–1173	

Real-time relative quantitations of *blaR1* and *blaZ* expressions were performed as previously described[18]. The *16SrRNA* gene, which was expressed at relatively the same level throughout the developmental cycle in WHO-2, was used as the control to normalize the quantity of a cDNA target to determine differences in the amount of total cDNA in a reaction[18,19]. The $\Delta\Delta C_t$ values were calculated as the following equation: $\Delta\Delta C_t = \Delta C_{t\ (treatment)} - \Delta C_{t\ (control)}$. The ΔC_t for the treated sample was then subtracted from the ΔC_t for the untreated control sample to generate $\Delta\Delta C_t$.

The mean of these $\Delta\Delta C_t$ measurements was then used to calculate the expression of the test gene ($2^{-\Delta\Delta Ct}$) relative to the reference gene and normalized to the untreated control as follows: Relative expression=$2^{-\Delta\Delta Ct}$. The evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the untreated control.

Statistical analysis Values are expressed as mean±SD and one-way ANOVA analysis followed by SNK (Student-Newman-Keuls) *t*-test was performed. $P<0.05$ was considered statistically significant.

Results

Effects of PS-DRz602 on colony forming of the WHO-2 strain The number of WHO-2 colonies on the Mueller-Hinton agar containing oxacillin (6 mg/L) was significantly decreased to 78.2%, 56.7%, and 37.8% of the control value in all anti-BlaR1 PS-DRz602-treated groups concentration-dependently. However, the growth of WHO-2 was neither influenced in the mismatched PS-DRz341-treated group nor affected in the control group without PS-DRz602 treatment (Figure 1).

Partial restoration of antibiotic susceptibility in the MRSA strain WHO-2 We found that the down-regulation of *blaR1* by the introduction of anti-*blaR1* PS-DRz602 can partially increase the susceptibility of WHO-2 to oxacillin (Table 2). The MIC of oxacillin was reduced from 32 to 8 μg/mL in the presence of 5, 10, and 15 μg/mL anti-*blaR1* PS-DRz. In contrast, the MIC of oxacillin for mismatched PS-DRz341 not targeting *blaR1* remained unchang-ed. Competence or electroporation alone did not affect the MIC of oxacillin on WHO-2 (Table 2).

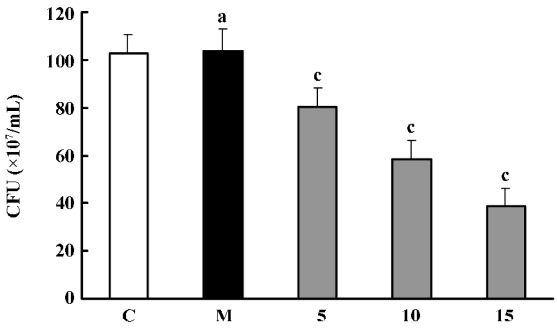

Figure 1. Effects of anti-*blaR1* PS-DRz602 on growth of WHO-2. C, control; M, 15 mg/L PS-DRz341; 5, 5 mg/L PS-DRz602; 10, 10 mg/L PS-DRz602; 15, 15 mg/L PS-DRz602. $^aP>0.05$, $^cP<0.01$ *vs* control group (mean±SD, $n=10$).

Real-time quantitation assays for *blaR1* and *blaZ* transcription We next determined whether the conversion of antibiotic resistance to antibiotic susceptibility in MRSA strain WHO-2 was accompanied by the inhibition of *blaR*1 and *blaZ* mRNA expression through anti-*blaR1* PS-DRz602.

An analysis of the melting curves of the PCR products for *blaR1*, *blaZ*, or *16SrRNA* showed a single-peak graph for all amplifications, indicating that a single PCR product was formed. This was confirmed by running 10 μL of each product on an ethidium bromide-stained 1% agarose gel.

The standard curves for *blaR1* or *blaZ* and *16SrRNA*

Table 2. MIC of oxacillin in the presence of PS-DRz147 for MRSA strain WHO-2 in broth culture. WHO-2 and MSSA strain ATCC 29213 (100 μL, 5×10^5 CFU/mL) was added to 96 well microtitre plates containing two-fold concentration increments of oxacillin. The plates were incubated at 35°C for 20 h. Then 10 μL of 1% triphenyl tetrazolium chloride (TTC) was added into each well and incubated for 1.5 h at 35°C. The MIC values were identified by color change. "+" represents competent cells or electroporation processing; "-" refers to non-competent cells or no electroporation processing.

