RESEARCH ARTICLE

oprM as a new target for reversion of multidrug resistance in *Pseudomonas aeruginosa* by antisense phosphorothioate oligodeoxynucleotides

Hui Wang[1], Jingru Meng[1], Min Jia[1], Xue Ma[1], Gonghao He[1], Jichen Yu[2], Rutao Wang[2], Hui Bai[1], Zheng Hou[1] & Xiaoxing Luo[1]

[1]Department of Pharmacology, School of Pharmacy, Fourth Military Medical University, Xi'an, Shaanxi, China; and [2]Xi'an Libang Pharmaceutical Company, Xi'an, Shaanxi, China

Correspondence: Xiaoxing Luo, Department of Pharmacology, School of Pharmacy, Fourth Military Medical University, 17 Changle West Rd, Xi'an 710032, Shaanxi, China. Tel./fax: +86 29 84774591; e-mail: xxluo3@fmmu.edu.cn

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Abstract

Multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) is one of the leading Gram-negative organisms associated with nosocomial infections. The increasing frequency of MDR-PA has represented a huge challenge in conventional antibacterial therapy. The loss of effectiveness of commonly used antibiotics calls for the immediate need to develop an alternative strategy for combating MDR-PA infections. The multiantibiotic resistance of MDR-PA is largely attributable to the production of multidrug efflux pumps, MexAB-OprM. OprM forms the antibiotic-ejecting duct and plays a crucial role in exporting incoming chemotherapeutic agents across the membranes. Disruption of the OprM expression may inhibit the function of multidrug efflux pumps and lead to restoration of MDR-PA susceptibility to antibiotics. In this study, we developed a novel anion liposome for encapsulating and delivering specific anti-*oprM* phosphorothioate oligodeoxynucleotide (PS-ODN617) and polycation polyethylenimine (PEI) complexes. The additions of the encapsulated anti-*oprM* PS-ODN617/PEI to MDR-PA isolates caused a significant reduction of *oprM* expression and inhibition of MDR-PA growth in the presence of piperacillin in a concentration-dependent manner. The encapsulated PS-ODN617 treatment also reduced minimal inhibitory concentrations of five most commonly used antibiotics to the sensitive margin values on MDR-PA clinical isolates, respectively. The results of present study firstly indicate that PS-ODN targeted to *oprM* can significantly restore the susceptibility of MDR-PA to existing antibiotics, which appears to be a novel strategy for treating MDR-PA infections.

Introduction

The prevalence and isolating rates of multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) has increased over the past decade and become a principal concern among hospitalized patients, especially those in intensive care unit (Karlowsky *et al.*, 2003; Obritsch *et al.*, 2004, 2005). The definition of MDR-PA was established as isolates intermediate or resistant to at least three drugs in the following classes: β-lactams, carbapenems, aminoglycosides and fluoroquinolones (Ohmagari *et al.*, 2005). Infections caused by MDR-PA are often severe and life threatening and are difficult to treat because of the limited susceptibility to antimicrobial agents, thus resulting in severe adverse outcomes (Carmeli *et al.*, 1999; Aloush *et al.*, 2006). The multidrug resistance of *P. aeruginosa* largely attributes to the production of multidrug efflux pumps, which lowers the intracellular drug concentration by exporting incoming chemotherapeutic agents across the membranes (Masuda *et al.*, 2000a, b). Among these efflux systems, MexAB-OprM and MexXY-OprM contribute to both intrinsic resistance and acquired resistance (Li *et al.*, 1995; Masuda *et al.*, 2000a, b). When the level of MexAB-OprM expression becomes relatively high, it results in overproduction of the MexAB-OprM pump complex and enhanced efflux of a broad range of antibiotics, such as quinolones, β-lactams, tetracycline, chloramphenicol and macrolides (Poole *et al.*,

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1993; Li *et al.*, 1995). Deletion of the *mexAB-oprM* multidrug efflux operon substantially compromised the β-lactam resistance and strongly decreased the invasive virulence of *P. aeruginosa* (Srikumar *et al.*, 1999; Hirakata *et al.*, 2002). As such, inhibition of MexAB-OprM is likely to be an effective approach to enhance the susceptibility of MDR-PA.

