Inhibition of AAC(6′)-Ib-Mediated Resistance to Amikacin in *Acinetobacter baumannii* by an Antisense Peptide-Conjugated 2′,4′-Bridged Nucleic Acid-NC-DNA Hybrid Oligomer

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Multiresistant *Acinetobacter baumannii*, a common etiologic agent of severe nosocomial infections in compromised hosts, usually harbors *aac(6′)-Ib*. This gene specifies resistance to amikacin and other aminoglycosides, seriously limiting the effectiveness of these antibiotics. An antisense oligodeoxynucleotide (ODN4) that binds to a duplicated sequence on the *aac(6′)-Ib* mRNA, one of the copies overlapping the initiation codon, efficiently inhibited translation *in vitro*. An isosequential nuclease-resistant hybrid oligomer composed of 2′,4′-bridged nucleic acid-NC (BNA^{NC}) residues and deoxynucleotides (BNA^{NC}-DNA) conjugated to the permeabilizing peptide (RXR)$_4$XB ("X" and "B" stand for 6-aminohexanoic acid and β-alanine, respectively) (CPPBD4) inhibited translation *in vitro* at the same levels observed in testing ODN4. Furthermore, CPPBD4 in combination with amikacin inhibited growth of a clinical *A. baumannii* strain harboring *aac(6′)-Ib* in liquid cultures, and when both compounds were used as combination therapy to treat infected *Galleria mellonella* organisms, survival was comparable to that seen with uninfected controls.

*A*cinetobacter baumannii is an opportunistic human pathogen, mainly nosocomial, that causes bacteremia, meningitis, urinary tract infections, pneumonia, and necrotizing fasciitis among other infections (1–4). Multidrug-resistant *A. baumannii* strains are increasingly found in hospitals, complicating treatment of the infections they cause (4). Antisense technologies could be a path for designing new therapeutic strategies to overcome this problem. Options include the silencing of one or more essential genes (5–12) or the silencing of one or more resistance genes to induce phenotypic conversion to susceptibility (13–16). In the latter case, the antisense compound would be administered in combination with the appropriate antibiotic. However, in spite of important advances, silencing of bacterial genes by antisense oligomers is far from reaching its full potential (10). The main antisense mechanisms of gene silencing include degradation of the target mRNA by double-stranded RNA (dsRNA)-specific RNase, RNase H, or RNase P and steric hindrance of translation (interference with assembly of the ribosome or translation arrest) (10, 17). Practical application of any of these strategies requires that the antisense compounds resist the action of the ubiquitous nucleases and reach the cytosol to exert their action.

There are numerous nuclease-resistant nucleotide analogs available that are adequate for different antisense strategies (10, 18, 19). For example, hybrid molecules containing locked nucleic acid and deoxyribonucleotide residues (LNA-DNA) in different configurations have been successfully utilized in bacteria and eukaryotes (16, 20–23). New analogs related to LNAs, the 2′,4′-bridged nucleic acid-NC (BNA^{NC}) analogs (Fig. 1), that exhibit advantages such as higher binding affinity to a cRNA and excellent single-mismatch discriminating ability, have been recently introduced (24). Furthermore, tests carried out in mice showed that BNA^{NC}-based antisense molecules have minimal toxicity (25).

The most successful strategy to guide antisense oligomers inside cells is through conjugation to cell-penetrating peptides, which consist of a small number (no more than 30) of amino
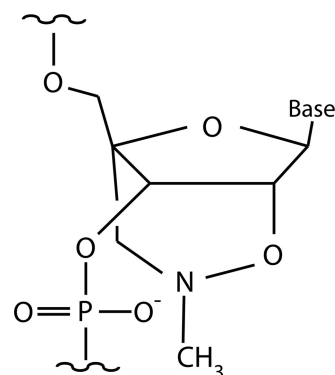

FIG 1 Chemical structure of a 2′,4′-BNA^{NC} residue.

acids, are amphipathic, and have a net positive charge (13, 18, 26, 27). LNA-DNA co-oligomers have been conjugated to cell-penetrating peptides and successfully used to inhibit gene expression (23). In particular, antisense oligonucleotide analogs conjugated to the (RXR)$_4$ or (RXR)$_4$XB peptides (R, arginine; X, 6-amino-

