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METHODS MANUSCRIPT

Assessment of configurations and chemistries of bridged nucleic acids-containing oligomers as external guide sequences: a methodology for inhibition of expression of antibiotic resistance genes

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Abstract

External guide sequences (EGSs) are short antisense oligoribonucleotides that elicit RNase P-mediated cleavage of a target mRNA, which results in inhibition of gene expression. EGS technology is used to inhibit expression of a wide variety of genes, a strategy that may lead to development of novel treatments of numerous diseases, including multidrug-resistant bacterial and viral infections. Successful development of EGS technology depends on finding nucleotide analogs that resist degradation by nucleases present in biological fluids and the environment but still elicit RNase P-mediated degradation when forming a duplex with a target mRNA. Previous results suggested that locked nucleic acids (LNA)/DNA chimeric oligomers have these properties. LNA are now considered the first generation of compounds collectively known as bridged nucleic acids (BNAs) – modified ribonucleotides that contain a bridge at the 2′,4′-position of the ribose. LNA and the second-generation BNA, known as BNA^{NC}, differ in the chemical nature of the bridge. Chimeric oligomers containing LNA or BNA^{NC} and deoxynucleotide monomers in different configurations are nuclease resistant and could be excellent EGS compounds. However, not all configurations may be equally active as EGSs. RNase P cleavage assays comparing LNA/DNA and BNA^{NC}/DNA chimeric oligonucleotides that share identical nucleotide sequence but with different configurations were carried out using as target the amikacin resistance *aac(6′)-Ib* mRNA. LNA/DNA gapmers with 5 and 3/4 LNA residues at the 5′- and 3′-ends, respectively, were the most efficient EGSs while all BNA^{NC}/DNA gapmers showed very poor activity. When the most efficient LNA/DNA gapmer was covalently bound to a cell-penetrating peptide, the hybrid compound conserved the EGS

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activity as determined by RNase P cleavage assays and reduced the levels of resistance to amikacin when added to *Acinetobacter baumannii* cells in culture, an indication of cellular uptake and biological activity.

Keywords: Rnase P; EGS technology; aminoglycoside; antibiotic resistance; Acinetobacter; antisense

Introduction

Antisense RNAs are known to regulate expression of numerous bacterial genes [1, 2]. Antisense technologies are a potential source of new drugs that interfere with translation of a target mRNA with high specificity, maximizing the therapeutic effect and minimizing side effects [3–6]. Several strategies are being pursued to inhibit expression of disease-associated genes including interfering with translation by steric hindrance or inducing mRNA degradation [3–8]. A promising antisense-based approach, "EGS technology," takes advantage of the property of RNase P to cleave a target RNA molecule if a short complementary oligoribonucleotide, known as external guide sequence (EGS), forms a bimolecular complex with the appropriate structure [8–10]. Bacterial RNase P is a ribonucleoprotein complex in which the RNA component, known as M1 subunit in *Escherichia coli*, is responsible for the catalytic activity [11]. The protein component, known as C5 in *E. coli*, acts as an essential cofactor *in vivo* [11]. The best-known role of RNase P is the maturation of tRNAs by a single endonucleolytic cleavage of the precursor tRNA at the 5′-end. However, more recent research showed that it also participates in the synthesis of other RNA molecules [9, 11–26]. The realization that the RNase P cleavage reaction had structural rather than sequence specificity and bimolecular complexes were substrates for RNase P as long as they had the appropriate structure [27–30] led to the idea that an antisense oligoribonucleotide (EGS) could be used as guide to induce bacterial RNase P to cleave a target RNA molecule (Fig. 1A)[10, 27, 29].

