Inhibition of Gene Expression and Growth by Antisense Peptide Nucleic Acids in a Multiresistant β-Lactamase-Producing Klebsiella pneumoniae Strain

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Klebsiella pneumoniae causes common and severe hospital- and community-acquired infections with a high incidence of multidrug resistance. The emergence and spread of β-lactamase-producing K. pneumoniae strains highlight the need to develop new therapeutic strategies. In this study, we developed antisense peptide nucleic acids (PNAs) conjugated to the (KFF),K peptide and investigated whether they could mediate gene-specific antisense effects in K. pneumoniae. No inner membrane permeabilization was observed with antisense PNAs when used alone. Antisense peptide-PNAs targeted at two essential genes, gyrA and ompA, were found to be growth inhibitory at concentrations of 20 μM and 40 μM, respectively. Mismatched antisense peptide-PNAs with sequence variations of the gyrA and ompA genes when used as controls were not growth inhibitory. Bactericidal effects exerted by peptide–anti-gyrA PNA and peptide–anti-ompA PNA on cells were observed within 6 h of treatment. The antisense peptide-PNAs specifically inhibited expression of DNA gyrase subunit A and OmpA from the respective targeted genes in a dose-dependent manner. Both antisense peptide-PNAs cured IMR90 cell cultures that were infected with K. pneumoniae (10⁴ CFU) in a dose-dependent manner without any noticeable toxicity to the human cells.

Klebsiella pneumoniae has emerged as a common cause of serious epidemic and nosocomial infections in hospitals, resulting in high morbidity and mortality (2). K. pneumoniae infections occur in almost all age groups, with urinary and respiratory tract infections being most commonly encountered. K. pneumoniae accounts for a significant proportion of hospital-acquired pneumonia, bacteremia, meningitis, septicemia, and soft tissue infections. Klebsiella neonatal infections are becoming a major concern of pediatricians, as septicemia and meningitis in newborns are now often caused by multidrug-resistant strains.

In addition to host factors, bacterial virulence determinants, such as production of capsular polysaccharides and the aerobactin-mediated iron uptake system contribute to the outcome of K. pneumoniae infections (20). K. pneumoniae is resistant to penicillins and expanded-spectrum cephalosporins through the production of β-lactamases that are encoded mainly by the blaSHV, blaTEM, and ampC genes. β-lactams such as imipenem and meropenem, which are highly resistant to hydrolysis by TEM, SHV, and AmpC β-lactamases remain effective antibiotic options (18). However, the emergence of carbapenem-resistant K. pneumoniae strains will have a serious impact on remaining therapeutic options (22). β-Lactamase-producing strains are clinically significant, as they are difficult to treat. Furthermore, these strains often acquire additional mechanisms of resistance, such as mutations in the gyrA gene and/or expression of efflux pumps (13).

The emergence of antibiotic-resistant bacteria and the slow progress in identifying new classes of antimicrobial agents call for research that will uncover novel therapeutic strategies. Peptide nucleic acid (PNA) is designed to incorporate the predictable recognition properties of nucleic acids and the chemical stability of peptides. The backbone of PNA carries 2-aminoethyl-glycine linkages in place of the regular phosphodiester backbone of DNA, and the nucleotide bases are connected to this backbone at the amino nitrogens through a methylene carbonyl linker (3, 16). PNA oligomers are found to form exceptionally strong complexes with complementary strands of DNA or RNA (4, 5). In vitro studies indicate that PNA could inhibit both transcription and translation of genes to which it has been targeted and holds promise as an antigen or antisense therapy (8, 15). However, as with other high-molecular-weight drugs, the delivery of PNA appears to be a general problem. The lipid bilayer, lipopolysaccharide, and peptidoglycan of the gram-negative bacteria act as major barriers to the entry of PNAS. When PNAS were conjugated with a cell wall-permeabilizing peptide such as KFFKFKFFK, their target-specific antisense effects were found to improve (7, 9, 21).

Sequence-specific PNAs can bind to complementary template DNA or mRNA and give rise to gene-specific silencing through blocking transcription or translation processes (17). In this study, we demonstrated that antisense peptide-PNAs targeted at genes essential for growth carried in the chromosomal DNA were able to inhibit the growth of β-lactamase-producing K. pneumoniae.

