siRNA-mediated gene silencing of MexB from the MexA-MexB-OprM efflux pump in *Pseudomonas aeruginosa*

Feng-Yun Gong1,3,#, Ding-Yu Zhang2,#, Jiang-Guo Zhang3, Li-Li Wang4, Wei-Li Zhan3, Jun-Ying Qi3 & Jian-Xin Song3,*

Departments of 1Infectious Diseases, 2Anesthesiology, Wuhan Pu-ai Hospital, Tongji Medical College, Huazhong University of Science and Technology, 3Department of Infectious Diseases, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei, 4Department of Respiratory Medicine, Central Hospital of Qingdao, Qingdao 266042, Shandong, China

To gain insights into the effect of MexB gene under the short interfering RNA (siRNA), we synthesized 21 bp siRNA duplexes against the MexB gene. RT-PCR was performed to determine whether the siRNA inhibited the expression of MexB mRNA. Changes in antibiotic susceptibility in response to siRNA were measured by the E-test method. The efficacy of siRNAs was determined in a murine model of chronic *P. aeruginosa* lung infection. MexB-siRNAs inhibited both mRNA expression and the activity of *P. aeruginosa* in vitro. In vivo, siRNA was effective in reducing the bacterial load in the model of chronic lung infection and the *P. aeruginosa*-induced pathological changes. MexB-siRNA treatment enhanced the production of inflammatory cytokines in the early infection stage (P < 0.05). Our results suggest that targeting of MexB with siRNA appears to be a novel strategy for treating *P. aeruginosa* infections. [BMB Reports 2014; 47(4): 203-208]

INTRODUCTION

The opportunistic pathogen *Pseudomonas aeruginosa* is a common cause of severe infections in hospitalized and immunocompromised individuals, affecting a wide range of organs and leading to potentially life-threatening complications. Treating *P. aeruginosa* infection is particularly challenging due to its inherent and acquired resistance to many of the available drugs. Indeed, multidrug resistant strains of *P. aeruginosa* have been clinically detected. A large study found *P. aeruginosa* drug resistant phenotypes uncovered a strong association with the bacteria’s efflux pump, MexA-MexB-OprM (1-3). The MexA-MexB-OprM pump is the key component of the excretory system and has wide substrate specificity. It has been hypothesized that inhibition or elimination of the inner membrane component, MexB, may strengthen antibiotic sensitivity of *P. aeruginosa*. Therefore, we sought to investigate the effect of silencing MexB gene expression by an RNA interference (RNAi) approach.

RNA-mediated interference (RNAi) was originally discovered as an antiviral mechanism in plants and other organisms, and has since been characterized as an evolutionarily conserved process for the specific suppression of gene expression. (4). siRNAs are 21-23 base pairs (bp) in length and have proven efficacious as exogenous agents to experimentally manipulate gene expression in cultured cell- and animal-based systems. Moreover, siRNAs are highly stable and have low toxicity (5, 6). While siRNA-based antibacterial methods are theoretically promising, very few studies to date have reported on such a strategy. One study achieved successful siRNA inhibition of the *Staphylococcus aureus* staphylococcalase gene (4), and demonstrated that siRNAs could effectively modulate virulence, drug resistance and pathopoiesis. Subsequent studies revealed that siRNAs possess distinctive sense-antisense regulatory capabilities and are dependent upon the RNA-binding protein Hfq (7, 8).

This study was designed to generate a siRNA against the MexB gene in *P. aeruginosa* and investigate the effects of silencing MexB on antibiotic sensitivity of cultured bacteria and pathogenic features in a rodent infection model. The findings from this study provide novel insights into the clinical potential for bacteria gene-targeting siRNA molecules, especially for *P. aeruginosa* infection.

RESULTS

siRNA targeting the MexB gene reduces MexB gene expression in PAO1

MexB gene expression in PAO1 was quantified by real time PCR after siRNA transfection. The 2-ΔΔCt method (9, 10) was used to determine the differences in normalized MexB gene expression induced by the various siRNAs. The results indicated that siRNA2 could significantly reduce the mRNA level...
of MexB, as compared to siRNAnon (P < 0.05). The siRNA1 had no effect on expression of the MexB gene (Fig. 1B), and thus was excluded from subsequent analysis.

The PAO1 strain carrying the siRNA2 exhibited a remarkable sensitivity to antibiotics, as compared to the strains carrying scrambled siRNA (Fig. 1A) or siRNA1.