The concept of using the antisense approach to combat antibiotic-resistant bacteria is revolutionary. Instead of targeting proteins or macromolecular complexes, as do traditional antibiotics, antisense oligomers target specific genes, rRNA or mRNA, and inhibit expression of the targeted sequence. Sequence data of the entire genome of pathogenic bacteria or from multiple isolates of a single pathogen have provided new insights into the microevolution of a species as well as new-generation targets for antimicrobials (Muzzi *et al.*, 2007). Base sequence-specific antisense antibiotics are now more attractive than ever. They can be rapidly synthesized, targeted to any gene of a known sequence and quickly modified to overcome any resistance that may arise (Geller, 2005).

The current study was undertaken to explore the use of a specific anti-*oprM* antisense phosphorothioate oligodeoxynucleotide (PS-ODN617) in inhibiting the expression of *oprM*, and thereafter leading to the restoration of susceptibility of MDR-PA to β-lactam antibiotics and fluoroquinolones. Moreover, we identified the potential of *oprM* as a new target for reversion of multidrug resistance in *P. aeruginosa*.

Materials and methods

Bacterial strains

Strains (MDR-PA070801, MDR-PA070802, MDR-PA070803, MDR-PA070804, MDR-PA070805) of MDR-PA (resistant to piperacillin, cefoperazone, ciprofloxacin, levofloxacin, imipenem and amikacin) used in this study were isolated from sputum of patients in the affiliated hospital of our university. All strains expressed *oprM*, which were confirmed by PCR detection (data not shown). The laboratory strain *P. aeruginosa* (ATCC 27853) was used as a reference strain for quality control measures.

Briefly, for preparation, 2 mL of overnight cultured MDR-PA was transferred to 200 mL of Mueller–Hinton broth medium from Beijing Land Bridge Technology Co. Ltd (Beijing, China) and incubated at 37 °C with moderate agitation (210 r.p.m.) until the $OD_{630\,nm}$ reached 0.55–0.65. Cells were then diluted to a concentration of 0.5×10^8 CFU mL^{-1}.

Chemicals

Piperacillin, cefoperazone, ciprofloxacin, levofloxacin, imipenem and amikacin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Polyethylenimine (PEI) was purchased from Sigma-Aldrich (St. Louis). Egg phosphatidylcholine (EPC) and polyethylene glycol 2000-hydrogenation phosphatidyl ethanolamine (HSPE-PEG_{2000}) were obtained from Xi'an Libang Pharmaceutical Co. Ltd (Xi'an, Shaanxi, China). Dimyristoyl phosphatidylglycerol (DMPG) was purchased from Lipoid (Ludwigshafen, Germany). All culture mediums were purchased from Beijing Land Bridge Technology Co. Ltd.

Antisense PS-ODNs

The sequence of the most active PS-ODNs in this study is AAACTCTTCTGGTAGGTG (PS-ODN617), which is complementary in sequence to nucleotides 600–617 of OprM mRNA in MDR-PA. The control-mismatched sequence of the antisense PS-ODN0701 is CGAGTCCCTTTTTACCAA, which was randomly aligned with same amount of bases. PS-ODNs were synthesized and purified by Aoke Biotechnology Limited-liability Company (Beijing, China).

Preparation of anionic liposome encapsulated with nanosized PS-ODN617/PEI complexes

PS-ODN/PEI complexes and anionic liposomes were prepared as described previously (Chen *et al.*, 2007). Briefly, nanosized PS-ODN/PEI complexes were formed at a molar ratio of PEI nitrogen to PS-ODN phosphate (ratio N/P) of 8 and an optimum charge ratio of 8.0 (+/−; PEI : PS-ODN, 1.1 : 1 w/w). Anionic liposomes composed of EPC, DMPG and HSPE-PEG_{2000} in a molar ratio of 14 : 0.9 : 1 were prepared using the film dispersion method as described previously (Meng *et al.*, 2009). The encapsulation efficiency was determined as the percentage of PS-ODN incorporated into the liposome relative to the initial total amount of PS-ODN.