TABLE 1 Oligodeoxynucleotides and analogs[a]

Name	Sequence	Length (bp)
ODN1	TTTTACTGCTGCGTAACATCGTTGCTG	20
ODN2	GTAACATCGTTGCTG	15
ODN3	GCGTAACATCGTTGC	15
ODN4	CTGCTGCGTAACATC	15
ODNS	GATGTTACGCAGCAG	15
ODNAP	AGCGGTAAGGCATCT	15
CPPBD4	(RXR)₄XB-Cys-SMCC-C6 amino-C+TGCT+GCGT+AACA+TC	15
CPPBDAP	(RXR)₄XB-Cys-SMCC-C6 amino-A+GCGG+TAAG+GCAT+CT	15

[a] ODNs are oligodeoxynucleotides; CPPBDs are permeabilizing peptide-conjugated 2′,4′-bridged nucleic acid residue and deoxynucleotide hybrid compounds. R, arginine; X, 6-aminohexanoic acid; B, β-alanine, +N, BNA^NC; SMCC, sulfosuccinimidyl-trans-4-(N-maleimidomethyl)cyclohexane-1-carboxylate.

hexanoic acid; B, β-alanine) were efficiently guided inside *A. baumannii* and showed biological activity (18, 28).

A. baumannii A155, a strain isolated from a urinary sample (29), harbors *aac(6′)-Ib*, the most common amikacin (AMK) resistance gene found in Gram-negative pathogens (30, 31). This gene is present in the chromosome as well as in integrons, transposons, plasmids, genomic islands, and other genetic structures, is broadly distributed among Gram-negative species, and is characterized as being highly heterogeneous at the N terminus (32). However, the *aac(6′)-Ib* allele present in the chromosome of *A. baumannii* A155 is found in numerous Gram-negative bacterial species. In this work, we show that a (RXR)₄XB-conjugated BNA^NC-DNA antisense co-oligomer that targets a duplicated region in the *aac(6′)-Ib* mRNA, one that includes the start codon and another that encompasses the codons specifying amino acids 7 to 11, induced susceptibility to AMK.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and permeabilizing peptide-conjugated oligonucleotide analogs. *A. baumannii* A155 is a multi-drug-resistant clinical strain isolated from a urine sample (29, 33). Plasmid pFC9 (34), which carries the *aac(6′)-Ib* gene, was used as the template for generating a linear DNA fragment consisting of the T7 promoter followed by *aac(6′)-Ib* that was used to synthesize the mRNA *in vitro* as described before (16). The oligonucleotides used as primers to generate the DNA template were 5′-TTGTAATACGACTCACTATAGGGAGAAA GCGCGTTACGCCGTGGGTCGATG and 5′-GGGTTAGGCATCACTG CGTGT. Antisense oligodeoxynucleotides (ODNs) were purchased from IDT (Integrated DNA Technologies) and (RXR)₄XB-Cys-SMCC-C6 amino-2′,4′-BNA^NC-DNA (R, arginine; X, 6-aminohexanoic acid; B, β-alanine) (CPPBD) from Bio-Synthesis Inc. (Table 1). The chemical structure of a 2′,4′-BNA^NC residue is shown in Fig. 1.

General procedures. The presence of the *A. baumannii* A155 *aac(6′)-Ib* allele in other Gram-negative bacteria was determined using BLAST (35). *In vitro* translation of AAC(6′)-Ib was carried out using an *Escherichia coli* S30 Cell-Free Extract System for Circular DNA kit (Promega). The reactions were performed as recommended by the supplier in the presence of 10 μCi (specific activity, 1,175 Ci/mmol) of [^{35}S]methionine (Perkin-Elmer) and, when indicated, 6.6 μM ODN or CPPBD compounds. The products were analyzed using sodium dodecyl sulfate-18% polyacrylamide gel electrophoresis (36). Gels were treated with En3hance (PerkinElmer) for 20 min, immersed in a solution containing 3.3% glycerol and 3.3% polyethylene glycol for 20 min, and dried, and radioactivity was detected on a phosphorimager (Cyclone Storage Phosphor system; Packard). Growth inhibition assays in the presence of (RXR)₄XB–BNA^NC-DNA oligonucleotide analogs were carried out by inoculating Mueller-Hinton broth (100 μl) with the additions indicated in the text in microtiter plates using a BioTek Synergy 5 microplate reader (37). Culture procedures were carried out at 37°C with shaking, and optical density at 600 nm (OD$_{600}$) was recorded every 20 min.