**MexB-targeting siRNA reduces the PAO1 bacterial load in host animals**

As shown in Fig. 2, the PAO1 siRNA2-infected mice (n = 8) had significantly less viable bacteria than either the PAO1 scrambled siRNA (n = 7) or no siRNA (siRNAnon)-infected (n = 7) mice at all time points evaluated (days 3, 5, and 7 post-infection) (P < 0.05 or P < 0.01, respectively). Meanwhile, the bacteria in the PAO1 siRNA2-infected group were significantly reduced over time (4 × 10^7 CFU/mL at day 3 vs. 3.2 × 10^5 CFU/mL at day 7; P < 0.05). There was no significant difference between the bacteria number of the PAO1 scrambled siRNA- and no siRNA (siRNAnon)-infected mice (P > 0.05).

**MexB-targeting siRNA reduces PAO1-induced microscopic pathologic changes in host lung tissues**

H&E staining revealed that obvious damage, edema, and hemorrhage occurred in the PAO1 no siRNA(siRNAnon)- and scrambled siRNA-infected lung tissues around the bronchus and lung parenchyma (Fig. 3). Similarly, the PAO1 siRNA2-infected lung tissues exhibited obvious damage around the surrounding of bronchus and lung parenchyma, but remarkably less edema than the negative controls at all time points examined (Fig. 3). Moreover, the microscope pathology score was significantly lower for the PAO1 siRNA2-infected group than for either the no siRNA (siRNAnon)- or scrambled siRNA-infected groups (P < 0.05).

**MexB-targeting siRNA reduces PAO1-induced cytokine production in host BAL fluid**

ELISA was used to detect the expression level of cytokines IL-1β and IL-12 in BAL fluid of mice infected with PAO1 carrying the various siRNAs. At days 3 and 5 post-infection, the siRNA2 group expressed much higher levels of IL-1β and IL-12 than the siRNAnon group or the scrambled siRNA group. However, at day 7, the siRNA2 group expressed much lower levels of all three cytokines than the siRNAnon group or the scrambled siRNA group (P < 0.05) (Fig. 4). Furthermore, the...
cystine levels in the siRNA-non group and the scrambled siRNA group were higher at day 7 than at days 3 and 5, but the difference was not statistically significant (P > 0.05).

**DISCUSSION**

*P. aeruginosa* remains a highly prevalent opportunistic pathogen of hospitalized individuals, and has emerged as a particularly challenging infection to treat due to its rapid development of drug resistance. Although carbapenems are active against *P. aeruginosa* and have been successfully used to treat acute exacerbations resulting from *P. aeruginosa* infection, they have also been reported as becoming less effective against *P. aeruginosa* over time (11, 12).

The genome of *P. aeruginosa* genome encodes many additional pumps of RND and other drug families. Of these, MexA-MexB-OprM and MexE-MexY-OprM mediate natural drug resistance and acquired drug-resistance (13). MexA-MexB-OprM was the first efflux pump system demonstrated as a natural drug-resistant mechanism for multiple antibiotics (14), and has since become the most widely studied among the efflux pumps. The efflux system has been implicated in the resistant phenotype (15).

To further investigate the protective antibiotic effects of the MexB subunit of the MexA-MexB-OprM efflux pump, we designed a study to evaluate siRNA-mediated gene silencing of MexB in the *P. aeruginosa* strain PAO1. This idea was based upon the previous study by Yanagiharal et al. (4), in which an siRNA molecule was delivered into methicillin-resistant *Staphylococcus aureus* to effectively silence the coagulase gene, produce methicillin sensitivity, and reduce virulence. In addition, a previous study by Greenfield et al. (16) demonstrated that siRNAs expressed by plasmid vectors were capable of killing bacteria cells that do not express the plasmid, indicating that prokaryote cells have an inherent RNAi regulation mechanism. More recently, Pérez-Martínez et al. (17) reported that a combination therapeutic strategy against *P. aeruginosa* using antibiotics and RNA interference of the rsmY and rsmZ genes effectively reduced biofilm formation and expression of the quorum sensing-related virulence factors.

In our study, we aimed to silence MexB gene expression by an siRNA approach. Two siRNAs were designed to target homologous sequences in exons of the MexB gene. These sequences were synthesized as double stranded DNA and inserted into the pGPU6/GFP/Neo-siRNA expression vector. The expression vector was introduced into the PAO1 strain by electroporation. Only one of the siRNAs targeting MexB, siRNA2, was effective in reducing MexB expression and affecting antimicrobial sensitivity, as evidenced by real time PCR and the antimicrobial susceptibility E-test, respectively. These results indicated that use of an siRNA molecule to silence MexB gene expression, thereby disrupting the expression and function of the MexA-MexB-OprM efflux pump, may be a useful method in decreasing drug resistance in *P. aeruginosa*. Meanwhile, evaluation of the MexB siRNA-silenced PAO1 bacteria in a mouse model showed that the siRNA molecule could reduce the pulmonary infectivity of *P. aeruginosa*, as evidenced by decreased viable bacteria numbers and PAO1-induced pathologic changes in the host lung.