Since oligoribonucleotides are extremely labile, for EGS technology to be a viable therapeutic option the EGSs must be composed of nuclease-resistant analogs that still elicit RNase P-mediated degradation of the target RNA molecule. A study including numerous analogs showed that chimeric oligonucleotides composed of locked nucleic acids (LNA) and deoxyribonucleotides (from here on referred to as LNA/DNA) efficiently induced RNase P-mediated cleavage of a target mRNA [31]. Furthermore, stability tests showed that they are not degraded when incubated in the presence of serum nucleases, *E. coli* intact cells or their soluble extracts [31–34]. LNA analogs (2′-O, 4′-methylene-β-D-ribofuranosyl nucleotides) are now considered the first generation of a group of compounds known as bridged nucleic acids (BNAs), analogs characterized for including a synthetically incorporated chemical structure at the 2′, 4′-position of the ribose (Fig. 1B). The second generation of BNA compounds, 2′-O,4′-aminoethylene-β-D-ribofuranosyl nucleotides, are known as 2′,4′- bridged nucleic acid-NC (BNA^{NC}) [35] (Fig. 1B) and in the very limited studies available they showed high binding affinity to RNA, robust antisense activity, excellent single-mismatch discriminating ability, and low toxicity [35–37]. Unlike other oligonucleotide analogs, LNA or BNA^{NC}-containing oligomers have higher hybridization affinity than regular oligoribonucleotides or oligodeoxyribonucleotides. It is estimated that each residue substitution increases the melting temperature of LNA/DNA:mRNA duplexes by about 4–6°C when compared to equivalent DNA:mRNA duplexes [38, 39]. Since the complex recognized by RNase P has stringent structural requirements, a higher affinity of binding does not necessarily correlate with better RNase P activation.

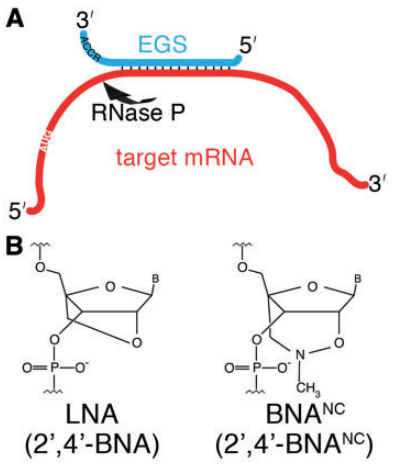

Figure 1: (A) Schematic representation of the interaction between an mRNA molecule (red) and an EGS (blue). The AUG sequence has been arbitrarily placed. The RCCA sequence is usually added to the antisense sequence in EGSs to mimic the 3′-end of the pre-tRNA and facilitate interaction with the RNase P. (B) Chemical structures of LNA and BNA^{NC} residues. Alternative nomenclature is shown in-between parentheses.

In this work we compared the efficiency of LNA/DNAs and BNA^{NC}/DNAs with the same sequence (isosequential) but with different configurations to induce RNase P-mediated cleavage of the *aac(6′)-Ib* mRNA at two different locations. This gene was selected as target because of its clinical relevance. It codes for the aminoglycoside 6′-N-acetyltransferase type Ib [AAC(6′)-Ib], which is present in over 70% of AAC(6′)-I-producing Gram-negative clinical isolates [40–44]. This enzyme is responsible for resistance to amikacin (AMK) and other aminoglycosides in several Gram-negative genera within the families of the *Moraxellaceae*, *Enterobacteriaceae*, *Pseudomonadaceae*, and *Vibrionaceae* [40, 41, 45]. Previous results showed that an RNA EGS expressed within the cell from a recombinant clone reduced the level of resistance to AMK [46]. Furthermore, isosequential LNA/DNAs had the same effect when introduced in a hyperpermeable *E. coli* mutant [31]. The results described here indicate that selected LNA/DNA gapmers exhibit robust EGS activity *in vitro* and when one of them was conjugated to a cell-penetrating peptide (CPP) it reduced the growth of *aac(6′)-Ib*-harboring *Acinetobacter baumannii* cells when administered in combination with AMK.

Materials and methods

Bacterial strains, plasmids, oligonucleotide, and permeabilizing peptide-conjugated oligonucleotide analogs

Acinetobacter baumannii A155 was isolated from a patient's urine. It is a multidrug-resistant clinical strain that includes the *aac(6′)-Ib* gene [37, 47]. *Escherichia coli* TOP10(pNW1) is a strain that contains the recombinant plasmid pNW1, an F′ derivative