MATERIALS AND METHODS

K. pneumoniae cell preparation. The β-lactamase-producing K. pneumoniae strain obtained from a hospital was grown in Muller-Hinton broth (MHB) or

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Luria-Bertani (LB) broth at 37°C on a shaker overnight. The β-lactamase-producing *K. pneumoniae* strain used in this study was resistant to amikacin, ampicillin, aztreonam, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, tazobactam, colistimethate, levofloxacin, and minocycline. This strain contained TEM-1, SHV-11, and AmpC type (DHA-I) β-lactamases that were confirmed by phenotypic tests and sequence analysis of the respective β-lactamase genes.

**Inhibition of bacterial growth.** PNAs were synthesized by Eurogentec and listed in Table 1. Overnight growth β-lactamase-producing *K. pneumoniae* cell cultures were diluted to $10^7$ CFU/ml in 100 μl MHB in a low-binding 96-well microtiter plate (Costar 7424), and antisense peptide-PNA, antisense PNA, or mismatched peptide-PNA were each added to a final concentration of 0, 2, 10, 20, 40, or 80 μM in the well. Microtiter plates were incubated at 37°C in a GENios spectrophotometer (TECAN Austria GmbH) which was set to shake the plate for 5 s at 3-min intervals for 12 h. Turbidities of the cultures were recorded at 550 nm every 3 min. Viable cell counts were determined at different time intervals on LB agar plates in triplicates. The plates were then incubated overnight at 37°C, and the colonies were enumerated by visual inspection.

**β-Galactosidase activity assay.** β-Galactosidase activity in liquid cultures was measured using the chromogenic LacZ substrate o-nitrophenyl-β-D-galactopyranoside (ONPG), as described by Miller (14).

**Nitrocin outer cell barrier permeabilization assay.** Outer membrane permeability was determined as described previously (1). Briefly, the β-lactamase-producing *K. pneumoniae* strain was prepared as described above. Permeabilization assays were carried out using 96-well microtiter plates with wells containing 100 μl of 5 mM HEPES (pH 7.4), 5 mM carbonyl cyanide m-chlorophenylhydrazone, and 20 μg/ml nitrocefin, and cells were added to reach an absorbance reading of 0.1 at 550 nm. Following the addition of the permeabilizing compounds at various concentrations, ranging from 0.1 to 60 μM, cell viability was determined as described previously (6). Free antisense PNAs targeted against gyrA and ompA genes are noninhibitory to cell growth when used at 0.1 μM to 1 μM concentrations. Permeabilization of the outer membrane was monitored using the chromogenic reporter molecule nitrocefin (6). Free antisense PNAs targeted against gyrA and ompA genes when added to cells in the presence of nitrocefin did not permeabilize the membrane, whereas the respective antisense peptide-PNA conjugates permeabilized the outer membrane rapidly. As seen in the nitrocefin assay, membrane permeabilization was much more rapid in the presence of the antisense peptide-PNA conjugates than when antisense PNA was used alone. Permeabilization of the outer membrane was found to be dose dependent, as reflected by the confluence of nitrocefin. When used at concentrations of 0.4 μM onwards, the rates of permeabilization of antisense peptide-PNA conjugates...
gates against gyrA and ompA through the outer membrane was exponential and reached a plateau within 3 min of addition (Fig. 2A and B, respectively). The results show that antisense PNAs when conjugated to the peptide were good membrane-permeabilizing agents.

Effect of growth exerted by antisense peptide-PNAs. The antisense potency of the peptide-PNA targeted at the essential gyrA gene encoding the DNA gyrase subunit A was evaluated. Growth of the β-lactamase-producing K. pneumoniae was evaluated by examining the inhibitory effects of various concentrations of peptide–anti-gyrA PNA compared to treatment with antisense PNA (anti-gyrA) or with the mismatched antisense peptide-PNA (Mis-peptide–anti-gyrA PNA). Growth was totally inhibited by a 15-mer anti-gyrA PNA when it was attached to the carrier peptide (KFF)3K and added to cells at 20 μM concentration (Fig. 3A). No growth inhibition was observed in cultures treated with anti-gyrA PNA which was not conjugated to the peptide (Fig. 3B). The effect of peptide–anti-gyrA PNA on gyrA transcripts in K. pneumoniae was observed by electrophoresis of amplicons after real-time reverse transcription-PCR (RT-PCR) (Fig. 3C). A dose-dependent gradual reduction of gyrA gene expression in K. pneumoniae cells was observed, and no expression was detectable when the antisense peptide-PNA was used above 20 μM. The mismatched peptide–anti-gyrA PNA with two nucleotide differences did not inhibit cell growth (Fig. 3D).