The interference effect of siRNA molecules is known to be able to affect the mammalian immune response and alter infection outcome and prognosis (15). In our study, we observed that siRNA-mediated silencing of MexB in PAO1 bacteria led to decreased neutrophil recruitment during the late stage of pulmonary infection, as indicated by changes in MPO activity. A similar trend was seen in the expressions of the IL-1b and IL-12 cytokines. The early neutrophil leukocytosis that was observed in all infected animals in our study demonstrated the natural host response to pulmonary infection. In chronic *P. aeruginosa* infections, however, the continued presence of activated neutrophils leads to inflammation-related damage by the persistent production of cytokines, chemotactic factors, reactive oxygen species, and protease (18, 19). Thus, the late stage decrease in neutrophils seen in the MexB-siRNA PAO1-infected mice indicates a beneficial quelling of the damaging inflammatory effects.

The MexA-MexB-OprM efflux pump plays an important part in multidrug resistance of *P. aeruginosa*, with overexpression leading to a strong drug-resistant phenotype to many kinds of antibiotics, such as quinolones, macrolides, and β-lactam (20). It has been suggested that the expression of MexA-MexB-OprM might be down-regulated when the peripheral environment is permissive to bacterial growth and survival (21). Thus, silencing of MexB gene expression was considered a promising mechanism by which antibiotic sensitivity could be induced. In this manner, the natural bacteria clearance functions of the T helper cells and associated inflammatory cytokines may be promoted to clear the *P. aeruginosa* pulmonary infection (3). In vivo, this event activated Th1 cell reactions and inflammatory cytokines production; moreover, the induced Th1 cytokimmunity was shown to be beneficial for the
model animal resisting PA pulmonary infection (3). Maintaining a balance between the particular cytokines that direct a Th1 response and those that direct a Th2 response may be critical for determining the outcome of the immune defense mechanisms employed to combat a particular type of infection. In chronic P. aeruginosa infection, a Th1-dominated response has been shown to be associated with better lung function than that mediated by a Th2-dominated response (22). Our research showed that siRNA molecule interference could promote and regulate the production of inflammatory cytokines, promote the organism’s resistance to PA pulmonary infection, and urge the host system to eliminate the pathogenic bacteria. In our study, after three and five days of PA infection, the IL-1β and IL-12 levels in the siRNA interference group were much higher than those in the siRNA non and the scrambled siRNA groups. Infiltrating leukocytes are a common feature of a variety of inflammatory conditions (23). IL-12 functions as an autocrine positive feedback system that amplifies the levels of IL-12 for the proliferation and activation of natural killer (NK) and Th1-type T cells. These cells often contribute to the pathogenesis of disease, but leukocyte recruitment undoubtedly is undoubtedly also critical for host clearance of the infectious pathogen (24, 25).

Ex vivo treatment of PSA with an siRNA molecule is a very long way off from the in vivo alteration of PSA using this pathogen-directed approach. The siRNA strategy described in this manuscript is not directly applicable to the clinical and, therefore, cannot be used to treat patients that have already been infected with PAO. The results, however, do suggest the potential benefit of developing a carrier for in vivo delivery of siRNA into PAO.

Future studies will need to address the choice of a high-performance siRNA molecule carrier that will be most efficacious and safe for human clinical applications.

MATERIALS AND METHODS

P. aeruginosa strain, animal host, and reagents

The P. aeruginosa strain PAO1 was supplied by the Laboratory Department of Tongji Medical Hospital (Huzhong University of Science and Technology, Wuhan, China). Specific pathogen-free (SPF) Balb/c mice (n = 60), six to eight-weeks old and weighting 20-30 g, were kept by the Experimental Animal Center in Tongji Medical College. The pGPU6/GFP/Neo-siRNA expression vector for short hairpin RNAs (shRNAs) was purchased from GenePharma (Shanghai, China). The objective gene, mexB, was 244 base pairs.

