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Antisense inhibition of gene expression and growth in gram-negative bacteria by cell-penetrating peptide conjugates of peptide nucleic acids targeted to *rpoD* gene

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ABSTRACT

Gram-negative bacteria (GNB) cause common and severe hospital- and community-acquired infections with a high incidence of multidrug resistance (MDR) and mortality. The emergence and spread of MDR-GNB strains limit therapeutic options and highlight the need to develop new therapeutic strategies. In this study, the peptide $(RXR)_4XB$- and $(KFF)_3K$-conjugated peptide nucleic acids (PPNAs) were developed to target *rpoD*, which encodes an RNA polymerase primary σ^{70} that is thought to be essential for bacterial growth. Their antimicrobial activities were tested against different clinical isolates of MDR-GNB in vitro and in infection models. The $(RXR)_4XB$- and $(KFF)_3K$- conjugated PNAs were bactericidal against different strains of MDR-GNB in concentration-dependent and sequence-selective manner, whereas a PPNA with a scrambled base sequence had no effect on growth. Among tested PPNAs, $(RXR)_4XB$ conjugate PPNA06 showed more potent and broad spectrum inhibition in multidrug-resistant *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, and *Shigella flexneri* in vitro and in vivo. The results were associated with suppression of *rpoD* mRNA and σ^{70} expression, as well as σ^{70} downstream regulated genes including *ftsZ, mazF, prfB*, *rpoS*, *seqA*, *turfB* and *ygjD*. The treatment of PPNA06 on mono- or multiple MDR-GBN infected human gastric mucosal epithelial cells demonstrated the complete inhibition on bacterial growth and no influence on morphology and growth of human cells. Also, PPNA06 did not show the induction of antibiotic resistance as compared with classical antibiotics in GNB. These findings firstly demonstrate that *rpoD* is potential target for developing antisense antibiotics, and indicate that peptide conjugates of anti-*rpoD* PNA are active against GNBs in vitro and in vivo. Our results offer a feasible strategy for treating MDR-GNB infections.

1. Introduction

Gram-negative bacteria pathogens represent a common cause of infections in community and hospital settings. According to the recent data from the U.S. National Nosocomial Infections Surveillance (NNIS) System, the clinical isolates of gram-negative bacteria are associated with 65%–80% of all cases of intensive care unit (ICU)–acquired pneumonia, 40%–60% of all ICU-acquired surgical-site infections, ~70% of all ICU-acquired urinary tract infections, and 25%–30% of all ICU-acquired bloodstream infections [1] and similar data are reported from China Nosocomial Pathogens Resistance Surveillance Study Group [2]. Unfortunately, multidrug-resistant gram-negative bacteria (MDR-GNB), including *Pseudomonas aeruginosa, Acinetobacter baumannii*, *Klebsiella pneumoniae,* extended-spectrum β-lactamase (ESBL)–producing or carbapenemase-producing Enterobacteriaceae and New Delhi metallo-beta-lactamase 1 (NDM-1) producing pathogens are increasingly being reported worldwide and the treatment of infections caused by these pathogens has become considerably more challenging, primarily because these organisms exhibit resistance to most commonly used antimicrobial agents, even to the most active carbapenems [3–5]. Therefore, infections caused by MDR-GNB are associated with considerable morbidity and mortality [6]. Furthermore, the co-colonization with multiple different species of MDR-GNB is identified in patients with high rates and therapeutic options are even more limited for those patients. The prevalence of co-colonization with different MDR-GNB, in turn, increases the risk of treatment failure, results in prolonged hospitalization, higher economic burden, and increases mortality [7]. Therefore, the need for developing new antimicrobials against MDR-GNB threat is urgent.