Table 1: Chimeric oligomers

Name	Sequence
RNAA	rCrGrArTrArTrGrArGrArTrCrGrArCrCrA
LDA1	+C+G+A+T+ATGAGATC+G+A+C+C+A
LDA2	+C+G+A+T+ATGAGATCG+A+C+C+A
LDA3	+C+G+A+T+ATGAGATCGA+C+C+A
LDA4	+C+G+ATATGAG+A+T+CGA+C+C+A
BDA1	*C*G*A*T*ATGAGATC*G*A*C*C*A
BDA2	*C*G*A*T*ATGAGATCG*A*C*C*A
BDA3	*C*G*A*T*ATGAGATCGA*C*C*A
BDA4	*C*G*ATATGAG*A*T*CGA*C*C*A
BDA5	*C*G*ATATGAGATCGA*C*C*A
RNAB	rArGrTrGrTrCrGrGrGrCrGrTrGrArCrCrA
LDB1	+A+G+T+G+TCGGGCGT+G+A+C+C+A
LDB2	+A+G+T+G+TCGGGCGTG+A+C+C+A
LDB3	+A+G+T+G+TCGGGCGTGA+C+C+A
CPPLDA2	$(RXR)_4$XB-Cys-SMCC-C6 amino-+C+G+A+T+ ATGAGATCG+A+C+C+A
CPPLDAP	$(RXR)_4$XB-Cys-SMCC-C6 amino- +A+G+C+G+ GTAAGGCAT+A+C+C+A

RNA, LD, or BD indicate that the EGS is an oligoribonucleotide, an LNA/DNA, or a BNA^{NC}/DNA, respectively. The following letter identifies the region targeted within the mRNA molecule. +, LNA residue, *BNA^{NC} residue. A nucleotide without a specific indication represents a deoxynucleotide. CPPLDA2 is the permeabilizer peptide-conjugated hybrid LNA/DNA chimeric oligomer. R, arginine; X, 6 aminohexanoic acid; B, beta-alanine; SMCC, sulfosuccinimidyl-trans-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

that includes a copy of *aac(6′)-Ib* [48]. LNA/DNA chimeric oligomers with the sequences CGATATGAGATCGACCA (region A) and AGTGTCGGGCGTGACCA (region B) with different LNA/DNA configurations (Table 1) were purchased from Exiqon. BNA^{NC}/DNA chimeric oligomers were purchased from Bio-Synthesis Inc. To generate the $(RXR)_4$XB-Cys-SMCC-C6 amino-LNA/DNA (R, arginine; X, 6-aminohexanoic acid; B, beta-alanine), the C6 amino-+C+G+A+T+ATGAGATCG+A+C+C+A (where + stands for LNA residue) was conjugated to $(RXR)_4$XB-Cys-SMCC by Bio-Synthesis Inc. Plasmid pNW1 [48] was used to amplify the DNA fragment that served as template for *in vitro* synthesis of *aac[6′)-Ib* mRNA.

General procedures

Plasmid DNA preparations were carried out by using the QIAspin miniprep kit (QIAGEN). PCRs were carried out using the HotStarTaq master mix kit (QIAGEN). The *aac(6′)-Ib* mRNA was synthesized *in vitro* using the MEGAscript high-yield transcription T7 kit (Thermo Fisher Scientific) according to the recommendations of the supplier. Labeling of the 5′-end was carried out in the dark using the 5′ EndTag Nucleic Acid Labeling system (Vector Laboratories) with the fluorescent dye cyanine5 maleimide (Lumiprobe). Secondary structure of the *aac(6′)-Ib* mRNA was determined using mfold [49].

RNase P preparation

RNase P was prepared by mixing *in vitro* synthesized M1 RNA and purified C5. M1 RNA was synthesized *in vitro* using a commercial kit (MEGAshortscript T7, Thermo Fisher Scientific) with pJA′, a plasmid that includes the *rnpB* gene under the control of a T7 promoter [50], as a template. The procedure for expression and purification of the C5 protein was modified from the previously described method [51]. A DNA fragment including the *rnpA* gene and a T7 was inserted into the *XbaI* site of pT7-5 [52] to generate the recombinant plasmid pRHC5. *Escherichia coli* BL21(DE3)(pLysE) was transformed with pRHC5, the transformant strain was cultured in Terrific broth [53] containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml), and expression of C5 was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD_{600} 0.6–0.8. The expressing cells were cultured overnight at 37°C, harvested by centrifugation (7000 r.p.m., 10 min), and resuspended in 0.02 volume of buffer (50 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 10 mM magnesium acetate, 0.15% dithiothreitol, 42% urea, and 0.2 mM Pefabloc protease inhibitor). The cells were then lysed by sonication, treated with DNase I for 30 min on ice, and the cell debris was removed by centrifugation at 8000 r.p.m. for 10 min (4°C). The soluble extract was then centrifuged at 30,000 × g for 30 min at 4°C, and the supernatant was collected and centrifuged for 2 h at 100,000 × g at the same temperature. After removing the supernatant, the pellet was resuspended in 10 ml of buffer (50 mM Tris-HCl (pH 7.5), 1 M NH_4Cl, 10 mM magnesium acetate, and 0.15% dithiothreitol), shaken at 4°C for 2 h and centrifuged again at 100,000 × g for 2 h (4°C). The supernatant was subjected to dialysis overnight against a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 10 mM $MgCl_2$, and 0.15% dithiothreitol at 20°C. After dialysis the supernatant was centrifuged at 15,550 r.p.m. for 30 min and the pellet was resuspended in a buffer containing 0.05 M sodium acetate (pH 7.2), 0.01 M $MgCl_2$, 7 M urea, and 0.15% dithiothreitol and applied to a Sephadex C50 (Amersham Biosciences) column. Elution was carried out with a linear gradient 0–0.5 M NaCl. The C5 protein eluted at 0.3 M NaCl, and the procedure yielded a solution that when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a 13.5 kDa corresponding to C5 and one minor band at a position corresponding to a larger molecular weight.