siRNA design and construction

The siRNA sequences were designed against PAO1 (26). All RNA oligonucleotides were synthesized by Shanghai Invitrogen Bio Co., Ltd. (Shanghai, China). The Silencer siRNA Construction kit was used to synthesize the siRNAs by in vitro transcription, according to the manufacturer’s instructions. Briefly, a CACC sequence was added to the N-terminal positive-sense strand template, which complemented the cohesive end in the plasmid expression vector digested with BbsI. In contrast, a GATC sequence was added to the N-terminal anti-sense strand template, which complemented the cohesive end in the plasmid expression vector digested with BamHI.

pGPU6/GFP/Neo-siRNA expression vectors were constructed and verified by Shanghai Invitrogen Bio Co., Ltd. The pGPU6/GFP/Neo-siRNA constructs were transformed into PAO1 via electroporation (PAO1-siRNA1, PAO1-siRNA2 and PAO1-Scrambled siRNA). The length of the siRNA was 21 base pairs. The sense sequence of siRNA1 was 5’-GATCCATGAGGTAGTATGGGATGTT-3’ and the anti-sense sequence was 5’-ACAGCTTGATGTGACAAAGG-3’. The sense sequence of siRNA2 was 5’-TGTCGAACTACATCGTTTCCA-3’ and the anti-sense sequence was 5’-TGTCGAACTACATCGTTTCCA-3’. The sense sequence of scrambled siRNA was 5’-GGTCACTACATCGTTTCCA-3’. The anti-sense sequence was 5’-GCTGAGGTAGTATGGGATGTT-3’. The numbering used was according to the wild type PAO MexA-MexB-OprM efflux pump target site (461, 1008, 1554, 740) in http://www.pseudomonas.com.

RNA extraction and real time RT-PCR

Total RNA was extracted from the siRNA-expressing PAO1 strain and PAO1 by using the Trizol reagent (Invitrogen), according to the manufacturer’s instructions. The isolated RNA was reverse transcribed using the RT-PCR kit (Toyobo, Osaka, Japan), and following the manufacturer’s protocol. Primers for MexB and srl (normalization gene) were designed as previously described (27), and were synthesized by Invitrogen (Shanghai, China). The length of the objective gene, mexB, was 244 base pairs. The forward primer sequence was 5’-GCTGAGGTAGTATGGGATGTT-3’ and the reverse primer sequence was 5’-GCCAGCAACACGTCTGTCG-3’. The length of the internal reference, srl, was 241 base pairs. The forward primer sequence was 5’-GCCAGCAACACGTCTGTCG-3’. The PCR amplification was carried out under the following thermal cycling conditions: 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. The PCR products were evaluated by 2% agarose gel electrophoresis and the
relative expression of MexB for all samples was computed.

Minimum inhibitory concentration (MIC) determination by E-test
According to the method described by Macia et al. (28), transformed PA01 cells with an optical density equivalent to McFarland turbidity standard of one were prepared, and 10 μL of the suspension was inoculated onto Mueller-Hinton agar plates that did not contain antibiotics. E-test strips were applied to the MH surface. The plates were read after 24 h incubation at 37°C under aerobic conditions. MIC values of CIP, MP and TZ were determined.

In vivo experiments
The siRNA PA01 strains and scrambled siRNA PA01 strains were immobilized in agarose beads as described by Taniguchi et al. (29). The suspensions of the siRNA PA01 strains and scrambled siRNA PA01 strain were then adjusted to 5 × 10^7 colony-forming units (CFU)/ml.

P. aeruginosa lung infection model and study design
The chronic P. aeruginosa lung infection model was established in 60 mice as previously described (23). The mice were randomly divided into four groups: the PA01-siRNA group (n = 15), the PA01-siRNA2 group (n = 15), the PA01-scrambled siRNA group (n = 15), and the normal group (n = 15). Animals were maintained and used in accordance with the China National Research Council recommendations and were provided food and water ad libitum.

Each mouse received 70 ml of sepharose beads containing 5 × 10^7 CFU of the respective PA01-siRNAs or PA01-scrambled siRNA and PA01. The incision was sutured with silk and healed without any complications. Starting on day 1 after surgical infection, an intra-abdominal injection of meropenem (100 mg/kg) was administered twice daily until sacrifice. The incision was sutured with silk and processed for evaluation by hematoxylin and eosin (H&E) staining.

A portion of the excised lung tissues were embedded in paraffin and processed for evaluation by hematoxylin and eosin (H&E) staining.

Cytokine production
Prior to lung excision, a bronchoalveolar lavage (BAL) was carried out, as previously described (30). The BAL samples were preserved at 4°C until use two hours later in enzyme-linked im-
munosorbent assay (ELISA; R&D Systems) to detect IL-1b and IL-12 cytokines, according to the manufacturer’s instructions.

