Internalization of Locked Nucleic Acids/DNA Hybrid Oligomers into *Escherichia coli*

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Abstract

Delivery inside the cells is essential for practical application of antisense technologies. The hybrid locked nucleic acid (LNA)/DNA <u>CAA</u>GTACTGT<u>TCC</u>A<u>CCA</u> (LNA residues are underlined) was labeled by conjugation to Alexa Fluor 488 (fLNA/DNA) and tested to determine its ability to penetrate *Escherichia coli* cells and reach the cytoplasm. Flow cytometry analysis showed that the fLNA/DNA was associated with 14% of cells from a stationary phase culture, while association with a labeled isosequential oligodeoxynucleotide was negligible. Laser scanning confocal microscopy confirmed that the fLNA/DNA was located inside the cytoplasm.

Key words: drug discovery; drug resistance; gene therapy; nucleic acids; microbiology

Introduction

Antisense-related technologies consist of the utilization of oligonucleotides to interfere with gene expression. Although one drug, fomivirsen, has been approved by the U.S. Food and Drug Administration[1] and a number of antisense drugs targeting a variety of diseases are presently in clinical trials,[2–4] several challenges, such as toxicity, nonspecific effects, or inability to penetrate the target, continue to slow progress. In particular, for applications against gram-negative bacteria, uptake by the target cell has been a problem because, although a few cases have been reported,[5,6] naked antisense compounds do not diffuse well inside most cells.[2,7] Numerous strategies have been developed to solve this limitation. The most successful of them being the conjugation of neutral oligonucleotide analogs to cell penetrating peptides[8,9] and the addition of cationic groups like *N*-(6-guanidinohexanoyl)piperazine to the phosphorodiamidate linker.[10] Cell penetrating peptide–phosphorodiamidate morpholino oligomers were shown to penetrate bacterial cells and inhibit gene expression by blocking ribosomal function[11] or acting as external guide sequences, which are short oligo-

mers that induce RNase P-mediated cleavage of a target RNA by forming a precursor tRNA-like complex.[12,13]

Hybrid oligomers composed of locked nucleic acids and deoxyribonucleotides (LNA/DNA) are a new generation of chemically modified oligonucleotides that exhibit high-affinity binding to complementary DNA or RNA,[14] are highly resistant to nuclease digestion,[15,16] and show low toxicity.[17,18] These compounds are being tested for treatment of numerous diseases and several clinical trials are under way.[18,19] Although utilization of LNA/DNA as antisense antibacterials has been very limited, one such compound was recently shown to be an efficient external guide sequence eliciting RNase P-mediated degradation of *aac(6′)-Ib* mRNA and concomitantly inducing a significant reduction in the levels of resistance to amikacin in a hyperpermeable *Escherichia coli* host.[15] As in most applications of antisense technologies to bacterial systems, uptake of LNA/DNA by the target cells is one of the major challenges. Attempts to conjugate LNA/DNA to cell-penetrating peptides have been largely unsuccessful, most probably due to their negatively charged nature. In our attempts to find a strategy to induce internalization of LNA/DNA co-oligomers, we found that these compounds

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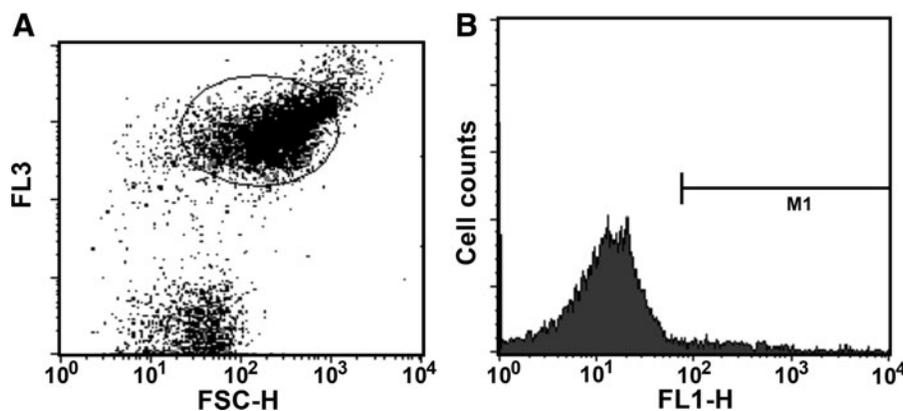
FIG. 1. Flow cytometry of fLNA/DNA cellular uptake. **(A)** Dot plot representing FSC-H (forward scattered) versus FL3. The inner circle represents the cells analyzed. **(B)** Histogram showing cell counters versus fluorescence from fLNA/DNA (FL1-H). M1 area represents percentage of cells containing Alexa Fluor 488.

can penetrate *E. coli* more efficiently than isosequential oligodeoxynucleotides.