Strain	Competent	Electroporation	DNAzyme	MIC(μg/mL)
ATCC29213	–	–	–	0.5
WHO-2	–	–	–	32
WHO-2	+	+	–	32
WHO-2	+	+	15 μg/mL DRz341	32
WHO-2	+	+	5 μg/mL DRz602	8
WHO-2	+	+	10 μg/mL DRz602	8
WHO-2	+	+	15 μg/mL DRz602	8

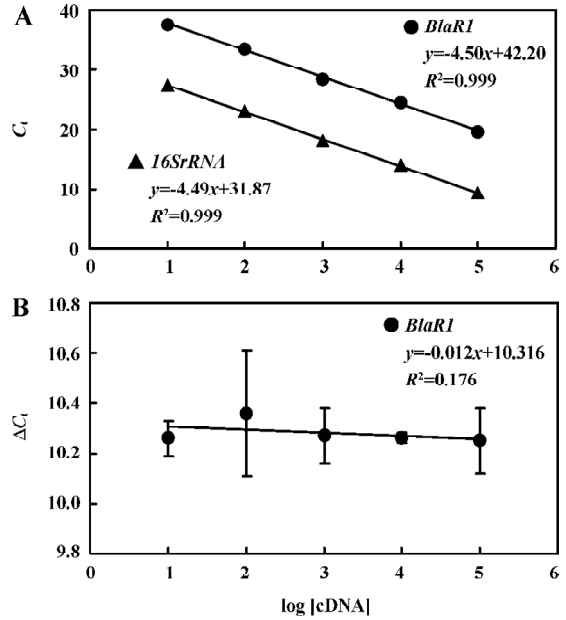

Figure 2. (A) Sandard curves for *blaR1* and *16SrRNA* using cDNA from the controls. Mathematical equation of the trends is shown as $y=mx+b$. Log represents $\log_{10}$; y axis represents the threshold cycle (C_t); x axis represents the log (cDNA) of each dilution used for the standard curve; m (-4.50 and -4.49 for *blaR1* and *16SrRNA*, respectively) represents the slope of the standard curve line (efficiency of the PCR reaction); R^2 represents the correlation coefficient of the standard curve line. (B) Relative PCR efficiency of amplification for *blaR1* and *16SrRNA* using the respective primers; y axis represents the difference in the threshold cycle (ΔC_t) obtained for the 2 PCR systems for the same cDNA dilution; x axis represents the log (cDNA) of each dilution used for both standard curves under comparison; 0.012 in the linear equation represents the slope of the log of cDNA for each dilution versus ΔC_t (value <0.1 indicates similar PCR efficiencies). n=3.

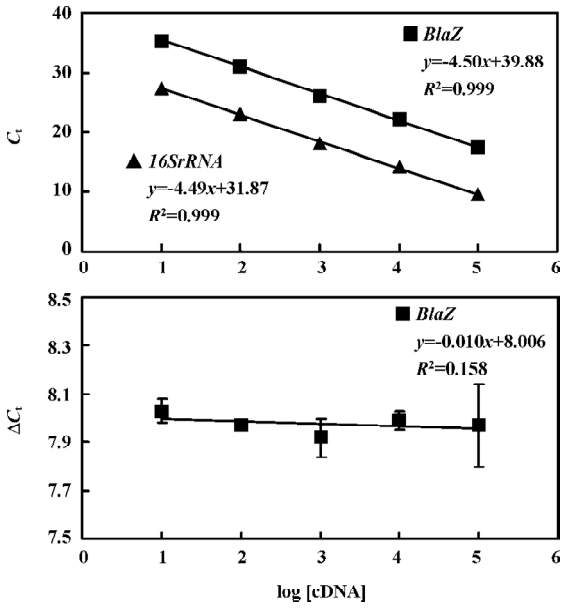

Figure 3. (A) Standard curves for *blaZ* and *16SrRNA* using cDNA from the controls. Mathematical equation of the trends is shown as $y=mx+b$. Log represents $\log_{10}$; y axis represents the threshold cycle (C_t); x axis represents the log (cDNA) of each dilution used for the standard curve; m (-4.50 and -4.49 for *blaZ* and *16SrRNA*, respectively) represents the slope of the standard curve line (efficiency of the PCR reaction); R^2 represents the correlation coefficient of the standard curve line. (B) Relative PCR efficiency of amplification for *blaZ* and *16SrRNA* using the respective primers; y axis represents the difference in the threshold cycle (ΔC_t) obtained for the 2 PCR systems for the same cDNA dilution; x axis represents the log (cDNA) of each dilution used for both standard curves under comparison; 0.010 in the linear equation represents the slope of the log of cDNA for each dilution versus ΔC_t (value <0.1 indicates similar PCR efficiencies). n=3.

