AntimiR-155 Cyclic Peptide−PNA Conjugate: Synthesis, Cellular Uptake, and Biological Activity

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Supporting Information

ABSTRACT: Efficient delivery of nucleic acids into cells still remains a great challenge. Peptide nucleic acids (PNAs) are DNA analogues with a neutral backbone and are synthesized by solid phase peptide chemistry. This allows a straightforward synthetic route to introduce a linear short peptide (a.k.a. cell-penetrating peptide) to the PNA molecule as a means of facilitating cellular uptake of PNAs. Herein, we have devised a synthetic route in which a cyclic peptide is prepared on a solid support and is extended with the PNA molecule, where all syntheses are accomplished on the solid phase. This allows the conjugation of the cyclic peptide to the PNA molecule with the need of only one purification step after the cyclic peptide−PNA conjugate (C9−PNA) is cleaved from the solid support. The PNA sequence chosen is an antimiR-155 molecule that is complementary to mature miR-155, a well-established oncogenic miRNA. By labeling C9−PNA with fluorescein isothiocyanate, we observe efficient cellular uptake into glioblastoma cells (U87MG) at a low concentration (0.5 μM), as corroborated by fluorescence-activated cell sorting (FACS) analysis and confocal microscopy. FACS analysis also suggests an uptake mechanism that is energy-dependent. Finally, the antimiR activity of C9−PNA was shown by analyzing miR155 levels by quantitative reverse transcription polymerase chain reaction and by observing a reduction in cell viability and proliferation in U87MG cells, as corroborated by XTT and colony formation assays. Given the added biological stability of cyclic versus linear peptides, this synthetic approach may be a useful and straightforward approach to synthesize cyclic peptide−PNA conjugates.

INTRODUCTION

Downregulation of RNA by chemically modified oligonucleotides (ODNs) has shown great promise in the clinic with recent FDA approvals of several ODN-based drugs for the treatment of rare orphan diseases. One such drug is Eteplirsen;1 a phosphorodiamidate morpholino oligomer (PMO)-based exon-skipping drug (EXONDYS 51) that is administered to children suffering from Duchenne muscular dystrophy (DMD). Surprisingly, Eteplirsen is simply modified with a short phosphorodiamidate morpholino oligomer (PMO)-based exon-skipping drug (EXONDYS 51) that is administered to children suffering from Duchenne muscular dystrophy (DMD). In vivo, a guanidium backbone-modified PNA was shown as an effective antisense molecule targeting EGFr mRNA.7 In addition, a pH-responsive PNA conjugate (pHLIP) was shown as an effective anticancer agent targeting the oncogenic miR-155 (antimiR PNA).5a

CPPs are widely used for the cellular delivery of therapeutic agents. Peptides, however, are prone to degradation by peptidases and proteases. To increase peptide stability, several approaches have been utilized, such as the use of unnatural amino acids, D-amino acids, and peptide cyclization.8 Peptide cyclization for PNA delivery was recently reported by forming a hairpin structure of a gamma-PNA−Tat peptide−gamma-PNA extended with a gamma-PNA overhang. Using this approach, one may design other CPPs on such a hairpin scaffold, thus interactions between the CPP and the PNA (as opposed to negatively charged ODNs).

In the context of cancer therapeutics, PNAs have been shown to target cancer cells by (1) modulating splicing at the pre-mRNA level1 and by (2) sequestering oncogenic miRNAs5 and mRNAs.

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avoiding the need for covalent peptide cyclization. In an earlier study, PNA−cyclic peptide conjugates were obtained on the solid support by the formation of an S–S bond by designing peptides with selected positions of cysteines on the peptide sequence.

Herein, we have devised a synthetic methodology to synthesize a cyclic peptide (C9) that is conjugated to the PNA via a classical amide bond where all syntheses are carried out on the solid support. This cyclic CPP was reported in the literature as the one that permeates cells by endocytosis and is by far more...
**Table 1. PNA Conjugates as antimiRs (antimiR-155) with/without FITC Labeling and Their Corresponding ESI Mass Assignments**

| name         | description          | construct (N’ to C’)                                      | mass (daltons) (calc) | mass (daltons) (found) |
|--------------|----------------------|----------------------------------------------------------|-----------------------|------------------------|
| dK4-PNA      | PNA conjugated to dK4 | 5′-ACCCCTATCACAATTAGCATTAA-3′-dK4                         | 6671.70               | 6674.20                |
| dK4-PNA-Scr  | scrambled PNA conjugated to dK4 | 5′-ACCCATCGTGATTCATATA-3′-dK4                          | 6671.70               | 6676.70                |
| dK4-PNA-FITC | PNA conjugated to dK4 and FITC | FITC-Ahx-5′-ACCCCTATCACAATTAGCATTAA-3′-dK4         | 7174.24               | 7177.56                |
| C9-PNA       | PNA conjugated to C9  | 5′-ACCCCTATCACAATTAGCATTAA-3′-C9                         | 7513.63               | 7516.5                 |
| C9-PNA-Scr   | scrambled PNA conjugated to C9 | 5′-ACCCATCGTGATTCATATA-3′-C9                            | 7513.63               | 7517.34                |
| C9-PNA-FITC  | PNA conjugated to C9 and FITC | FITC-Ahx-5′-ACCCCTATCACAATTAGCATTAA-3′-C9    | 8022.22               | 8018.76                |

*C9 = cyclic-(F−f−Φ−r−r−r−γE)K and Ahx = 6-aminohexanoic acid

**Figure 1.** miR-155 expression following incubation of PNA conjugates (0.5 μM) in U87MG cells for 24 h at 37 °C. miR-155 is shown in comparison to scrambled PNA controls. **P** value < 0.01.

effective for cellular delivery than classical CPPs such as Tat and nona-arginine (R9). We have conjugated C9 to a PNA sequence that targets the mature form of oncogenic miRNA-155. In this report, we have studied the cellular uptake and biological activity of C9−PNA in glioblastoma cancer cells (U87MG).