RNA extraction and real-time reverse transcription (RT)-PCR

The total RNA was extracted from the bacterial culture with Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

The cDNA of *oprM* was synthesized, respectively, by RT from 1 μg of each RNA sample. Briefly, 1 μg of total mRNA from each sample was mixed with 0.1 μg random primer, 4 μL of 2.5 mM dNTP mix and sterile water, and then heated to 65 °C for 5 min followed by cooling on ice for at least 1 min. Then, 1 μL 0.1 M DTT, 4 μL 5 × RT buffer, 0.5 μL 40 U μL^{-1} RNase inhibitor and 0.5 μL 200 U μL^{-1} SuperScript III reverse transcriptase (Invitrogen) were subsequently added to the mixture to obtain a final volume of 20 μL. The samples were then incubated at 25 °C for 5 min, at 50 °C for 45 min and followed by 70 °C for 15 min.

The resulting cDNA was amplified by real-time PCR (Bioer line-gene K, Bioer Technology Co. Ltd, China) using the following gene-specific oligonucleotide primers: *oprM*

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primers corresponding to nucleotides 935–952 bp (5′-CGTT CTTCCCGAGCATCA-3′) and 1029–1047 bp (5′-CAACCA GGAACCCGAACC-3′), respectively, yielded a 93-bp product. Primers for *16S rRNA*, an internal housekeeping gene in MDR-PA, corresponding to nucleotides 317–339 bp (5′-GAG ACACGGTCCAGACTCCTAC-3′) and 436–456 bp (5′-CTT ACTGCCCTTCCTCCCAAC-3′), yielded a 140-bp product. The PCR was run using SYBR Green I. And 12.5 μL of 2 × SYBR Premix Ex Taq^{TM} (TaKaRa, China)1 μL of each primer (5 μM), 5 μL of sample cDNA and 5.5 μL sterile water were mixed to obtain a final volume of 25 μL PCR reagents mixture. Each plate included its own negative controls: no template controls (where all the reaction reagents, except for cDNA, were used). The thermal cycling conditions involved an initial denaturation step at 95 °C for 2 min, 40 cycles at 94 °C for 20 s, 56 °C for 20 s and 72 °C for 20 s. The melting curves of the PCR products were acquired by a stepwise increase of the temperature from 70 to 99 °C (temperature transition 0.4 °C s^{-1}).

The cDNA of control group was fivefold series diluted and the analysis was performed as follows: for each sample, a difference in the cycle threshold values (ΔC_t) was calculated for *oprM* by taking the mean C_t of duplicate tubes and subtracting the mean C_t of the duplicate tubes for *16S rRNA* measured on an aliquot from the same RT reaction: $\Delta C_t = C_t(oprM) - C_t(16S\ rRNA)$.

Relative expression of *oprM* mRNA was calculated using the comparative $\Delta\Delta C_t$ method. *16S rRNA* gene, which is expressed at relatively the same level throughout the developmental cycle in the bacteria, 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. 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 a $\Delta\Delta C_t$. The mean of these $\Delta\Delta C_t$ measurements was then used to calculate the expression of *oprM* ($2^{-\Delta\Delta C_t}$) relative to *16S rRNA* and normalized to the untreated control as follows: relative expression $= 2^{-\Delta\Delta C_t}$. Analyses of gene expression data using the $2^{-\Delta\Delta C_t}$ method have appeared in the literatures (Winer *et al.*, 1999; Schmittgen *et al.*, 2000). The evaluation of $2^{-\Delta\Delta C_t}$ indicates the fold change in gene expression relative to the untreated control.

Bacterial growth assay and susceptibility test

The fresh culture of MDR-PA070801 was diluted to a concentration of 1.5×10^8 CFU mL^{-1} and then 180 μL diluted bacteria broth medium was mixed with 20 μL liposome-encapsulated phosphate-buffered saline (PBS), 20 μL liposome-encapsulated mismatched PS-ODN (100 μg mL^{-1}), 20 μL free PEI (5.5 μg mL^{-1}), 20 μL free PS-ODN617 (100 μg mL^{-1}) or 20 μL liposome-encapsulated PS-ODN617 (3, 10, 30 or 100 μg mL^{-1}), respectively. The cultures were incubated at 37 °C with moderate agitation (210 r.p.m.) for 6 h. Fifty microliters of diluted cells was spread onto Mueller–Hinton agar, which contained 150 μg mL^{-1} of piperacillin. Plates were incubated for 48 h at 37 °C. The number of colonies was counted for plates with > 10 and < 500 colonies. The total CFUs per sample were determined by correcting the colony count for the dilution factor.