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Antisense antibacterial strategy is a revolutionary concept for silencing essential genes at mRNA level by antisense nucleic acid analogs, such as phosphorothioate oligodeoxynucleotides (PS-ODN), peptide nucleic acid (PNA), phosphorodiamidate morpholino oligomer (PMO), and locked nucleic acid (LNA) [8,9]. Of these nucleic acid analogs, PNA oligomers form strong complexes with complementary strands of DNA or RNA and are quite stable against nucleases and proteases, as well as low toxicity [10,11]. Therefore, PNA antisense oligomers have been studied as bacterial growth inhibitors [12,13]. The conjugation of various cell penetrating peptides (CPP) to the PNA has led to dramatically increased cell permeability and uptake of PNA into bacteria [11]. These molecules have been applied successfully against different species such as *S. aureus, Escherichia coli, K. pneumonia*. A range of essential genes in bacteria have been validated as potential targets, e.g. the *acpP* encoding the essential fatty acid biosynthesis protein in *E. coli*, *S. enterica serovar Typhimurium and Burkholderia cepacia* complex [10,14]; *fabI* encoding enoyl-acyl carrier protein reductase in *E. coli* and *S. aurea*; 16S rRNA in *E. coli* [11].

However, most of researchers focused on one potential target gene in limited strains of one bacterial species [12–14]. Few reports describe promising gene targets that have potential for broad spectrum antisense growth inhibition in different bacterial species. Thus, identification of gene targets for broad spectrum antisense inhibition would aid the development of new anti-gram-negative agents that could relieve the exacerbating therapeutic consequences caused by MDR-GNB infections [15].

Bacterial DNA-dependent RNA polymerase (RNAP) is a key enzyme of the transcription process, and is a final target in many regulatory pathways that control gene expression in all bacterial cells. Bacterial RNAP exists in two forms: core and holoenzyme. The core enzyme consists of five subunits: α-dimer (α2), β, β′, and ω. Although it is catalytically active, the core enzyme is incapable of initiating transcription efficiently and with specificity. For this, it requires a sixth, dissociable subunit called a σ factor, which reversibly associates with the core RNAP complex to form a holoenzyme. The vast majority of σ factors belong to the so-called σ70 family, reflecting their relationship to the principal σ factor of *E. coli*, σ^{70}. Members of the σ^{70} family play key roles in initiation, being directly involved in promoter recognition, DNA melting, and promoter escape and clearance [16]. Multiple members of the σ^{70} family have since been discovered in most bacteria, with up to 63 encoded by a single gene, *rpoD* [17]. Sequence alignments of the σ^{70} family members reveal that they shares four regions of sequence homology, designated 1 to 4 and highest conservation is found in regions 2 and 4, which are involved in binding to RNA polymerase, recognizing promoters and melting DNA strands [18]. The blockade of activity of sigma factor 70 by anti-sigma factor 70 Rsd and AsiA could shuts down global gene transcription [19]. These features imply that *rpoD* might be a potential target gene for antisense approach and the blockade of *rpoD* may cause broad spectrum of antibiotic effects.

2. Materials and methods

2.1. Bacterial strains

E. coli MG 1655 was a gift from Professor Liping Zhao (Shanghai Jiaotong University, Shanghai, China). Clinical isolates of MDR-*Salmonella enterica* (*S. enterica*) and MDR-*Shigella flexneri* (*S. flexneri*) strains were obtained from Tangdu Affiliated Hospital (Fourth Military Medical University, Xi'an, China). *E. coli* (ATCC 25922), ESBLs-*E. coli* (ATCC 35218), MDR-*E. coli*, and ESBLs-*Klebsiella pneumoniae* (*K. pneumoniae*) were collections preserved in our lab. All strains were grown in Mueller-Hinton broth (Land Bridge Technology Co. Ltd., Beijing, China) and grown overnight at 37 °C with shaking for MIC analysis or for preparation of inocula used to infect mice.

2.2. PPNA synthesis

The cell penetrating peptides (CPP) of $(KFF)_3K$ and $(RXR)_4XB$ were synthesized and purified at Genotide, Inc. (Xi'an, China). PNAs were synthesized and purified at Panagene Inc. (Daheon, Korea). Then conjugates of CPP and PNA were synthesized by manual coupling chemistry and purified at Panagene Inc. (Daeheon, Korea) to form a CPP conjugated PNA (PPNA).

2.3. PNA target site selection and anti-rpoD PPNA optimization

Sequence alignment of gene *rpoD* was performed by BLAST to decide the region that showed the highest sequential homology among gram-negative species (including *E. coli*, *S. enterica*, *K. pneumoniae*, and *S. flexneri*). And secondary structure of *rpoD* mRNA predicted by RNA structure 4.6 software and paring parameters referenced by Oligo Walk 5.0 program were used to decide the targeting sites that showed best binding affinity. Four potential target sites located in the region 1 of *rpoD* were chosen for the synthesis of complementary antisense PNAs at 12-base length. All PNAs used are covalently conjugated with the cell penetrating peptide $(KFF)_3K$ (in which K is lysine and F is phenylalanine) at the carbon terminus (corresponding to the 3′ end of a conventional oligonucleotide). The effective target site was verified by modified minimal inhibitory concentration (MIC) assay of PPNA in four *E. coli* strains (Table S1).