were generated using cDNA from WHO-2 (Figures 2, 3). To demonstrate that the PCR efficiencies for the target and the control genes were approximately equal, the values of the 2 standard curves were used to determine the absolute value of the slope of the log of cDNA for each dilution versus ΔC_t (difference in the cycle threshold obtained for the 2 PCR systems for the same cDNA dilution) for the respective dilution. This validation experiment involved pairwise com-

Table 3. Relative mRNA expression level of *blaR1* in different treated groups. C_t refers to cycle numbers when the fluorescence reaches fluorescence threshold. $\Delta\Delta C_t=(C_{t\text{-}blaR1}-C_{t\text{-}16s})_{treatment}-(C_{t\text{-}blaR1}-C_{t\text{-}16s})_{control}$. [a]$P$>0.05, [b]$P$ <0.05, [c]P<0.01 *vs* control group (mean±SD, n=3).

Group	Concentration (mg/L)	$C_{t\text{-}blaR1}$	*16srRNA* C_t	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta Ct}$
Control	0	15.69±0.05	8.19±0.04	7.50±0.06	0±0.06	1.00 (0.96-1.04)
PS-DRz341	15	14.78±0.06	7.20±0.03	7.58±0.07	0.08±0.07	0.95 (0.90-0.99)[a]
PS-DRz602	5	15.96±0.08	8.16±0.07	7.80±0.11	0.31±0.11	0.81 (0.75-0.87)[b]
PS-DRz602	10	14.44±0.08	5.96±0.05	8.48±0.09	0.98±0.09	0.51 (0.48-0.54)[c]
PS-DRz602	15	16.98±0.05	7.79±0.04	9.20±0.06	1.70±0.06	0.31 (0.30-0.32)[c]

Table 4. Relative mRNA expression level of *blaZ* in different treated groups. C_t refers to cycle numbers when the fluorescence reaches fluorescence threshold. $\Delta\Delta C_t=(C_{t\cdot blaZ}-C_{t\cdot 16s})_{treatment}-(C_{t\cdot blaZ}-C_{t\cdot 16s})_{control}$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control group (mean±SD, $n=3$).

Group	Concentration (mg/L)	$C_{t\cdot blaZ}$	*16srRNA* C_t	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta Ct}$
Control	0	14.15±0.05	8.18±0.03	5.97±0.06	0±0.06	1.00 (0.96-1.04)
PS-DRz341	15	13.14±0.03	7.21±0.03	5.93±0.04	-0.05±0.04	1.03 (1.01-1.06)[a]
PS-DRz602	5	14.43±0.10	8.17±0.02	6.27±0.10	0.29±0.10	0.82 (0.76-0.88)[b]
PS-DRz602	10	12.76±0.11	5.97±0.01	6.79±0.11	0.81±0.11	0.57 (0.53-0.62)[c]
PS-DRz602	15	14.93±0.16	7.66±0.11	7.27±0.19	1.30±0.19	0.41 (0.36-0.46)[c]

parisons between *blaR1* and *16SrRN*A or *blaZ* and *16SsRNA*. The slope was 0.012 and 0.010 for each experiment, respectively, indicating approximately equal amplification efficiencies between *blaR1* or *blaZ* and *16SrRNA* (Figures 2, 3).

Comparative transcription of a single gene for *blaR1* and *blaZ* The change in the transcription of the target genes (*blaR1* and *blaZ*) normalized to *16SrRNA* was monitored in the different concentration groups. As expected, the transcriptional change of *blaR1* in the differently treated groups was coincidental to that of *blaZ*. Compared with the control group, the relative transcription of *blaR1* in 3 groups (5, 10, 15 mg/L PS-DRz602) was decreased to 81%, 51%, and 31% of the control values, respectively, in a concentration-dependent manner (Table 3). Interestingly, the blockade of the transcription of *blaR1*, a sensor transducer gene, led to the concentration-dependent repression of its downstream gene *blaZ* to 82%, 57%, and 41% of the control values, respectively (Table 4).