To determine the MDR-PA070801 growth in the broth medium, the cell dilution was mixed, respectively, with liposome-encapsulated PBS, liposome-encapsulated mismatched PS-ODN (100 μg mL^{-1}), free PEI (5.5 μg mL^{-1}), free PS-ODN617 (100 μg mL^{-1}) or liposome-encapsulated PS-ODN617 (3, 10, 30 or 100 μg mL^{-1}). One hundred microliters of mixture containing 150 μg mL^{-1} piperacillin was added into a 96-well microtiter plate and the culture was incubated at 37 °C with agitation at 120 r.p.m. The OD of each well was measured at different time points using a microplate reader (Bio-Rad Laboratories, Tokyo, Japan) at 630 nm.

The minimal inhibitory concentrations (MICs) of piperacillin, cefoperazone, ciprofloxacin, levofloxacin, imipenem and amikacin for MDR-PAs were determined using a twofold microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Briefly, serial dilutions of antibiotics in Mueller–Hinton broth were prepared and bacteria culture was mixed with encapsulated PBS liposome, encapsulated mismatched PS-ODN liposome, free PEI or free PS-ODN617, encapsulated PS-ODN617 liposome, respectively. The mixture (50 μL) was then added to each tube to achieve a final inoculum of 5×10^5 mL^{-1}. Incubation was processed at 37 °C for 20 h. Afterwards, 100 μL of 1% triphenyl tetrazolium chloride (TTC, a colorimetric indicator) was added into each tube and cells were further incubated for 3 h at 37 °C. The lowest concentration of antibiotics that resulted in complete inhibition of bacterial growth was indicated by the TTC-based MIC. Lack of bacterial growth shown as no color change from the colorimetric indicator would demonstrate that antibiotics had an inhibitory effect.

Statistical analysis

Values are expressed as mean ± SD, and one-way ANOVA analysis followed by Student-Newman-Keuls *t*-test was performed. A value of $P < 0.05$ was considered to be statistically significant.

Results

Real-time quantitation assays for *oprM* expression

To ascertain whether anti-*oprM* PS-ODN617 inhibits the target gene, the encapsulated anti-*oprM* PS-ODN617/PEI

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Table 1. Relative mRNA expression of *oprM* of MDR-PA070801 detected by real-time PCR

Groups	Concentrations ($\mu g\,mL^{-1}$)	$C_{t\ oprM}$	$C_{t\ 16SrRNA}$	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta C_t}$
Control	0	16.41 ± 0.04	9.49 ± 0.02	6.92 ± 0.06	0	1
L-M	100	16.49 ± 0.06	9.64 ± 0.07	6.86 ± 0.04	0.06 ± 0.01	$1.04 \pm 0.01^{*}$
L-PBS	0	16.52 ± 0.14	9.66 ± 0.08	6.86 ± 0.06	0.05 ± 0.005	$1.04 \pm 0.04^{*}$
F-PEI	5.5	16.50 ± 0.02	9.56 ± 0.02	6.94 ± 0.04	0.02 ± 0.002	$0.98 \pm 0.01^{*}$
F-ODN617	100	16.90 ± 0.05	9.62 ± 0.03	7.29 ± 0.04	0.37 ± 0.01	$0.77 \pm 0.009^{**}$
L-ODN617	3	18.00 ± 0.07	10.00 ± 0.04	8.00 ± 0.03	1.08 ± 0.04	$0.47 \pm 0.01^{***}$
L-ODN617	10	19.10 ± 0.06	10.20 ± 0.05	8.90 ± 0.01	1.98 ± 0.04	$0.25 \pm 0.008^{***}$
L-ODN617	30	19.52 ± 0.05	10.26 ± 0.01	9.27 ± 0.04	2.35 ± 0.05	$0.19 \pm 0.007^{***}$
L-ODN617	100	20.34 ± 0.05	10.43 ± 0.06	9.91 ± 0.02	2.99 ± 0.07	$0.12 \pm 0.006^{***}$