The primary target of fluoroquinolones in gram-negative bacteria is DNA gyrase, a type II topoisomerase required for DNA replication and transcription. DNA gyrase is a tetrameric enzyme composed of two A and two B subunits. It catalyzes the negative supercoiling of DNA and is therefore essential for maintenance of DNA topology. In the gram-negative organisms, resistance to fluoroquinolones has been shown to be associated most frequently with alterations in gyrA (12). In this study, the PNA which was designed to be complementary to the gyrA gene present in the coding strand will bind directly to

FIG. 1. LacZ expression and inhibition with antisense peptide-PNA conjugate in K. pneumoniae. The values indicate relative enzyme activities in K. pneumoniae cultures growing in MHB. For antisense inhibition, the lacZ gene in K. pneumoniae was induced with 50 μM IPTG and assayed at A420 with the substrate ONPG. Anti-lacZ, antisense peptide-PNA targeted at lacZ gene; Mis-lacZ, mismatched peptide-PNA targeted at lacZ gene.

FIG. 2. Kinetics of outer membrane permeabilization by different concentrations of antisense peptide-PNAs. (A) Peptide–anti-gyrA PNA was included in the reactions at 0.1 μM (●), 0.2 μM (+), 0.3 μM (○), 0.4 μM (▲), 0.5 μM (●), and 1 μM (◆) concentrations. Anti-gyrA PNA alone without peptide conjugation was included at 1 μM concentration (+). (B) Peptide–anti-ompA PNA was included in the reactions at 0.1 μM (●), 0.2 μM (+), 0.3 μM (○), 0.4 μM (▲), 0.5 μM (●), and 1 μM (◆) concentrations. Anti-ompA PNA alone without peptide conjugation was included at 1 μM concentration (+).
the mRNA, leading to degradation of the message and subsequent inhibition of translation. The failure to synthesize DNA gyrase subunit A, which is needed for DNA replication, resulted in inhibition of growth.

A 16-mer peptide–anti-ompA PNA conjugate targeted at the major outer membrane protein A (OmpA) prevented cell growth when it was used at a 40 μM concentration (Fig. 4A). When anti-ompA PNA was not conjugated to a peptide, growth was not inhibited and no change in OmpA protein expression was observed (Fig. 4B and C). Total inhibition of ompA gene and protein expression was observed by real-time RT-PCR and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively (Fig. 4C). Characterization of the OmpA protein was conducted using matrix-assisted laser desorption ionization–time of flight (mass spectrometry) analysis, and the data were compared to the NCBI database for sequence matches. The mismatched peptide–anti-ompA PNA which differed in 2 nucleotides from the antisense peptide–anti-ompA PNA was found not to be growth inhibitory (Fig. 4D). These results indicate that the (KFF)₅K peptide was needed to carry the two antisense PNAs into K. pneumoniae. Such peptide-PNA conjugates open up new possibilities for anti-infective drug development. OmpA is highly represented in the bacterial cell wall, is conserved among the Enterobacteriaceae, and is involved in bacterial virulence and growth. OmpA is one of the major outer membrane proteins that assembles into the outer membrane via an N-terminal eight-transmembrane amphipathic β-barrel region, with the C-terminal region being retained in the periplasm (19). Functions attributed to OmpA include maintenance of structural cell integrity and a role in bacterial conjugation, bacteriophage binding, and cell growth (11). It also contributes to the ability of the gram-negative bacteria to invade mammalian cells (10).

**Effect of peptide-PNA conjugate on cell viability.** In *Escherichia coli*, the cationic peptide with the sequence KFFKFF KFKK was previously shown to be effective in carrying PNA (as peptide-PNA conjugate) across the membrane barrier (9). The antisense peptide-PNA conjugates targeting the gyrA and
ompA genes were evaluated for their antibacterial efficacy against the multiresistant β-lactamase-producing *K. pneumoniae* strain in liquid culture. The bacteriostatic versus bactericidal effects of gene inhibition were studied by determining the number of CFU in cultures treated with antisense peptide-PNAs. To do this, aliquots were taken at multiple time points during antisense peptide-PNA treatment, and the number of viable cells in the sample was indicated by determining the number of CFU.

After incubation for 3 h, the numbers of viable cells in the culture were significantly reduced in the presence of the antisense peptide-PNA conjugates compared with cell cultures treated with PNA alone. After 6 h of incubation, numbers of CFU were determined by dilution and plating on LB agar. No CFU was observed when cultures were treated with antisense *gyrA* and *ompA* peptide-PNAs at concentrations above 40 μM. This inhibition was not observed in cell cultures treated with antisense mismatched PNA conjugates targeted at the respective genes (data not shown). When the respective antisense PNAs which were not conjugated to peptides were used, no bactericidal effect was observed (Fig. 5).