Infection assays. *A. baumannii* cells were collected by centrifugation and resuspended in phosphate-buffered saline (PBS) (7.2 pH) or in PBS (7.2 pH) with the indicated additions as described before (38). Bacterial inocula were estimated spectrophotometrically at 600 nm. The injection site was swabbed with ethanol immediately prior to injection using a syringe pump (New Era Pump Systems, Inc., Wantagh, NY) with a 26-gauge by half-inch needle to deliver 5-μl inocula containing 5×10^5 (± 0.5 log) *A. baumannii* A155 cells into the hemocoel at the last left proleg. Ten healthy randomly selected final-instar *G. mellonella* larvae (Grubco, Fairfield OH), weighing 250 mg to 350 mg, were used for each group ($n = 30$) in experiments performed in triplicate. If >2 deaths occurred in either the PBS-injected or the no-injection control groups, that trial was omitted. After injection, the larvae were incubated at 37°C in the dark. Survival was assessed at 24-h intervals over 120 h with removal of dead caterpillars at time of inspection. The survival curves were plotted using the Kaplan-Meier method. A P value of ≤0.05 was considered statistically significant for the log-rank test of survival curves (SAS Institute Inc., Cary, NC).

RESULTS

AAC(6′)-Ib is a ubiquitous aminoglycoside-modifying enzyme that confers multiresistance to aminoglycosides, including AMK, to the majority of AAC(6′)-I-producing Gram-negative clinical isolates (30, 39). The purpose of this work was to inhibit production of this enzyme in the *A. baumannii* A155 clinical strain using antisense BNA^NC-DNA co-oligomers complementary to the region of initiation of translation. We selected BNA^NC-DNA molecules as antisense molecules because of their high affinity of binding to the sense molecule, their specificity, which may lead to a highly efficient and selective inhibitory effect, and their low toxicity (24, 25).

Since numerous variants of *aac(6′)-Ib* have been identified, several of them differing at the N terminus (32, 40), we decided to find out if a potential antisense molecule that inhibits expression of the resistance gene could have applications beyond this isolate. For this, we determined if the *aac(6′)-Ib* allele found in *A. baumannii* A155 is present in other Gram-negative clinical isolates or is unique to this strain. A BLAST comparison of the nucleotides in the sequence of the complete gene plus 16 nucleotides upstream of the start codon showed 27 identical sequences and 39 that have 100% coverage of the sequence and 99% identity (see Table S1 in the supplemental material). This allele of the gene is found in chromosomes and plasmids in diverse strains of Gram-negative species, including *Salmonella enterica* serovar Typhimurium, *Klebsiella pneumoniae*, *K. oxytoca*, *E. coli*, *E. fergusonii*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *A. baumannii*, *Shigella flex-*
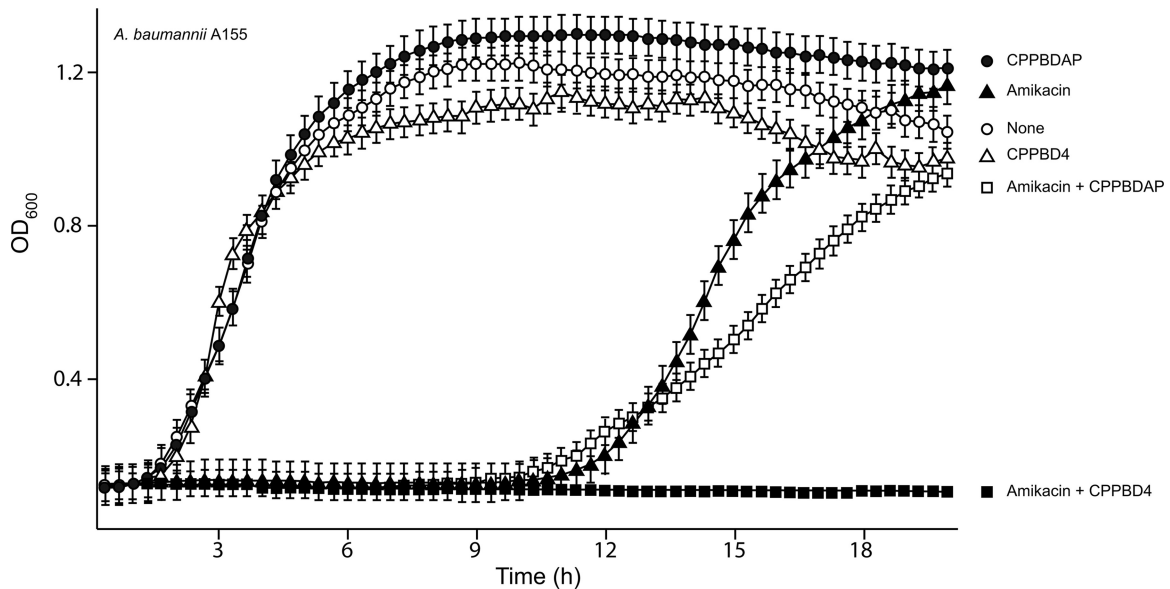
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ODN1                                           GTCGTTGCTACAATGCGTCG-5'
                          GTCGTTGCTACAATGCGTCG-5'

