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Inhibiting the growth of methicillin-resistant *Staphylococcus aureus* in vitro with antisense peptide nucleic acid conjugates targeting the *ftsZ* gene

Shumei Liang [a], Yunyan He [b], Yun Xia [a,*], Huijuan Wang [a], Lipeng Wang [a], Rui Gao [a], Meilan Zhang [a]

[a] *Department of Clinical Laboratory, the First Affiliated Hospital of Chongqing Medical University, 1 You Yi Road, Yuzhong District, Chongqing 40016, China*
[b] *Department of Clinical Laboratory, Chongqing Zhongshan Hospital, Chongqing, China*

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SUMMARY

Background: The increasing emergence of clinical infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) challenges existing therapeutic options and highlights the need to develop novel treatment strategies. The *ftsZ* gene is essential to bacterial cell division.
Methods: In this study, two antisense peptide nucleic acids (PNAs) conjugated to a cell-penetrating peptide were used to inhibit the growth of MRSA. PPNA1, identified with computational prediction and dot-blot hybridization, is complementary to nucleotides 309–323 of the *ftsZ* mRNA. PPNA2 was designed to target the region that includes the translation initiation site and the ribosomal-binding site (Shine–Dalgarno sequence) of the *ftsZ* gene. Scrambled PPNA was constructed with mismatches to three bases within the antisense PPNA1 sequence.
Results: PPNA1 and PPNA2 caused concentration-dependent growth inhibition and had bactericidal effects. The minimal bactericidal concentrations of antisense PPNA1 and PPNA2 were 30 μmol/l and 40 μmol/l, respectively. The scrambled PPNA had no effect on bacterial growth, even at higher concentrations, confirming the sequence specificity of the probes. RT-PCR showed that the antisense PPNAs suppressed *ftsZ* mRNA expression in a dose-dependent manner.
Conclusion: Our results demonstrate that the potent effects of PNAs on bacterial growth and cell viability were mediated by the down-regulation or even knock-out of *ftsZ* gene expression. This highlights the utility of *ftsZ* as a promising target for the development of new antisense antibacterial agents to treat MRSA infections.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), a horrific superbug, has become an overwhelming pathogenic threat to human health.[1] The high rate of MRSA infection is a great burden to the clinical treatment and control of nosocomial infections. Currently, linezolid and vancomycin are used as the first-line drugs for the treatment of serious MRSA infections. However, several linezolid-resistant strains have been reported.[2] Patients with MRSA bloodstream infections and high minimum inhibitory concentrations (MICs) for vancomycin have a greater likelihood of treatment failure and mortality.[3] The strong resistance of MRSA to antibiotics makes clinical treatment difficult and has created an urgent demand for the discovery and development of new antibacterial agents directed toward novel targets to avoid cross-resistance to licensed antibiotics.

The processes of bacterial cell division are promising targets for new antibacterial drugs.[4–7] *ftsZ* is the most conserved of all known bacterial cell-division genes and encodes the bacterial proto-ring protein FtsZ, and so plays a key role in bacterial cell division. It is the first protein to localize at the site of incipient division and functions as a GTP-dependent guanosine triphosphatase, like the tubulin protein in eukaryotic cells. It recruits the other cell-division proteins in an orderly way to form the Z ring, which leads the constriction of the cell membrane, and coordinates the whole process of cell division. Because it has a pivotal role in initiating the whole process of cell division, FtsZ is an attractive target for

* Corresponding author. Tel.: +86 023 89012742; fax: +86 023 89012513.
E-mail address: xiayun12cn@aliyun.com (Y. Xia).

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antibacterial drug development. Several synthetic and natural inhibitors of FtsZ have been suggested for the treatment of antibiotic-resistant bacterial infections, such as *S. aureus* and *Escherichia coli*.[8–11]

Exploiting the antisense mechanism is a way to silence the expression of a target gene at the mRNA level in a sequence-specific manner. Antisense antibacterial agents are short (about 10–20 bases). Peptide nucleic acids (PNAs), locked nucleic acids (LNAs), and phosphorodiamidate morpholino oligomers (PMOs)[12,13] are synthetic nucleic acid analogues that inhibit targeted gene expression at the level of DNA replication or transcription.[14,15] PNAs, which lack a negative charge and are quite stable against nucleases, exert potent antisense effects, while they are not susceptible to active efflux from the cell. These features make them attractive candidates as antisense antimicrobial agents. However, a major hurdle in the development of PNAs as antimicrobials is the inefficient delivery of PNAs to cells.[16] Numerous studies have demonstrated that conjugating a cell-penetrating peptide (CPP) to a PNA dramatically improves its antisense activity.[17] Currently, the peptide $(RXR)_4XB$ is most commonly used in prokaryotic organisms to enhance the entry of PNAs.