In vitro RNase P assays

The EGSs were tested by preincubating 5′-end-labeled *aac(6′)-Ib* mRNA (5 pmol) and the EGS (10 pmol) at 25°C for 30 min in a volume of 3 µl before adding this mixture to a solution containing 2.5 pmol of M1 RNA, 70 pmol of C5 protein, 20 mM HEPES-KOH (pH 8.0), 400 mM ammonium acetate, 10 mM magnesium acetate, and 5% glycerol that had been preincubated at 37°C for 15 min in a final volume of 7 µl [54]. After combining both solutions the mix was incubated at 37°C for the times indicated. The reaction was stopped by the addition of 1 volume of phenol-chloroform followed by ethanol precipitation. The pellet was resuspended in 10 µl DEPC-treated water, mixed with 10 µl of 2 x gel loading buffer, and analyzed by 5% denaturing TBE-PAGE along with labeled RNA Century Marker or RNA Century Marker-Plus (ThermoFisher) as described before [31]. Fluorescence was detected on a Storm 860 Molecular Imager (Molecular Dynamics) and the signal in each band was quantified using Image J [55].

Growth inhibition assays

Inhibition of cell growth was performed in Mueller–Hinton broth (100 μl) with the additions indicated in the text in microtiter plates using the BioTek Synergy 5 microplate reader [56, 57]. Cultures were carried out at 37°C with shaking and optical density at 600 nm (OD_{600}) was recorded every 20 min.

Results and discussion

Activity of EGSs with different LNA/DNA configurations

A characteristic of RNase P is that its specificity is determined by the structure rather than the sequence of the substrate RNA

molecule [27]. This property led to development of diverse bimolecular substrates composed of an antisense EGS and the target RNA [10]. The need for development of nuclease resistant analogs capable of forming the appropriate structure for a target RNA to be cleaved by RNase P led to the identification of LNA/DNAs as functional EGSs [31]. Since LNA-containing oligomers significantly modify the hybridization affinity of isosequential oligoribonucleotides or oligodeoxyribonucleotides, the configuration of LNA/DNAs to be utilized as EGSs may be critical for their efficiency in eliciting RNAse P-mediated cleavage of the target mRNA. EGSs usually consist of two regions, an oligonucleotide antisense to the target mRNA followed by the ACCA sequence at the 3′-end. This latter sequence interacts with the UGG sequence within the P15-loop of the M1 RNA component of the *E. coli* RNase P facilitating recognition of the substrate [13, 58]. We determined before that LNA/DNAs that had LNA residues only at the 3′-end showed very poor activity as EGSs. However, the activity drastically improved when 5′-end residues were also substituted [31]. In this work we tested the impact of the number of residues modified at the 3′-end when the number of LNA residues at the 5′-end is constant. We assessed the activities of two sets of isosequential oligomers with a different number of substitutions at the 3′-end that are complementary to two regions within the *aac(6′)-Ib* mRNA that have previously been identified as available to interact with RNA EGSs *in vitro* (Fig. 2) [46, 48, 59]. The configurations of the chimeric oligonucleotides tested are shown in Table 1.