ODN2                                           GTCGTTGCTACAATG-5'
                          GTCGTTGCTACAATG-5'

ODN3                                           CGTTGCTACAATGCG-5'
                          CGTTGCTACAATGCG-5'

ODN4                                           CTACAATGCGTCGTC-5'
                          CTACAATGCGTCGTC-5'

Ab A155    GTTATGG AGCAG CAACGATGTTACGC AGCAG CAACGATGTTACGC AGCAG GGCAGTCGCC...
                    M     L  R  S  S    N     D  V  T  Q     Q     G  S  R...
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FIG 2 Nucleotide sequences targeted by antisense ODNs. The nucleotide sequences around the initiation codon of the *A. baumannii* (*Ab*) A155 *aac(6')-Ib* gene are shown at the bottom of the figure. The duplicated sequences are within red and blue boxes. The sequences of the ODNs tested and the locations of antisense regions are shown at the top.

neri, Aeromonas allosaccharophila, and *Burkholderia cepacia*. In addition, sequences targeted by some of the oligonucleotides tested are also present in other alleles of this gene such as those present in InV117, an integron found in a ca. 150-kbp *Vibrio cholerae* O1 biotype El Tor plasmid (34), and in In116, an integron described in *Morganella morganii* (41).

Inhibition of translation in vitro. We first tested oligodeoxynucleotides (ODNs) complementary to a region encompassing the start codon where there is a 19-bp tandem duplication (Fig. 2). As a consequence, each antisense ODN was able to bind to two regions, one of which included the initiation codon (Fig. 2). Cell extracts were incubated in the presence of the mRNA with or without addition of the ODNs. The *in vitro* synthesis reaction produced two protein bands of ca. 20 and 25 kDa, respectively, and a smaller one of ca. 15 kDa (Fig. 3). Although we do not know why we systematically observed these products, we think that the

FIG 3 *In vitro* activities of ODNs. Cell-free translation reactions were carried out as described in Materials and Methods in the absence (−) or presence (+) of the indicated ODNs at 6.6 μM. The products were processed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the radioactivity was detected with a phosphorimager. A control lacking *aac(6')-Ib* mRNA was included (−). (A) "AS" stands for "antisense," and the numbers indicate the ODN numbers corresponding to those shown in Fig. 2. "P" and "S" stand for ODNs antisense to a *phoA* sequence and sense sequence, respectively. (B) "AS" stands for "antisense." "C4" and "CP" stand for "CPPBD4" and "CPPBDAP," respectively.

ca. 25-kDa band corresponds to the full protein whereas the ca. 20- and 15-kDa proteins may be products of premature termination. Figure 3A shows that all four ODNs tested were robust inhibitors of translation of the *aac(6')-Ib* mRNA but ODN1 and ODN4 showed the strongest activity. Since ODN4 was the shorter of the two ODNs, we selected this compound to carry out further assays.