Discussion

Because of the emergence and spread of resistance genes[9–12,20], MRSA has been the cause of major outbreaks and epidemics among hospitalized patients, with high mortality and morbidity rates. Recently, some schemes have shown both a dramatic rise in the total numbers of cases of *S aureus* bacteremia reported annually and an increase in the proportion of such cases that involve MRSA (from 2% in 1990 to >40% in the early 2000s)[21]. However, the emergence of vancomycin-resistant MRSA and treatment failure of MRSA infections has led to the urgent need for alternative anti-MRSA therapies.

The different antisense approaches have demonstrated the feasibility of using antisense oligonucleotides or oligonucleotide analogs in the treatment of bacterial infections, and the promising results have been observed by researchers *in vitro* and *in vivo*[22,23]. Sarno *et al* demonstrated that selectively-designed antisense oligonucleotides could bind to AAC(6')-I-type acetyltransferase (aminoglycoside 6'-*N*-acetyltransferase type Ib) mRNA, mediate RNase H digestion, and thereafter decrease the level of resistance to amikacin in *Escherichia coli*[24]. The application of peptide-PNA (peptide nucleic acid) conjugates, antisense phosphorodiamidate morpholino oligomer, or antisense DNA analogs showed a significant inhibition of gene expression in *E Coli*, respectively, and effectively reversed the multiple drug resistance of *E coli*[25–27]. When PS-ODN (phosphorothioate oligodeoxynucleotide), targeting a specific region of the mycobacterium aspartokinase (*ask*) gene in *Mycobacterium smegmatis* were utilized in combination with ethambutol, the results showed a significant inhibition of growth of drug-resistant strains of *M smegmatis*[28]. The inhibition of glutamine synthetase activity with antisense oligonucleotides to glutamine synthetase mRNA also held back the replication of *Mycobacterium tuberculosis*[29]. Resistance to β-lactam was reversed by the blockade of the expression of *bla* in the presence of antisense peptide nucleic acid in *E coli*[30] and resistance to vancomycin was fully reversed when an *Enterococcus faecalis* isolator harbored recombinant shuttle vectors containing a *vanH* promoter-*vanA* antisense gene cassette[31]. The peptide nucleic acid treatment was effective in rescuing 100% of infected animals[23]. Antisense antibiotics have been proposed as a new hope for bacteria infection therapy through targeting specific genes in bacteria[32].

Our previous study indicated that a phosphorothioate oligodeoxynucleotide ODN6087 could inhibit *mecR1* and *mecA* expression and partially restore the susceptibility of a MRSA strain[33]. The current study demonstrated that PS-DRz602 targeted to the sequence of *blaR*1 mRNA significantly inhibited the resistance of MRSA strain WHO-2 to oxacillin, together with the reduction of *blaZ* mRNA

expression. The colony forming of WHO-2 was decreased to 78.2% (5 mg/L), 56.7% (10 mg/L), and 37.8% (15 mg/L) of the control values, respectively, in a concentration-dependent manner after PS-DRz602 treatment. However, anti-*blaR1* PS-DRz602 had limited activity to reverse the susceptibility of MRSA strain WHO-2 in the present study. The reason for the insufficient restoration of susceptibility is that we delivered the PS-DRz to competent *S aureus* only once by electroporation. Although the delivery efficiency was estimated at around 9×10^8 transformants/micrograms in all groups, PS-DRz was not retained in the culture medium constantly and bacterial proliferation diluted the PS-DRz. As demonstrated, the MIC of oxacillin to WHO-2 were reduced from 32 to 8 μg/mL, which was still 2-folds higher than the margin value (2 μg/mL) of oxacillin sensitivity to *S aureus* and the inhibitory efficiency of the anti-*blaR1* PS-DRz602 at 78.2%–37.8% of control values. However, if the highly-efficient delivery system is applied and the antisense PS-DRz is sustainable throughout proliferation, then a high efficiency of anti-*blaR1* PS-DRz will be achieved.

Since BlaR1 is an upstream regulatory element for the initiation of BlaZ expression via the inactivation of BlaI, it was found that BlaZ expression was consequently greatly inhibited after the blockade of BlaR1. Although there is no direct evidence to prove the cleaving activity of PS-DRz602 to the mRNA substrates of BlaR1 precisely in WHO-2, the mismatched anti-*blaR1* PS-DRz341 had no effects on the growing of WHO-2, as well as no influence on the expression of BlaR1, and β-lactam mRNA would provide indirect evidence to support the selectivity of PS-DRz602. In addition, in the cytoplasm of cells, a full length of mRNA often has a secondary structure. If the target sites of DNAzymes are within the secondary structure of the RNA, DNAzymes may still be ineffective[34,35]. Some studies have shown that DNAzymes work well in the *in vitro* system, but do not work in the whole cell[35,36]. Mitchell *et al* and Patzel *et al* found that without any prior screening, the initially synthesized DNAzyme sequence specifically reduced the target gene expression[34,37].