Bacteria were treated with free PBS (control), encapsulated PBS liposome (L-PBS), free PEI (F-PEI), encapsulated mismatched PS-ODN (L-M), free ODN617 (F-ODN617) or encapsulated ODN617 with different concentrations (L-ODN617).
C_t refers to cycle numbers when the fluorescence reaches the fluorescence threshold. $\Delta\Delta C_t = (C_{t\cdot mecA} - C_{t\cdot 16s})_{treatment} - (C_{t\cdot mecA} - C_{t\cdot 16S})_{control}$.
All data presented are representative of the mean ± SD of three independent experiments.
$^{*}P > 0.05$,
$^{**}P < 0.05$,
$^{***}P < 0.01$ vs. control.

complexes were applied to MDR-PA070801 and the expression of *oprM* mRNA of MDR-PA070801 was detected with real-time PCR. The values of the two standard curves were used to determine the absolute value of the slope of the log[cDNA] vs. ΔC_t (difference in the cycle threshold obtained of the two PCR systems with the same cDNA dilution) to the respective dilution. This validation experiment involved pairwise comparisons between *oprM* and *16S rRNA*. The slope was 0.015, demonstrating approximately equal PCR amplification efficiencies between *oprM* and *16S rRNA*. Analysis of the melting curves of the PCR products for each sample of each gene showed a single-peak graph for all amplicons, indicating that a single PCR product was formed. This was confirmed by running 5 μL of each product on 1% agarose gel. The negative controls did not show any amplification product (data not shown).

Compared with the control group, the relative expression of *oprM* in anti-*oprM* PS-ODN617-treated groups (3, 10, 30 and 100 $\mu g\,mL^{-1}$) was decreased in a concentration-dependent manner to 47%, 25%, 19% and 12% of control values, respectively. The relative expression of *oprM* in free PS-ODNs617 group was 77% compared with the control group. Moreover, no detectable changes were observed in the empty liposome, liposome-encapsulated mismatched PS-ODN and free PEI-treated groups (Table 1). This result demonstrates that the encapsulated antisenses not only enter the bacterial cells but also interact with the target mRNA.

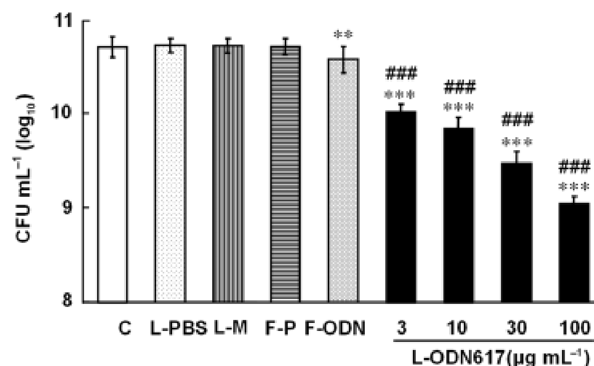

Fig. 1. Effects of anti-*oprM* PS-ODN617 on the growth of MDR-PA070801 colonies. The encapsulated PS-ODN617 was added to cell cultures containing 1.5×10^{8} CFU mL^{-1} MDR-PA070801 to a final concentration of 3, 10, 30 or 100 $\mu g\,mL^{-1}$, with addition of an equal volume of free PBS as a control (C), encapsulated PBS liposome (L-PBS), 100 $\mu g\,mL^{-1}$ encapsulated mismatched PS-ODN (L-M), 5.5 $\mu g\,mL^{-1}$ free PEI (F-P) and 100 $\mu g\,mL^{-1}$ free PS-ODN617 (F-ODN). Aliquots of each culture were collected at 6 h, diluted and inoculated on solid agar containing 150 $\mu g\,mL^{-1}$ of piperacillin. The number of CFU was calculated from the number of colonies growing on plates. The data were shown as mean ± SD of 10 samples, which are from five experiments with duplicate samples in each experiment. $^{**}P < 0.05$, $^{***}P < 0.01$ vs. control; $^{###}P < 0.01$ vs. F-ODN.