Materials and Methods

Bacterial strains oligomers

E. coli TOP10 F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) 80*lacZ*ΔM15 Δ*lacX74nupG recA1araD139* Δ(*ara-leu*)7697 *galE15 galK16 rpsL*(Str^R) *endA1* λ$^-$ was used as host to test internalization of oligomers. Alexa Fluor 488-conjugated CAAGTACTGT TCCACCA (fLNA/DNA; LNA residues are underlined) and Alexa Fluor 488-ODN (fODN), isosequential, were used for quantification of internalization by flow cytometry and by laser scanning confocal microscopy (LSCM).

Quantification and visualization of internalized oligomers

Uptake of fLNA/DNA and fODN was quantified by flow cytometry using cells in a stationary phase that have been incubated in Lennox Luria (L) broth[20] for 18 h at 37°C. Cells were washed with phosphate-buffered saline solution and incubated 15–30 min with phosphate-buffered saline and fLNA/DNA or fODN. After washing the cells, the membrane-specific stain, FM5-95, was added to a final concentration of 5 μg/mL and the samples were analyzed by flow cytometry and laser screening confocal microscopy (LSCM). Viability of cells was assessed by treating the cells as described above, but omitting the FM5-95 treatment and exposing the cells to propidium iodide. Flow cytometry data were analyzed using the CellQuest software. Visualization of incorporation of fLNA/DNA by LSCM was done in a Zeiss Pascal or Zeiss Meta confocal microscope (Zeiss) as described before.[15] Statistical analysis was carried out performing an unpaired two-tailed t-test with Welchs correction. $p < 0.05$ was considered statistically significant.

Results and Discussion

Internalization of nuclease resistant oligonucleotide analogs into bacterial cells is one of the stumbling blocks in the application of antisense technologies to prokaryotic systems. Although significant progress has been made on internalization of peptide nucleic acids and phosphorodiamidate morpholino oligomers by conjugating them to cell penetrating peptides,[9,21–25] strategies to induce cell penetration by negatively charged analogs remain elusive. Since LNA/DNA oli-

gonucleotide analogs have been recently shown to be active as external guide sequences,[15] they are promising compounds to be used as antibacterials. However, before they are seriously considered as candidates for development of new antimicrobials, a strategy for delivery inside the cells must be found. Interestingly, LNA containing oligonucleotides have been recently shown to be delivered into diverse eukaryotic cells without using transfection agents, in a process called gymnosis.[26,27] We tested if an LNA/DNA co-oligomer could be internalized by *E. coli* cells using two complementary experimental techniques, flow cytometry and LSCM. An LNA/DNA co-oligomer was labeled with the fLNA/DNA and mixed with *E. coli* cells in the stationary phase. The percentage of cells that took up fLNA/DNA was determined by flow cytometry. Figure 1 shows the dot plot and histogram of a typical experiment, of which seven repeats were carried out. As control, six assays were performed exposing the *E. coli* cells to fODN. Figure 2 shows that while it is clear that the fODN did not associate with cells, 13.96% ± 5.12% of them were associated with fLNA/DNA. Although these results show a significant difference of behavior of both oligomers when they get in contact with untreated *E. coli* cells, they do not provide enough information to know the location of the fLNA/DNA with respect to
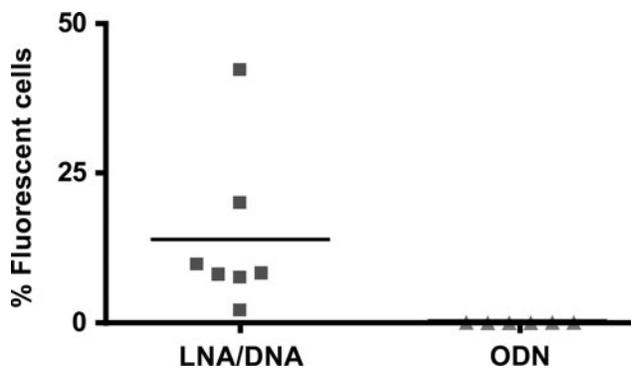