A first assay using EGSs targeting region A (Fig. 2) in which the reaction was incubated for 2 h showed that all LNA-containing gapmers were able to elicit high levels of cleavage (Fig. 3A). We also tested a mixmer, which induced markedly lower cleavage levels (Fig. 3A). It was also observed that all EGSs induced cleavage at the expected location but also at an additional target location (see Fig. 3A). This phenomenon has been observed before and could occur due to specific binding to other location of the RNA molecule favored by the enhanced affinity conferred by the presence of LNA monomers [31]. The 3D structure of the mRNA could expose a region that, although not perfectly complementary, is available for interaction with the EGS and the high thermostability of the complex between the mRNA and the LNA-containing oligomer permits a productive interaction. To further compare the efficiency of the different EGSs, cleavage assays were carried out at different incubation times. Figure 4A and Supplementary Fig. 1S show that the kinetics of mRNA cleavage are similar for the RNAA and the gapmer LDA3 (3 LNA residues at the 3′-end) while the gapmer LDA2 (4 LNA residues at the 3′-end) was marginally more efficient. The rate of degradation dropped significantly in the case of the gapmer LDA1 (5 LNA residues at the 3′-end) and as expected, the mixmer only minimally induced cleavage of the substrate mRNA.

Another set of experiments was carried out with EGSs targeting region B (Fig. 2). We showed before that the sequence targeted was amenable to interaction with an RNA EGS although its predicted structure was in part forming a stem with another region of the mRNA molecule [46]. As we suspected based on the predicted structure, the efficiency of degradation at this location was lower when compared to that of region A (Fig. 4B). Furthermore, the RNAB EGS showed lower activity when compared to the levels presented by LDB2 and LDB3 (4 and 3 LNA residues at the 3′-end). This may be the result of the increase in hybridization affinity exhibited by LNA-containing oligomers as compared to RNA and DNA. As it happened with region A, addition of one more substitution at the 3′-end, i.e. 5 LNA residues at this end, results in an EGS with reduced activity (LDB1, Fig. 4B).

The results of both sets of experiments suggest that independent of the quality of the targeted region, the activity—as EGSs of gapmers composed of LNA and DNA residues—shows some dependence on the number of LNA residues at the 3′-end. At least in the cases analyzed here, while the activity as EGSs of analog oligonucleotides is similar when 3 or 4 LNAs are located at the 3′-end, adding a fifth substitution significantly reduced their ability to induce RNase P-mediated degradation of the