Since therapeutic use of oligonucleotides on bacterial systems requires the oligomers to be resistant to nucleases and to reach the cytoplasm, we designed a bridged nucleic acid-NC (BNA^NC) DNA (BNA^NC-DNA) hybrid oligomer with the permeabilizing peptide (RXR)₄XB covalently bound to the 5′ end (CPPBD4; Table 1). A compound with the same BNA^NC-DNA configuration but with a nucleotide sequence antisense to a region of *phoA* was used as a negative control (CPPBDAP; Table 1). CPPBD4 was able to inhibit translation of the *aac(6')-Ib* mRNA in the cell-free system with the same efficiency as that observed when the antisense molecule was DNA (Fig. 3B). As expected, addition of CPPBDAP to the reaction did not affect translation (Fig. 3B).

Inhibition of AMK resistance of *A. baumannii* A155 cells in culture. The ability of CPPBD4 to inhibit expression of resistance to AMK in *A. baumannii* A155 (MIC, 12 μg/ml) was assessed by performing growth curve experiments in the presence or absence of the antisense compound. Figure 4 shows that *A. baumannii* A155 cells cultured in the presence of AMK displayed a longer lag time than cells that were cultured in the absence of the antibiotic but reached the same OD₆₀₀ at the stationary phase and displayed approximately the same doubling time. Figure 4 also shows that addition of CPPBD4 or CPPBDAP did not significantly affect the growth of *A. baumannii* A155 cells. However, when CPPBD4 was added to cultures containing AMK, there was complete growth inhibition for the duration of the experiment. Conversely, addition of the control CPPBDAP did not significantly modify the growth curve. These results strongly suggest that the peptide permeabilizer-conjugated BNA^NC-DNA compounds reach the cell cytosol and that CPPBD4 interferes with expression of resistance to AMK.

Effect of CPPBD4 in an experimental infection. The potential capability of CPPBD4 combined with AMK for use as a therapeutic agent against *A. baumannii* A155 was examined using the *G. mellonella* virulence model, which has been extensively validated for the study of host-pathogen interactions and to determine the

FIG 4 Effect of CPPBD4 on resistance to AMK. *A. baumannii* A155 was cultured in 100 μl Mueller-Hinton broth in microtiter plates at 37°C, with the additions indicated in the figure, and the OD_{600} was determined every 20 min. CPPBD compounds were added at 0.5 μM and AMK at 4 μg/ml.

efficacy of antibiotic treatments against numerous pathogens, including *A. baumannii* (38, 42–44). Figure 5 shows that infection with *A. baumannii* A155 bacteria resulted in a significant increase in mortality compared with larvae that were not injected with the bacteria or were injected with sterile PBS. Similar virulence results were observed in those groups injected with CPPBD4. In the group treated with AMK, high mortality was also observed, although with a delay with respect to the negative control. This delay seems to correspond with the extension of the lag time observed in cultures when AMK was added to the media (see Fig. 4). The

group treated with the combination of AMK plus CPPBD4 showed a significant increase in survival rates comparable to those seen with the controls injected with PBS or not injected. As expected, the group treated with AMK plus CPPBDAP showed high levels of mortality similar to those observed in the presence of AMK. The results described in this section show that the new hybrid analogs composed of BNA^{NC} and DNA, when conjugated to a permeabilizing peptide, can reach the *A. baumannii* cytosol and efficiently exert an antisense effect in tests using the *G. mellonella* model of infection.