The antisense PNAs described in this paper are very promising compounds for antimicrobial development against β-lactamase-producing *K. pneumoniae* strains. Antisense PNAs targeted against essential genes are potential targets for new antimicrobial development and this approach enables one to find out which genes in *K. pneumoniae* are susceptible targets for more conventional antimicrobial development.

Treatment of *K. pneumoniae*-infected epithelial cells with antisense peptide-PNAs. To investigate an in vivo model of *K. pneumoniae* infection, IMR90 monolayer cells were infected with a β-lactamase-producing *K. pneumoniae* strain. The morphology of the IMR90 monolayer cells was monitored. At 6 h postinfection, cells in the monolayer were observed to round up (Fig. 6A). Complete cell death was observed after overnight infection (Fig. 6A).

To evaluate the antibacterial potential of antisense *ompA* and *gyrA* peptide-PNAs against the growth of *K. pneumoniae* in
the presence of eukaryotic cells, IMR90 cell cultures were infected with $10^4$ CFU of the β-lactamase-producing K. pneumoniae strain, and antisense peptide-PNAs were added 1 h postinfection. This system can be viewed as a very simple model for the growth of an extracellular pathogen in eukaryotic cells in the presence of antisense peptide-PNAs. The antisense peptide-PNA did not visibly affect IMR90 cell growth at the highest concentration tested (80 μM) (data not shown). When antisense peptide-PNAs at 20 μM or higher were added to K. pneumoniae-infected cells, no change in cell morphology was observed and the cells remained healthy after overnight incubation (Fig. 6B). No live bacteria were detected from the PNA-treated samples in an agar CFU test, confirming that these antisense peptide-PNA targets are bactericidal and serve as a very simple model for the growth of an extracellular pathogen in eukaryotic cells in the presence of antisense peptide-PNAs.

FIG. 5. Bactericidal effects of antisense peptide-PNAs targeting gyrA and ompA. The number of CFU was calculated at different time points. Antisense peptide-PNAs were added to K. pneumoniae cultures at 2 (■), 10 (▲), 20 (×), 40 (+), and 80 (●) μM concentrations and compared to the culture containing PNA alone at 80 μM (★).

FIG. 6. (A) Microscopy of epithelial cells infected with K. pneumoniae. (1) Normal healthy IMR90 cells in monolayer; (2) IMR90 cell morphology was changed (rounded cells) after 6 h of infection with bacteria; (3) after overnight infection, complete cell death was observed. (B) Antisense peptide-PNA treatment of IMR90 cell lines infected with a β-lactamase-producing K. pneumoniae strain. The images show IMR90 cell cultures grown in MEM and 10% fetal calf serum. IMR90 cell cultures were treated with different concentrations of different antisense peptide-PNAs (10, 20, or 40 μM) after K. pneumoniae infection was allowed to proceed for 1 h. Complete cell death of the IMR90 monolayer was observed for the cultures not treated with PNA after overnight incubation.
as valuable targets for antigenic therapeutic drugs against multiresistant K. pneumoniae strains. The observed antimicrobial effects of antisense peptide-PNA within a cell culture system broaden the potential scope of the approach and raise the prospects for PNA-based antibacterial drugs. However, of concern are delivery, potency, toxicity, and pharmacokinetics of antisense peptide-PNA conjugates in humans infected with antibiotic-resistant bacteria.

In conclusion, antisense peptide-PNAs targeted to essential genes of the human pathogen, a β-lactamase-producing K. pneumoniae strain, were shown to inhibit growth of the bacteria in vitro and in infected IMR90 epithelial cells. Treatment of K. pneumoniae cells with 20 μM peptide–anti-graA PNA was shown to be bactericidal, as no viable cells were detected after 6 h of infection. Higher concentrations of antisense peptide-PNA (at 40 μM) targeted at the ompA gene were able to inhibit K. pneumoniae growth. From this study, DNA gyrase subunit A and OmpA were established to be essential for bacterial growth and thus could serve as good anti-infective targets. Dose-dependent growth inhibition of K. pneumoniae was observed, with both of the antisense peptide-PNAs targeting the respective genes. No inhibition of growth was observed when control antisense PNAs with sequence variations or PNAs without the peptide were administered. Future experimentation with peptide-conjugated PNAs will need to be carried out with mice and primates to evaluate the potency, toxicity, and pharmacokinetics of PNAs for prevention and treatment of β-lactamase-producing K. pneumoniae infections.

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REFERENCES