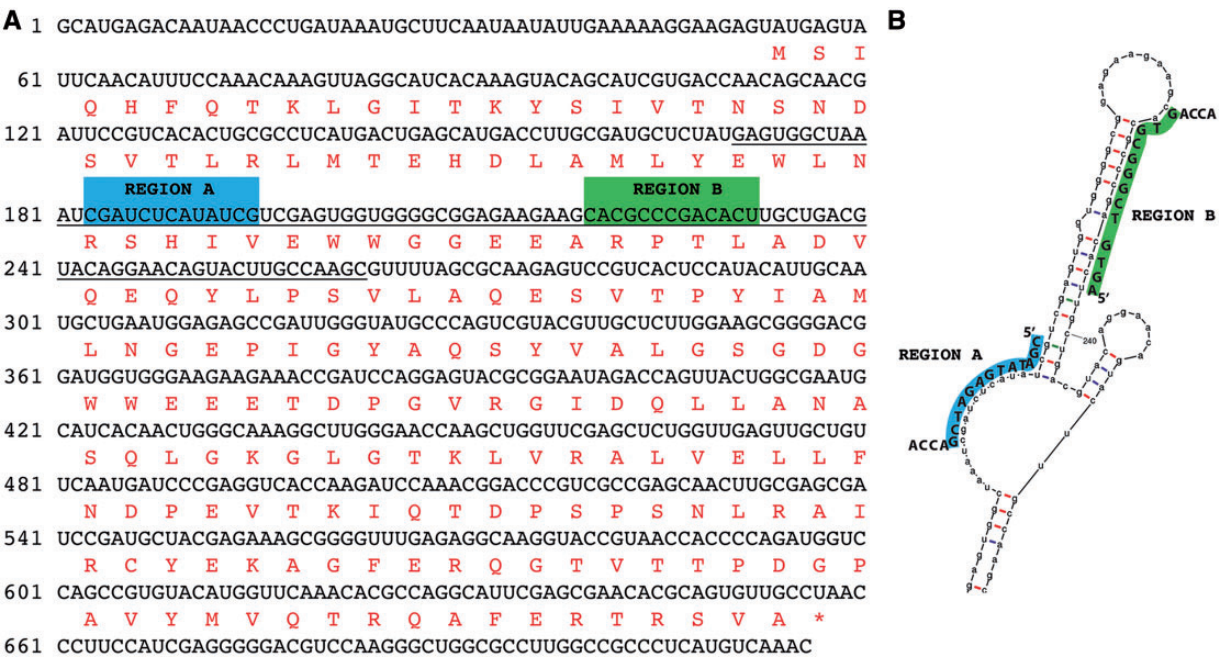

Figure 2: *aac(6′)-Ib* mRNA target regions. (A) Sequence of the *aac(6′)-Ib* mRNA highlighting regions A and B. (B) Predicted structure of the fragment containing the 2 regions selected as targets (underlined in part A).

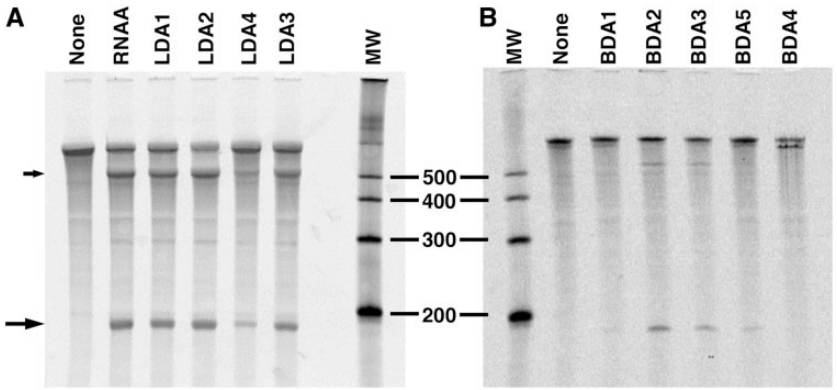

Figure 3: RNase P-mediated cleavage of labeled *aac(6′)-Ib* mRNA. The RNase P cleavage reactions were carried out as described in section "Materials and Methods" in the presence of the EGSs indicated on top. Panels A and B show the cleavage results when LNA/DNA (A) or BNA/DNA (B) oligomers were tested. In panel A, a reaction in the presence of the RNAA EGS was also included as control. The large arrow indicates the location of the expected product. The small arrow shows the location of a secondary product (see discussion in the text). MW, molecular size standards. The nucleotide size of the marker bands is shown between the gels.