FIG. 2. Association of *Escherichia coli* cells with fLNA/DNA or fODN. The percentage of cells associated with fluorescence is compared for the fLNA/DNA (■) and fODN (▲). The averages, shown by horizontal lines were fLNA/DNA, 13.96 ± 5.12, fODN, 0.05 ± 0.01. The p value is 0.0349, $p < 0.05$ is considered significant.

FIG. 3. Internalization of fLNA/DNA. **(A)** Cells in the stationary phase were incubated with fLNA/DNA as described in Materials and Methods, washed with phosphate-buffered saline, stained with FM5-95, and analyzed by laser scanning confocal microscopy. **(B)** Z-stack from a typical cell with internalized fLNA/DNA.
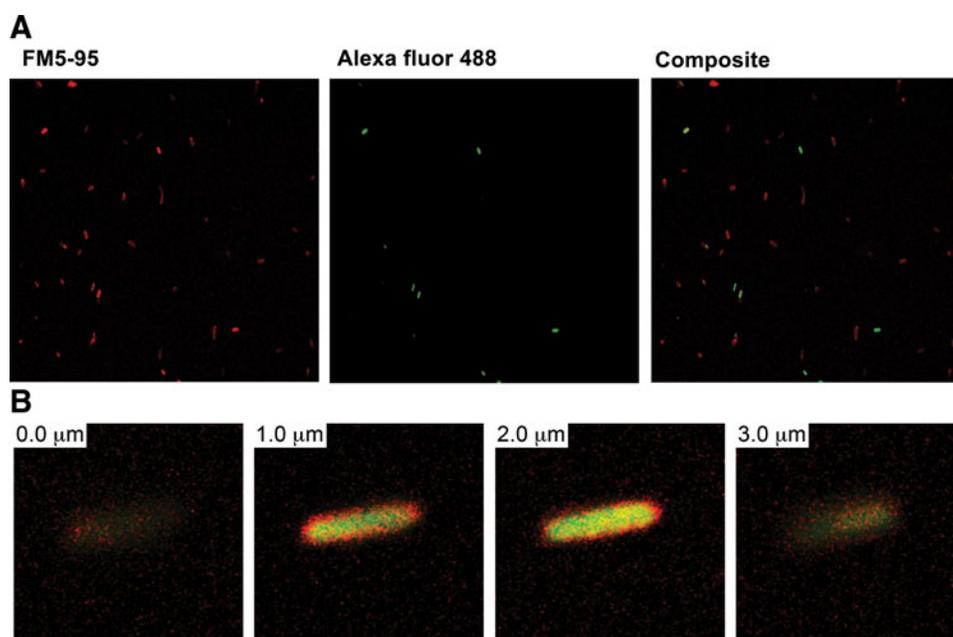
the cell. To determine if the fLNA/DNA molecules penetrated the cell wall and reach the cytosol or simply remained associated to membranes or stayed within the periplasmic space, *E. coli* cells were examined by microscopy after being exposed to the labeled compound. Figure 3 shows that the fLNA/DNA localizes within the cytoplasmic compartment in numerous cells. To discard the possibility that those cells that show internalization of fLNA/DNA are dead, and therefore more permeable to the entry of the oligomer, a control experiment was carried out exposing the cells to propidium iodide, a membrane impermeant dye that is excluded from viable cells, but reaches and intercalates into the DNA if cells are dead. The results showed that no more than 2% of the cells were dead in each culture.

The results shown here indicate that hybrid LNA/DNA compounds are good candidates as antisense antimicrobials. However, strategies to increase the efficiency of delivery inside the cells must be developed. Furthermore, future research must address questions like the role played by the length and configuration of the LNA/DNAs in efficiency of internalization as well as the physiologic state of the recipient cells.

Acknowledgments

Author Disclosure Statement

No competing financial interests exist.

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