Reversal of antibiotic resistance of MDR-PA070801 strain by encapsulated anti-*oprM* PS-ODN617

It was found that the downregulation of *oprM* mRNA expression by the addition of anti-*oprM* PS-ODN617 was correlated with the susceptibility restoration of MDR-PA strain to piperacillin. The number of MDR-PA070801 colonies on the Mueller–Hinton agar containing piperacillin (150 $\mu g\,mL^{-1}$) decreased significantly to 20.2%, 13.7%, 5.7% and 2.1% of the control value in all encapsulated anti-*oprM* PS-ODN617-treated group concentration dependently. The CFU of MDR-PA070801 was only slightly inhibited by free PS-ODN617 treatment (Fig. 1). Additionally, in

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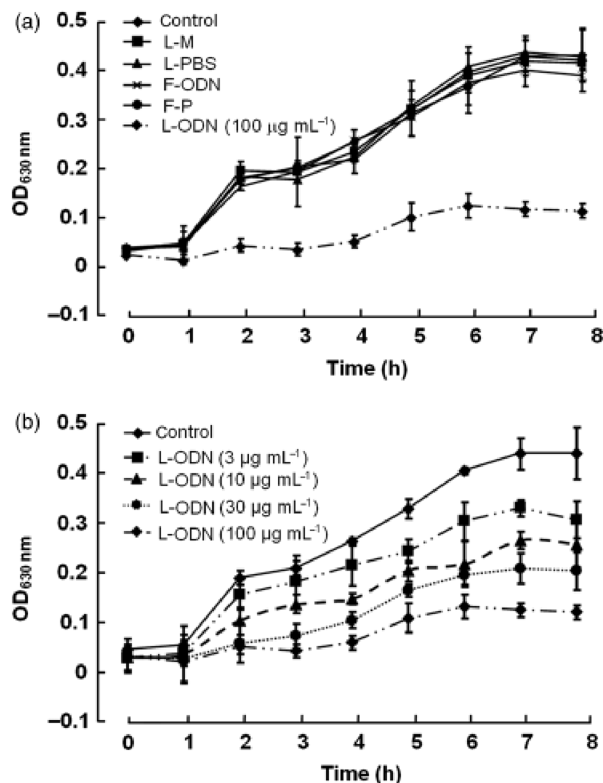

Fig. 2. Effect of anti-*oprM* PS-ODN617 on growth of the MDR-PA070801 in liquid culture medium. The cells were cultured in liquid medium containing 150 μg mL^{-1} of piperacillin. Growth of MDR-PA070801 in different groups was monitored by OD measurements. (a) Bacteria were treated with free PBS (control), encapsulated mismatched PS-ODN (L-M, 100 μg mL^{-1}), encapsulated PBS liposome (L-PBS), free PEI (F-P, 5.5 μg mL^{-1}), free PS-ODN617 (F-ODN, 100 μg mL^{-1}) or encapsulated PS-ODN617 (L-ODN, 100 μg mL^{-1}). (b) Bacteria were treated with a final concentration of 3, 10, 30 or 100 μg mL^{-1} of encapsulated PS-ODN617, respectively, with addition of an equal volume of free PBS as a control. The data were shown as mean ± SD of 10 samples, which are from five experiments with duplicate samples in each experiment.

piperacillin (150 μg mL^{-1})-containing liquid medium, the growth of MDR-PA070801 was also inhibited in encapsulated PS-ODN617-treated groups in a concentration-dependent manner. However, the growth of MDR-PA070801 was not influenced by treatment with empty liposome, liposome-encapsulated mismatch PS-ODN and free PEI (Fig. 2).