DISCUSSION

Although the most obvious consequence of the increase in the number of multiresistant bacterial species is the complication of treatment of infectious diseases, the problem also threatens medical procedures such as surgery, cancer treatment, transplants, prosthetic replacements, care for premature infants, and some dentistry procedures (45). Part of the management of this problem could be the extension of the useful life of existing antibiotics by finding inhibitors of the resistance mechanisms or their expression (30, 46, 47). Here we tested one of the latest oligonucleotide analogs, a hybrid oligomer composed of 2′,4′-bridged nucleic acid-NC residues and deoxynucleotides conjugated to the permeabilizing peptide (RXR)$_4$XB, as an antisense inhibitor of resistance to AMK mediated by AAC(6′)-Ib, one of the most widespread aminoglycoside-modifying enzymes (30, 39). We used the clinical *A. baumannii* A155 strain (29, 37), which carries an *aac(6′)-Ib* allele that has been characterized as possessing a sequence duplication encompassing the initiation codon. As determined by BLAST analysis, this is a quite common variant present in most Gram-negative species. We took advantage of the duplication to design antisense oligonucleotides that target this sequence and therefore can bind simultaneously to two regions in the same mRNA molecule. ODNs antisense to this region were robust inhibitors of expression of the gene *in vitro*. On the basis of these results, we speculate that the mechanism of inhibition of
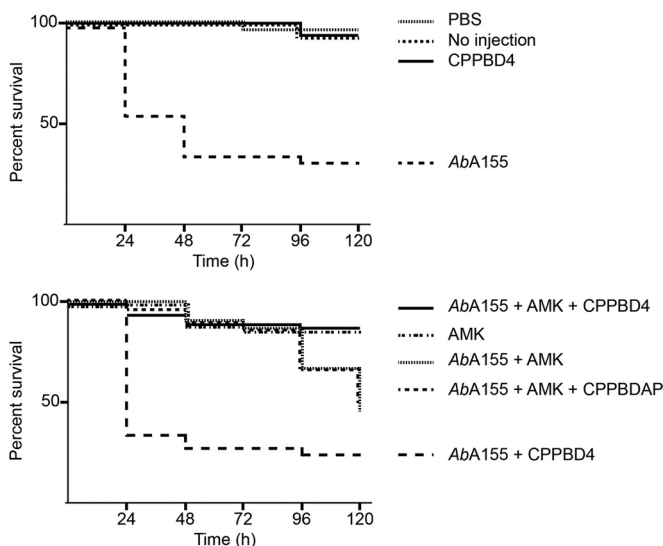

FIG 5 *G. mellonella* infection and treatment assays. Final-instar larva groups of 10 individuals were injected with the components shown in the figure, and a control group was not injected ("No injection"). The concentrations of the injected components were 10 mg AMK/kg of body weight and 0.5 μM CPPBD4 and CPPBDAP. The larvae were incubated at 37°C in the dark, and survival was recorded at 24-h intervals over 120 h.

resistance to AMK is interference with ribosome assembly and/or steric hindrance of translation. Although we cannot discount a level of contribution of RNase H-mediated mRNA degradation, previous results obtained testing LNA-DNA co-oligomers with various configurations that showed that only gapmers with at least 6 contiguous deoxynucleotides induce significant RNase H activity (48–50) would discourage interpretation of this as a significant mechanism.

Since it has been shown that a successful solution to the problem of cellular uptake of oligonucleotide analogs, such as peptide nucleic acids or phosphorodiamidate morpholino oligomers, was their conjugation to permeabilizing peptides (13, 51), a BNA^{NC}-DNA hybrid co-oligomer with the most active sequence, that of ODN4, was conjugated to the (RXR)$_4$XB peptide. This compound, CPPBD4, was then tested for its ability to reduce the levels of resistance to AMK of *A. baumannii* A155 cells. In combination with AMK, CPPBD4 showed sequence-specific inhibition of growth of *A. baumannii* A155 cells in culture and a reduction of their virulence in tests in the *G. mellonella* infection model. These results, taken together with those indicating that BNA-based compounds exhibit low toxicity for the host (25), suggest that hybrid oligomers composed of 2′,4′-bridged nucleic acid-NC residues and deoxynucleotides conjugated to permeabilizing peptides can be a viable option to develop antisense therapeutics. Previous studies showed that there are several variables that affect the activity of antisense compounds. For example, the efficiency of LNA-DNA hybrid compounds as antisense molecules is dependent on the configuration of the residues (16) and the efficiency of antisense peptide-phosphorodiamidate morpholino oligomers has been shown to be dependent on the composition of the permeabilizing peptide (52). Therefore, our future experiments will include examining a variety of permeabilizing peptides and oligomer configurations that will permit identification of the best compounds to treat different Gram-negative species.

ACKNOWLEDGMENTS

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