In this study, antisense oligonucleotides targeting the *fts*Z gene were designed and peptide-conjugated PNAs (PPNAs) were synthesized to evaluate their inhibitory effects on MRSA growth in vitro.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The MRSA strain CY-11 was isolated from a clinical sample, identified with the VITEK 2 Compact System (bioMérieux, Lyon, France), and confirmed to be *mec*A gene-positive with PCR. The *mec*A gene was amplified by PCR with the forward primer 5′-GGTACTGCTATCCACCCTCAAA-3′ and the reverse primer 5′-TTACGACTTGTTGCATACCATCA-3′.[18] The PCR product was sequenced and compared to known *mec*A gene sequences in GenBank. Antibiotic sensitivity tests showed that this strain is resistant to oxacillin (MIC ≥4 mg/l), benzylpenicillin (MIC ≥0.5 mg/l), erythromycin (MIC ≥8 mg/l), gentamicin (MIC ≥16 mg/l), ciprofloxacin (MIC ≥8 mg/l), clindamycin (MIC ≥8 mg/l), levofloxacin (MIC ≥8 mg/l), tetracycline (MIC ≥16 mg/l), and moxifloxacin (MIC ≥8 mg/l), according to the standards of the Clinical and Laboratory Standards Institute (CLSI, USA, 2013).

2.2. Screening the effective antisense oligomers

2.2.1. Full-length fts*Z* gene cloning and in vitro transcription

The genomic DNA of MRSA CY-11 was extracted with a TIANamp Bacteria DNA Kit, based on affinity columns (Tiangen, Beijing, China), according to the manufacturer's protocol. The full-length *fts*Z gene (GenBank accession number **NC_002952**) was amplified by PCR with the forward primer 5′-CCAATAAAAC-TAGGAGGAAA-3′ and the reverse primer 5′-ACCGATTAACGTCTTG-TTCTTCTT-3′. The PCR product was sequenced and cloned into the pGEM-T Easy vector (Promega, Fitchburg, WI, USA), generating the recombinant plasmid designated GEM-T–*fts*Z. In the recombinant plasmid, the full-length *fts*Z gene was correctly inserted downstream from the T7 promoter, and the recombinants were selected and linearized with *Spe*I (Promega). *fts*Z mRNA was transcribed in vitro with Riboprobe Systems-T7 (Promega) in the presence of digoxigenin–recombinant 11-uridine-5′-triphosphate (DIG–rUTP; Roche), according to the manufacturer's instructions. Briefly, a 20-μl reaction was set up containing 4 μl of Transcription Optimized 5× Buffer, 2 μl of 100 mM DL-Dithiothreitol (DTT), 0.5 μl of Recombinant RNasin Ribonuclease Inhibitor, rATP, rGTP, rATP, and rUTP (2.5 mM each), 3.75 μg of linearized template DNA, and 1 μl of T7 RNA polymerase. Nuclease-free water was added to a final volume of 20 μl. After incubation at 37 °C for 3 h, the DNA was removed by digestion with DNaseI for 30 min. To prepare the RNA transcripts for dot-blot hybridization (described below), UTP and DIG–UTP were added to the transcription reaction in a ratio of 13:7. The RNA transcripts were purified, quantified, and stored as aliquots in nuclease-free water at −80 °C until analysis.

2.2.2. Antisense oligonucleotide probe design, computational screening with prediction software, and dot-blot hybridization

The secondary structure of the *fts*Z mRNA was predicted with Mfold software and the RNA structure 5.5 program, based on minimal free energy theory. In total, 10 plausible target sites lacking obvious stable secondary structures, such as stem-loops or hairpins, were selected as antisense oligonucleotide probes. One oligonucleotide from the sense strand sequence was used as the negative control. The sequences of the antisense oligonucleotide probes and their parameters are listed in Table 1. These 11 oligonucleotides were hybridized to the *fts*Z transcription product with dot-blot hybridization (DIG Luminescent Detection Kit, Roche), according to the manufacturer's instructions, to evaluate their binding activity to the *fts*Z mRNA.

Table 1
Probe sequence for screening the effective antisense oligonucleotides

Probe sequence (5′–3′)	Target site[a]	Parameters[b]			
		Overall ΔG	Oligo–self ΔG	Oligo–oligo ΔG	Tm (°C)
GTGGTCAATCATTCGGTTTA	80–99	−18.3	0	−3.9	67.9
CGTGTTAATTTTTCACCGAT	181–200	−12	0	−3.8	62.1
CCGCCACCCATACCAGAAGT	304–323	−16.6	0	−2.4	76.3
CACCAACAGTTAATGCGCCC	372–391	−10.6	0	−7	70.4
TGTCAACGATATCTAATAAA	504–523	−11	0	−5.6	50.2
TGTACACCTTGGCGTAACAC	565–584	−10.3	0	−5.8	68.5
TGAGATACCTTGTACACCTT	575–594	−15.4	0	−6	67.4
TTCTACCGCTCTATTTTCAC	698–717	−15.2	0	−2.8	68.9
ATGACTCGCCACCAGTAATA	789–808	−13.9	0	−2.6	67.8
TAGTTGTATGCGTTCTTTCA	1086–1105	−12.2	0	−3.4	68.4
GCTGCAGAGGAATCTCGTGA	Control sequence	ND	ND	ND	ND

ND, not determined.
[a] Numbering from the first base of the gene *fts*Z.
[b] ΔG means free energy. Index for each parameter: overall ΔG <−10 kcal/mol; duplex ΔG <−25 kcal/mol; oligo–self ΔG ≥−1.1 kcal/mol; oligo–oligo ΔG ≥−8 kcal/mol; Tm >50 °C.