Statistical analysis
All data are expressed as mean ± standard error of the mean (SEM); SPSS statistical software, version 13.0, was used to conduct all statistical analyses. Intergroup comparisons were carried out by the Chi-square (χ²) test. A P value less than 0.05 indicated statistical significance.

ACKNOWLEDGEMENTS
This study was supported by a grant from the National Natural Science Foundation of China (No. 30873189).

REFERENCES
1. Poole, K. (2005) Efflux-mediated antimicrobial resistance. J. Antimicrob. Chemother. 56, 20-51.
2. Coban, A. Y., Ekinci, B. and Durpinar, B. (2004) A multi-drug efflux pump inhibitor reduces fluoroquinolone resistance in Pseudomonas aeruginosa isolates. Chemotherap. 50, 22-26.
3. Hong, W., Song, Z., Givskov, M., Doring, G., Werlitzsch, D., Mathee, K., Rygaard, J. and Holst, N. (2001) Pseudomonas aeruginosa mutations in lasI and rhlI quorum sensing systems result in milder chronic lung infection. Microbiology 147, 1105-1113.
4. Yanagihara, K., Tashiro, M. and Fukuda, Y. (2006) Effects of short interfering RNA against methicillin-resistant Staphylococcus aureus coagulase in vitro and in vivo. J. Antimicrob. Chemother. 57, 122-126.
5. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Potent and genetic interference by double stranded RNA in caenorhabditis elegans. Nature 391, 806-811.
6. Takeuchi, K., Kiefer, P., Reimmann, C., Keel, C., Dubuis, C., Rolli, J., Vorholt, J. A. and Haas, D. (2009) Small RNA-dependent Expression of Secondary Metabolism Is Controlled by Krebs Cycle Function in Pseudomonas fluorescens. J. Biol. Chem. 284, 34976-34983.
7. Aria, H. (2007) Mechanism of RNA silencing by Hfq-binding small RNAs. Curr. Opin. Microbiol. 10, 134-139.
8. Sonnleitner, E. and Haas, D. (2011) Small RNAs as regulators of primary and secondary metabolism in Pseudomonas species. Appl. Microbiol. Biotechnol. 91, 63-79.
9. Waqner, J. G. and Roth, R. A. (2000) Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. Pharmacol. Rev. 52, 349-374.
10. Reiniger, N., Lee, M. M., Coleman, F. T., Ray, C., Golan, D. E. and Pier, G. B. (2007) Resistance to Pseudomonas aeruginosa chronic lung infection requires cystic fibrosis transmembrane conductance regulatormodulated interleukin-1 (IL-1) release and signaling through the IL-1 receptor. Infect. Immun. 75, 1598-1608.
11. Li, X. Z., Zhang, L. and Poole, K. (2000) Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resist-
siRNA-mediated gene silencing of MexB
Feng-Yun Gong, et al.

12. Sanchez, P., Rojo, F. and Martinez, J. L. (2002) Transcriptional regulation of MexR, the repressor of Pseudomonas aeruginosa MexAB-OprM multidrug efflux pump. FEMS Microbiol. Lett. 207, 63-68.

13. Moser, C., Jensen, P. O., Kobayashi, O., Hougén, H. P., Song, Z., Rygaard, J. and Khazarzmi, A. H. by N. (2002) Improved outcome of chronic Pseudomonas aeruginosa lung infection is associated with induction of a Th1-dominated cytokine response. Clin. Exp. Immunol. 127, 206-213.

14. Strieter, R. M., Standiford, T. J. and Huffnagle, G. B. (1996) “The good, the bad and the ugly”, The role of chemokines in models of human disease. J. Immunol. 156, 3583-3586.

15. Koedel, U., Frankenberg, T., Kirschnek, S., Obermaier, B., Häcker, H., Paul, R. and Häcker, G. (2009) Apoptosis Is Essential for Neutrophil Functional Shutdown and Determines Tissue Damage in Experimental Pneumococcal Meningitis. PloS Pathog. 5, e1000461.

16. Wu, C. M., Cao, J. L., Zheng, M. H., Ou, Y., Zhang, L., Zhu, X. Q. and Song, J. X. (2008) Effect and Mechanism of Androgapholide on the Recovery of Pseudomonas aeruginosa Susceptibility to Several Antibiotics. J. Int. Med. Res. 36, 178-186.

20. Greenfield, T. J., Franch, T., Gerdes, K. and Weaver, K. E. (2001) Antisense RNA regulation of the par post-segregational killing system: structural analysis and mechanism of binding of the antisense RNA, RNAI and its target, RNAI. Mol. Microbiol. 42, 527-537.