Restoration of antibiotic susceptibility of MDR-PA clinical isolates to clinical commonly used antibiotics

The results presented in Table 2 revealed that the MIC of piperacillin for the laboratory strain *P. aeruginosa* (ATCC 27853), which was used as a reference strain for quality

Table 2. MICs of piperacillin for piperacillin-sensitive strain ATCC 27853 and clinical isolated strain MDR-PA070801 in the presence or absence of anti-*oprM* PS-ODN617

Groups	Concentrations of PS-ODN (μg mL^{-1})	MICs of piperacillin (μg mL^{-1})
ATCC 27583	0	4
Control	0	1024
L-PBS	0	1024
F-PEI	0	1024
L-M	100	1024
F-ODN617	100	512
L-ODN617	3	128
L-ODN617	10	32
L-ODN617	30	8
L-ODN617	100	4

L-PBS, encapsulated PBS group; L-M, encapsulated mismatched PS-ODN group; F-PEI, 5.5 μg mL^{-1} free PEI group; F-ODN, 100 μg mL^{-1} free PS-ODNs617 group; L-ODN, encapsulated PS-ODN617 group. The experiment was repeated three times.
The MIC of piperacillin to *Pseudomonas aeruginosa*: sensitive, MIC $\leq$ 64 μg mL^{-1}; resistant, MIC $\geq$ 128 μg mL^{-1}.

control measures, was 4 μg mL^{-1}, while the MIC of piperacillin for MDR-PA070801 was 1024 μg mL^{-1}. Furthermore, encapsulated anti-*oprM* PS-ODN617 lowered the MICs of piperacillin against MDR-PA070801 concentration dependently. At concentrations of 10, 30 and 100 μg mL^{-1}, the encapsulated PS-ODN617 reduced MICs of piperacillin from 1024 μg mL^{-1} to 32, 8 and 4 μg mL^{-1}, respectively, which reached sensitive margin value of piperacillin to MDR-PA070801 on the basis of the interpretive criteria recommended by CLSI and represented the restoration of MDR-PA070801 susceptibility to piperacillin. The MIC value of piperacillin was merely decreased from 1024 μg mL^{-1} in the control group to 512 μg mL^{-1} in the free PS-ODN617-treated group, which suggested that free PS-ODN617 could not efficiently penetrate into MDR-PA070801 cells. The wrapped mismatched PS-ODN (100 μg mL^{-1}), free PEI (5.5 μg mL^{-1}) or empty liposome did not alter MICs of piperacillin on MDR-PA070801.

The results reported above prompted us to examine whether this observation could be extended to other strains of current MDR-PA clinical isolates and to other class of antibiotics. We obtained four other clinical MDR-PA isolates from an affiliated hospital of our university. These five clinical MDR-PA isolates all showed the strong resistance to most common β-lactam antibiotics piperacillin and cefoperazone, quinolone antibiotics levofloxacin and ciprofloxacin, carbapenems antibiotics imipenem as well as aminoglycosides amikacin (control groups). The encapsulated PS-ODN617 (100 μg mL^{-1}) treatment could reduce MICs of all six antibiotics from 4–1024 to 0.5–32 μg mL^{-1}, respectively (Table 3). The results revealed that encapsulated

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Table 3. MICs of piperacillin, ciprofloxacin, levofloxacin, cefoperazone, imipenem and amikacin in the presence/absence of anti-*oprM* PS-ODN617 encapsulated in anionic liposome for five clinical isolated MDR-PA strains in broth culture

	MIC (μg mL^{-1})											
	Piperacillin		Ciprofloxacin		Levofloxacin		Cefoperazone		Imipenem		Amikacin	
Strains	Control	ODN617	Control	ODN617	Control	ODN617	Control	ODN617	Control	ODN617	Control	ODN617
070801	1024	4	8	0.5	32	2	512	16	512	4	512	8
070802	>1024	8	4	1	16	2	256	8	128	4	1024	32
070803	1024	4	16	1	64	1	256	16	512	16	256	16
070804	256	32	32	1	32	1	128	4	256	2	128	16
070805	512	8	8	1	32	2	256	8	256	8	128	16