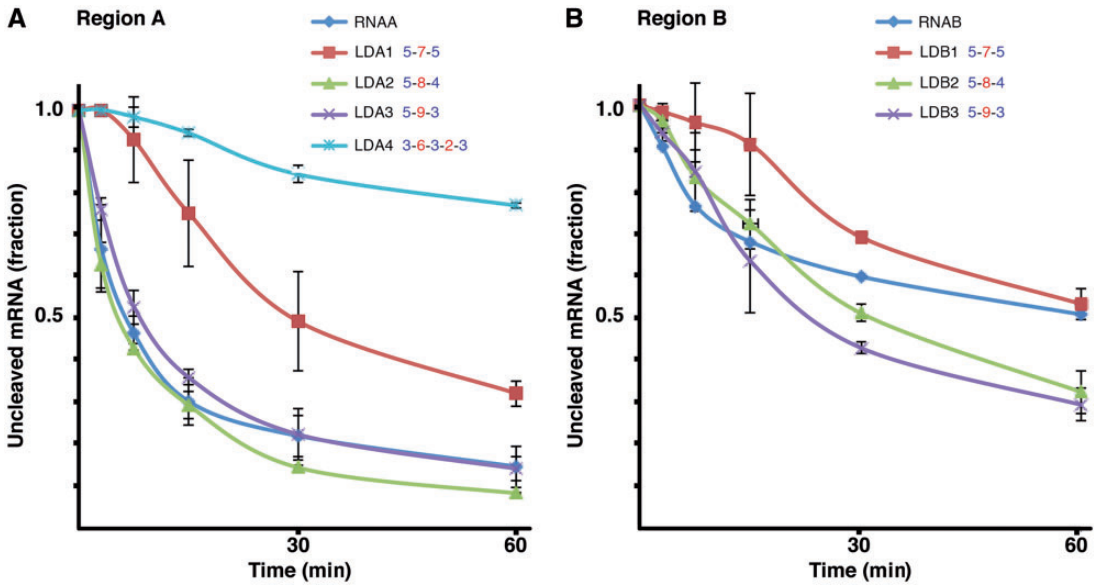

Figure 4: Time courses of RNase P-mediated cleavage of labeled *aac(6′)-Ib* mRNA. Reactions using the indicated EGSs were stopped at different times, subjected to 5% denaturing TBE–PAGE, and the bands corresponding to products and uncleaved mRNA were quantified using ImageJ. The configuration of the different EGSs (see Table 1) is schematically shown on the upper right of the figure. The number of nucleic acid (red) and locked nucleic acid (blue) residues are shown. Values are the average and standard deviation of duplicates. (A) Region A. (B) Region B.

aac(6′)-Ib mRNA. Whether this is a general characteristic of LNA/DNA oligonucleotides when used as EGSs will be confirmed when more molecules and regions are tested.

Comparison of the EGS activity of oligomers including LNA or BNA^{NC} analogs

Since the second generation of BNA compounds, BNA^{NC} (Fig. 1B), is now available, we also generated chimeric oligomers containing these analogs and tested their efficiency as EGSs. We recently showed that antisense oligonucleotide analogs composed of BNA^{NC} and DNA residues efficiently interfered with gene expression by steric hindrance when used as antisense compounds targeting the translation initiation region of *aac(6′)-Ib* [37]. Therefore, we decided to compare the activity of LNA/DNA with that of BNA^{NC}/DNA oligomers as EGSs. Figure 3B shows that all BNA^{NC}-containing compounds assayed, BDA1, BDA2, BDA3, BDA4, BDA5 (Table 1), were unable to elicit cleavage of the target mRNA at levels comparable to those found when testing LNA/DNAs. These results show that small changes in the chemistry of the analogs can result in dramatic differences in the activity of the oligomers as EGSs. It will be of interest to investigate the behavior of subsequent generations of BNAs as they become available. BNA^{NC}-containing oligomers have shown improved properties, such as higher stability and lower toxicity [36, 60], when compared to LNA-containing compounds. Although it seems that BNA^{NC}-containing gapmers are not good EGS candidates, efforts to seek better BNA analogs are under way [60] and it is most probable that some of them will exhibit enhanced EGS activity.

EGS activity of CPPLDA2

It is well known that effective penetration of oligonucleotides and oligonucleotide analogs is one of the challenges for their effective use as therapeutics [61]. Although LNA/DNAs show a

degree of gymnotic uptake by some bacterial species, the levels of internalization seem not enough for effective EGS activity [62]. A strategy to increase the internalization efficiency is to conjugate them to CPPs [37, 63–66]. Chemical conjugation and purification of negatively charged oligonucleotide analogs with CPPs, which are usually cationic, has been quite difficult [67]. However, LNA/DNAs or BNA^{NC}/DNAs have now been successfully conjugated to CPPs and the conjugates were able to inhibit gene expression by steric hindrance [37, 68]. Therefore, we conjugated one of the two most potent EGSs, LDA2, to the $(RXR)_4XB$ peptide. As shown in Fig. 5, the resulting compound, CPPLDA2, acted as an EGS in an *in vitro* RNase P cleavage reaction, albeit with a slightly lower efficiency than the cognate oligomer LDA2 (compare Figs. 4A and 5). This result indicates that linking the penetrating peptide to the oligonucleotide analog does not significantly impair the ability of the gapmer to elicit RNase P-mediated degradation of the target mRNA. In the past, phosphorodiamidate morpholino oligonucleotide EGSs conjugated to a permeabilizer peptide efficiently inhibited expression of Gram-negative and Gram-positive genes [69–71]. Conversely, tests to assess LNA/DNAs bound to CPPs were delayed due to difficulties in the chemical conjugation and purification of negatively charged oligomers with the cationic CPPs [67]. The possibility of conjugating CPPs to LNA/DNAs and the results shown in this section open new opportunities to optimize and enhance the activity of these compounds as EGSs not only in bacteria but also in eukaryotic cells.