To further optimize the anti-*rpoD* PPNA, de novo design of most potent PPNA selected from target site screening was performed in ways of (i) adding glycine spacer between PNA and CPP; (ii) conjugating CPP at the 5′ end of PNA chain; (iii) shortening PNA length; (iv) upstream shifting of antisense PNA on selected target site of *rpoD* mRNA; and (v) utilization of CPP $(RXR)_4XB$ (in which R is arginine, X is 6-aminocaproic acid and B is β-alanine) for PNA delivery into bacterial cells. The potency of optimized anti-*rpoD* PPNA was determined by MIC and minimum bactericidal concentration (MBC) assays (Table S2).

2.4. Bacterial susceptibility assays

Minimal inhibitory concentration (MIC) of each PPNA was measured according to the broth microdilution method of the CLSI [20]. The minimum bactericidal concentration (MBC) values were determined by plating 10 μl samples from the wells with no visible turbidity onto Mueller-Hinton agar plates. The least concentration showing no visible growth on agar subculture was taken as MBC value. To determine the kill curve, triplicate bacterial cells in logarithmic growth were diluted to 10^5 CFU/ml and incubated with added anti-*rpoD* PPNA at 37 °C in 96-well microtiter plate. Samples (10 μl/time) were taken at different time point (0, 3, 6, and 9 h) during culture, plated onto Mueller-Hinton agar in appropriate diluted concentrations, grown at 37 °C overnight, and numbers of colonies were counted.

2.5. RT-PCR

To detect the expression of *rpoD* and *rpoD* controlled downstream genes including *seqA*, *ftsZ*, *mazF*, *prfB*, *rpoS*, *tufB*, and *ygjD* in bacteria, total RNA from ESBLs-*E. coli*, MDR-*A. baumanni*, MDR-*S. enterica* and MDR-*S. flexneri* was extracted respectively using RNeasy Mini Kit (QIAGEN China Co. Ltd, Shanghai, China) according to the manufacturer's protocol, and was reverse-transcribed using PrimeScript RT reagent Kit with DNA Eraser (Takara Bio Inc, Kyoto, Japan). PCR was performed with the Premix Taq RT-PCR System (Takara Bio Inc, Kyoto, Japan) according to the manufacturer's instructions. Specific primers for all genes indicated above were listed in Table S3. Amplification was performed in a Gradient thermal cycler (BioRad laboratories Inc., Hercules, CA, USA) under the following condition: denaturation at 95 °C for 3 min for the first cycle and for 30 s thereafter, annealing at 55 °C for 30 s, and extension at 72 °C for 40 s for 32 repetitive cycles. Final extension was at 72 °C for 10 min. 16S rRNA was used as an internal control. The PCR products were analyzed by electrophoresis on a 1% agarose gel.

2.6. Western blotting

Anti-*rpoD* PPNA treated bacterial cells and untreated controls were lysed using lysis buffe (Dingguo Biotech Co, Ltd, Beijing, China) containing lysozme (100 μg/mL) and PMSF (1 mM). Cell lysates were quantitated, resolved, blotted and visualized essentially as described by Luo [21]. Equal amounts of protein were loaded and separated on 12% SDS-polyacrylamide gel and were then transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA). The bacterial RNAP σ^{70} monoclonal antibodiy (Abcam, Cambridge, MA, USA) was used at dilution of 1:1000 for overnight blotting at 4 °C; the secondary horseradish peroxidase-conjugated anti-mouse antibody was used at dilution of 1: 2000.

2.7. Epithelial cell infection assay

Gastric mucosal epithelial cells were plated in 96-well culture dishes (Falcon, Franklin Lakes, NJ, USA) at a concentration of 1.5×10^4 cells/ml in a volume of 200 μl and grown at 37 °C for 48 h in Dulbecco's minimal essential medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum in 95% air-5% CO_2. For