The concentration of encapsulated anti-*oprM* PS-ODN617 liposome was 100 μg mL^{-1}. The experiment was repeated three times.
The MIC of piperacillin to PA: sensitive, MIC $\leq$ 64 μg mL^{-1}; resistant, MIC $\geq$ 128 μg mL^{-1}.
The MIC of ciprofloxacin to PA: sensitive, MIC $\leq$ 1 μg mL^{-1}; intermediate, MIC = 2 μg mL^{-1}; resistant, MIC $\geq$ 4 μg mL^{-1}.
The MIC of levofloxacin to PA: sensitive, MIC $\leq$ 2 μg mL^{-1}; intermediate, MIC = 4 μg mL^{-1}; resistant, MIC $\geq$ 8 μg mL^{-1}.
The MIC of cefoperazone to PA: sensitive, MIC $\leq$ 16 μg mL^{-1}; intermediate, MIC = 32 μg mL^{-1}; resistant, MIC $\geq$ 64 μg mL^{-1}.
The MIC of imipenem to PA: sensitive, MIC $\leq$ 4 μg mL^{-1}; intermediate, MIC = 8 μg mL^{-1}; resistant, MIC $\geq$ 16 μg mL^{-1}.
The MIC of amikacin to PA: sensitive, MIC $\leq$ 16 μg mL^{-1}; resistant, MIC $\geq$ 32 μg mL^{-1}.

anti-*oprM* PS-ODN617 lowered the MICs of piperacillin against five clinical MDR-PA isolates, and most of which reached sensitive margin values of these antibiotics to MDR-PA strains on the basis of the interpretive criteria recommended by the CLSI (Table 3).

Discussion

A rapid rise in MDR-PA infections in both community and hospital is a threat that clinicians worldwide will be fighting in the years to come and that limits the therapeutic options available (D'Agata, 2004; Obritsch *et al.*, 2005). New approaches for discovering the next generation of antibiotics are urgently required to combat the rise in bacteria that are resistant to current drugs (Payne, 2008).

There is an expectation that the next generation of drugs to combat the increasing threat from multiple antibiotic-resistant bacteria will come from the information explosion generated by bacterial genomics and proteomics. As substitution for the old whole-cell screening, target-based screening in antibacterial agent optimization and development requires that genes essential for pathogen survival and multidrug resistance should be identified and tested as targets before specific compounds are designed or screened from chemical libraries (Taylor *et al.*, 2002; Woodford *et al.*, 2009). The development of antisense antibacterials has shown some promise to reverse, for example, amikacin resistance (Sarno *et al.*, 2003), chloromycetin resistance (Gao *et al.*, 2005) and multidrug efflux in *Escherichia coli* (White *et al.*, 1997) and glycopeptide resistance in enterococci (Torres Viera *et al.*, 2001). Interrupting the expression of resistance genes in this manner could restore susceptibility to key antibiotics, which would be coadministered with the antisense compound. This would extend the lifespan of existing antibiotics, which offer clinically proven therapies, and are often cheaper, more effective or less toxic than the alternatives (Geller, 2005).

Four resistant-modulation-division efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) have been identified in *P. aeruginosa*; two of these, MexAB-OprM and MexXY-OprM, play a role in resistance to several antibiotics, including aminoglycosides, tetracycline, chloramphenicol, quinolones, β-lactams, novobiocin, macrolides and trimethoprim (Poole *et al.*, 1993; Li *et al.*, 1995; Masuda *et al.*, 2000a, b). Overproduction of MexAB-OprM is frequent in clinical settings (Ziha-Zarifi *et al.*, 1999). MexAB-OprM-overproducing strains may represent up to 46% of *P. aeruginosa* isolates and MexAB-OprM overexpression in *P. aeruginosa* may be responsible for therapeutic failures (Hocquet *et al.*, 2007; Boutoille *et al.*, 2009). Hybridization studies with an *oprM* probe have revealed that *oprM* is highly conserved in all serotypes of *P. aeruginosa* (Bianco *et al.*, 1997). The essential biological role OprM played in induction of MDR and highly molecular conservation meet the desirable qualities for its potential as new target.

Our present study demonstrated that PS-ODNs617 targeting the OprM mRNA reduced the expression level of OprM mRNA significantly and consequently, restored the sensitivity of MDR-PA strains. MICs of six clinically commonly used antibiotics, piperacillin, cefoperazone, ciprofloxacin, levofloxacin, imipenem and amikacin on MRD-PA clinical isolates were significantly reduced to the sensitive margin level (Table 3). The results obtained in these experiments are encouraging, as our findings support the notion that OprM mRNA is a promising target for developing new

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antisense oligomer-based drugs to combat the emerging infectious diseases caused by *P. aeruginosa* in the near future.

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