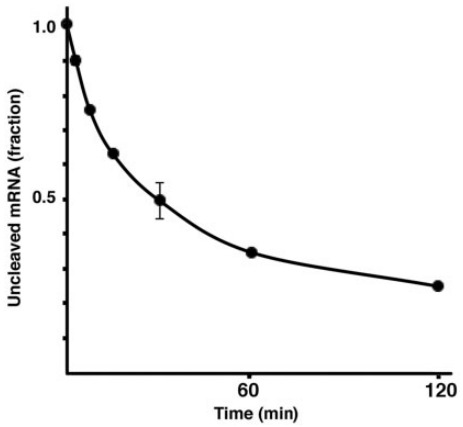

Figure 5: Time course of RNase P- mediated cleavage of labeled *aac(6′)-Ib* mRNA in the presence of CPPLDA2. The RNase P cleavage reactions were carried out as described in section "Materials and Methods" in the presence of the CPPLDA2 for the indicated times. Analysis and quantification were carried out as in Fig. 4. Values are the average and standard deviation of duplicates.

Reduction of *A. baumannii* A155 AMK resistance level induced by CPPLDA2

The ability of CPPLDA2 to inhibit expression of resistance to AMK in cells in culture was determined by monitoring bacterial growth in AMK containing growth medium in the presence of CPPLDA2. The bacteria tested were an *E. coli* laboratory strain harboring a recombinant clone including *aac(6′)-Ib* and *A. baumannii* A155, a clinical isolate naturally carrying the gene. Controls were cultures containing only AMK or the antibiotic and a peptide-oligomer with a scrambled sequence (CPPLDAP). Figure 6 shows that *A. baumannii* A155 cells cultured in the presence of CPPLDA2 grew to a lower OD_{600} value when compared to the control cultures. Conversely, no effect was found in the case of *E. coli* TOP10(pNW1) (not shown). These results indicate that CPPLDA2 produces a modest but consistent effect of reducing the level of AMK resistance in *A. baumannii* A155. However, the same compound did not modify the resistance phenotype of *E. coli*. Although this latter result is disappointing, the effect observed when testing *A. baumannii* A155 is encouraging. Further studies will have to be carried out to enhance the level of inhibition of expression of resistance. The nature of the CPP conjugated to the EGS could be important for mediating penetration of enough compound molecules inside the cells. Further work analyzing different CPPs could permit us to improve internalization with a concomitant enhancement of the gene expression inhibition activity. Of course other mechanisms could be responsible for the lack of activity observed in *E. coli* such as the cytosol not being appropriate for the RNase P cleavage of the nonnatural substrates. In summary, the results indicate that CPP covalently bound to LNA/DNA EGS with a determined configuration could be a viable strategy to inhibit expression of

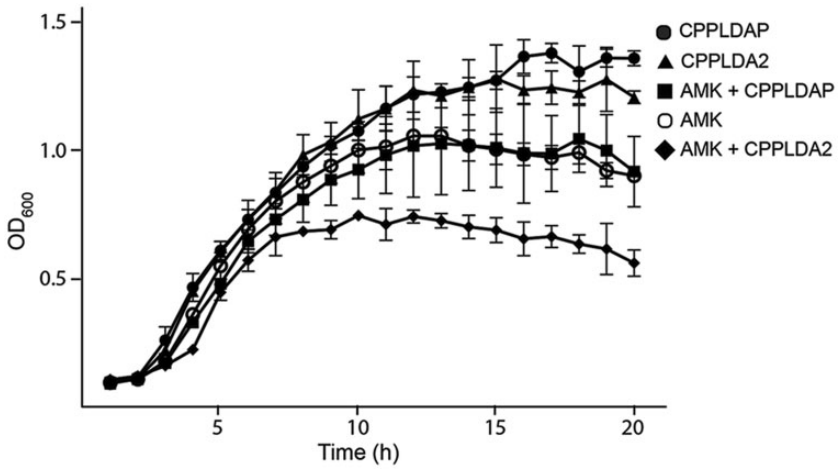

Figure 6: Effect of CPPLDA2 on resistance to AMK. *Acinetobacter baumannii* A155 cultures were carried out in microtiter plates at 37°C, with the additions indicated in the figure, and the OD600 was determined every 20 min. AMK was added at 25 μg/ml and the oligomers at 5 μM. Values are the average and standard deviation of duplicates.

aac(6′)-Ib. However, the results also show that more systematic analyses of EGS configuration and chemistries as well as conjugation to CPPs with different characteristics must be carried out. Furthermore, the differences in the results obtained with two different bacteria show that the tests should include several Gram-negative pathogens.

Supplementary data

Supplementary data is available at *Biology Methods and Protocols* online.

Conflict of interest statement. None declared.

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