In conclusion, the results of our present study suggested that the blockade of the *blaR1-blaZ* signaling pathway via a DNAzyme was an alternative strategy to the reverse phenotype of antibiotic resistance of MRSA, and BlaR1 may be an attractive target for antimicrobial agent development.

Acknowledgement

We are grateful to Prof Yue MA (Chinese National Center for Surveillance of Antimicrobial Resistance, Beijing, China) for providing the WHO-2 strain.

References

1 Aires de Sousa M, de Lencastre H. Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. FEMS Immunol Med Microbiol 2004; 40: 101–11.
2 Zetola N, Francis JS, Nuermberger EL, Bishai WR. Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging threat. Lancet Infect Dis 2005; 5: 275–86.
3 Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, *et al*. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying panton-valentine leukocidin genes: worldwide emergence. Emerg Infect Dis 2003; 9: 978–84.
4 Eady EA, Cove JH. Staphylococcal resistance revisited: community-acquired methicillin resistant *Staphylococcus aureus* — an emerging problem for the management of skin and soft tissue infections. Curr Opin Infec Dis 2003; 16: 103–24.
5 Crum NF. The emergence of severe, community-acquired methicillin-resistant *Staphylococcus aureus* infections. Scand J Infec Dis 2005; 37: 651–6.
6 Tumbarello M, de Gaetano Donati K, Tacconelli E, Citton R, Spanu T, Leone F, *et al*. Risk factors and predictors of mortality of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia in HIV-infected patients. J Antimicrob Chemother 2002; 50: 375–82.
7 Crowcroft NS, Catchpole M. Mortality from methicillin resistant *Staphylococcus aureus* in England and Wales: analysis of death certificates. BMJ 2002; 325: 1390–1.
8 Srinivasan A, Dick JD, Perl TM. Vancomycin resistance in *Staphylococci*. Clin Microbiol Rev 2002; 15: 430–8.
9 Archer GL, Bosilevac JM. Signaling antibiotic resistance in *Staphylococci*. Science 2001; 291: 1915–6.
10 Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. J Clin Invest 2003; 111: 1265–73.
11 Safo MK, Zhao Q, Ko TP, Musayev FN, Robinson H, Scarsdale N, *et al*. Crystal structures of the BlaI repressor from *Staphylococcus aureus* and its complex with DNA: insights into transcriptional regulation of the bla and mec operons. J Bacteriol 2005; 187: 1833–44.
12 Golemi-Kotra D, Cha JY, Meroueh SO, Vakulenko SB, Mobashery S. Resistance to beta-lactam antibiotics and its mediation by the sensor domain of the transmembrane BlaR signaling pathway in *Staphylococcus aureus*. J Biol Chem 2003; 278: 18 419–25.
13 Emilsson GM, BreakerRR. Deoxyribozymes: new activities and new applications. Cell Mol Life Sci 2002; 59: 596–607.
14 Khachigian LM. Catalytic DNAs as potential therapeutic agents and sequence-specific molecular tools to dissect biological function. J Clin Invest 2000; 106: 1189–95.
15 Bignardi GE, Woodford N, Chapman A, Johnson AP, Speller DC. Detection of the mec-A gene and phenotypic detection of resistance in *Staphylococcus aureus* isolates with borderline or low-level methicillin resistance. J Antimicrob Chemother 1996; 37: 53–63.
16 Yazdankhah SP, Sorum H, Oppegaard H. Comparison of genes involved in penicillin resistance in *Staphylococci* of bovine origin. Microb Drug Resist 2000; 6: 29–36.
17 Augustin J, Gotz F. Transformation of *Staphylococcus epidermidis* and other staphylococcal species with plasmid DNA

by electroporation. FEMS Microbiol Lett 1990; 54: 203–7.
18 Eleaume H, Jabbouri S. Comparison of two standardisation methods in real-time quantitative RT-PCR to follow *Staphylococcus aureus* genes expression during *in vitro* growth. J Microbiol Methods 2004; 59: 363–70.
19 Goerke C, Bayer MG, Wolz C. Quantification of bacterial transcripts during infection using competitive reverse transcription-PCR (RT-PCR) and LightCycler RT-PCR. Clin Diagn Lab Immunol 2001; 8: 279–82.
20 Dzidic S, Bedekovic V. Horizontal gene transfer-emerging multidrug resistance in hospital bacteria. Acta Pharmacol Sin 2003; 24: 519–26.
21 Johnson AP, Pearson A, Duckworth G. Surveillance and epidemiology of MRSA bacteraemia in the UK. J Antimicrob Chemother 2005; 56: 455–62.
22 Chopra I. Prospects for antisense agents in the therapy of bacterial infections. Expert Opin Investig Drugs 1999; 8: 1203–8.
23 Tan XX, Actor JK, Chen Y. Peptide nucleic acid antisense oligomer as a therapeutic strategy against bacterial infection: proof of principle using mouse intraperitoneal infection. Antimicrob Agents Chemother 2005; 49: 3203–7.
24 Sarno R, Ha H, Weinsetel N, Tolmasky ME. Inhibition of aminoglycoside 6'-*N*-acetyltransferase type Ib-mediated amikacin resistance by antisense oligodeoxynucleotides. Antimicrob Agents Chemother 2003; 47: 3296–304.
25 Good L, Awasthi SK, Dryselius R, Larsson O, Nielsen PE. Bactericidal antisense effects of peptide-PNA conjugates. Nat Biotechnol 2001; 19: 360–4.
26 Geller BL, Deere J, Tilley L, Iversen PL. Antisense phosphorodiamidate morpholino oligomer inhibits viability of *Escherichia coli* in pure culture and in mouse peritonitis. J Antimicrob Chemother 2005; 55: 983–8.
27 White DG, Maneewannakul K, von Hofe E, Zillman M, Eisenberg W, Field AK, *et al*. Inhibition of the multiple antibioc resistance (mar) operon in *Escherichia coli* by antisense DNA analogs. Antimicrob Agents Chemother 1997; 41: 2699–704.
28 Rapaport E, Levina A, Metelev V, Zamecnik PC. Antimycobacterial activities of antisense oligodeoxynucleotide phosphorothioates in drug-resistant strains. Proc Natl Acad Sci USA 1996; 93: 709–13.
29 Harth G, Zamecnik PC, Tang JY, Tabatadze D, Horwitz MA. Treatment of *Mycobaterium tuberculosis* with antisense oligonucleotides to glutamine synthetase mRNA inhibits glutamine synthetase activity, formation of the poly-*L*-glutamate/glutamine cell wall struture, and bacterial replication. Proc Natl Acad Sci USA 2000; 97: 418–23.
30 Good L, Nielsen PE. Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA. Nat Biotechnol 1998; 16: 355–8.
31 Torres Viera C, Tsiodras S, Gold HS, Coakley EP, Wennersten C, Eliopoulos GM, *et al*. Restoration of vancomycin susceptibility in *Enterococcus faecalis* by antiresistance determinant gene transfer. Antimicrob Agents Chemother 2001; 45: 973–5.
32 Geller BL. Antibacterial antisense. Curr Opin Mol Ther 2005; 7: 109–13.
33 Meng JR, Hu BQ, Liu J, Hou Z, Meng J, Jia M, *et al*. Restoration of oxacillin susceptibility in methicillin-resistant *Staphylococcus aureus* by blocking the MecR1-mediated signaling pathway. J Chemother 2006; 18: 360–5.
34 Patzel V, Steidl U, Kronenwett R, Haas R, Sczakiel G. A theoretical approach to select effective antisense oligodeoxyribonucleotides at high statistical probability. Nucleic Acids Res 1999; 27: 4328–34.
35 Cairns MJ, Hopkins TM, Witherington C, Wang L, Sun LQ. Target site selection for an RNA-cleaving catalytic DNA. Nat Biotechnol 1999; 17: 480–6.
36 Cairns MJ, Sun LQ. Target-site selection for the 10–23 DNAzyme. Methods Mol Biol 2004; 252: 267–77.
37 Mitchell A, Dass CR, Sun LQ, Khachigian LM. Inhibition of human breast carcinoma proliferation, migration, chemoinvasion and solid tumour growth by DNAzymes targeting the zinc finger transcription factor EGR-1. Nucleic Acids Res 2004; 32: 3065–9.