**RESULTS AND DISCUSSION**

Peptide cyclization on the solid support followed by on-resin PNA synthesis was accomplished as described in Scheme 1.

The peptide of choice is 9-mer that is designed in the following manner: the amino acid at the C-terminus is L-lysine with its ε-amine protected with a trityl group. This allows the extension of the peptide via the α-amine and post on-resin cyclization, the introduction of the PNA monomers at the ε-amine following trityl group deprotection. The remaining 8-mer peptide is the peptide that is then cyclized on the solid support. This cyclic peptide has several features: (1) it has a hydrophobic (Flϕ, where ϕ = 1-2-naphthylalanine, F = L-phe, and f = D-phe) and a hydrophilic (Rrτ, four arginines with alternating chirality of L and D) region; (2) it contains both natural and unnatural amino acids (e.g., 1-2-naphthylalanine); and (3) it has been reported to be about 20-fold more efficient as a CPP in comparison to classical CPPs such as Tat and nona-L-arginine (R9). Cyclization on the resin is carried out using standard coupling reagents (2-(1H-7-azabenzotriazol-1-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU), hydroxybenzotriazole (HOBT), and diisopropylethylamine (DIPEA)).

As a PNA sequence, we chose the one that is fully complementary to the mature miR-155 oncogenic miRNA. In addition, we designed a scrambled sequence to serve as a control (Table 1). This scrambled sequence consists of the same amount of four PNA monomers (as anti-miR155 PNA) and the sequence was found not to have a full complementarity to any human transcript, as verified by BLAST analysis. An additional control was synthesized that consisted of four D-lysines as the CPP. There were two criteria for this choice: (1) this peptide has four positive charges in physiological pH as does the cyclic C9 peptide (four Arg); (2) this peptide is also stable in biological medium because of the chirality (D) of all four amino acids. As an additional control, dK4−PNA was also prepared with the scrambled PNA sequence (Table 1). dK4−PNA conjugates have also been previously reported as cancer cell-permeable at a 1 μM concentration. Fluorescein isothiocyanate (FITC) labeling at the N-terminus of both C9−PNA and dK4−PNA was carried out to allow us to follow the cellular uptake of both PNAs that differ only in their peptide sequence (Table 1).

miR-155 is highly expressed in a variety of cancers. We chose glioblastoma cells (U87MG) as the means to follow antimiR activity and cellular uptake of the PNA conjugates. These cells express high levels of miR-155 and are not easy to transfect. To evaluate the antisense (antimiR) activity of the PNA conjugates, we followed the effect of PNA conjugate...
incubation with U87MG cells by following the expression levels of miR-155. As shown by Gait and co-workers, PNA antimiRs sequester mature miRNA in cells. Thus, isolating miRNAs from cells after PNA treatment is anticipated to result in low levels of isolated miRNA because of PNA sequestration (and not miRNA degradation). This is related to the chemistry of PNA that does not evoke RNAse H activity.

U87MG cells were treated with either C9−PNA or dK4−PNA (0.5 μM). Scrambled PNAS were also tested as controls (C9−PNA-Scr or dK4−PNA-Scr). After 24 h, total miRNA was isolated and cDNA was prepared from 1 μg of total miRNA using the qScript microRNA cDNA Synthesis Kit. The expression of miR-155 was then evaluated by quantitative reverse transcription polymerase chain reaction (in triplicates, Figure 1).

In comparison to scrambled controls, both PNA conjugates were shown to sequester miR-155, with the C9−PNA conjugate showing over 80% decrease in miR-155 in comparison to the scrambled control.

Next, we examined cell viability by the XTT assay after treating U87MG cells with 1 μM of PNA conjugates (Figure 2). U87MG cells were incubated with PNA conjugates for 72 h at 37 °C in triplicates in 96-well plates. As shown in Figure 2a, over an 80% reduction in cell viability was observed for cells treated with C9−PNA in comparison to the C9−PNA-Scr control. Both C9−PNA and C9−PNA-Scr had negligible effects on the viability of THESCs uterus (immortalized fibroblast) cells (Figure 2b) as well as on normal uterus human fibroblast cells produced from a patient (Nf08 uterus, Figure S9).

To examine the effect on cell survival and proliferation, we seeded U87MG glioblastoma cells in six-well plates, in the presence of either dK4−PNA and C9−PNA or scrambled controls (dK4−PNA-Scr and C9−PNA-Scr). After 2 weeks, the plates were fixed and stained, and the colonies were counted (in triplicates). *P value < 0.05.

Figure 2. Cell viability for U87GM cells and THSCs cells as determined by the XTT assay. (a) U87GM cells were treated with 1 μM PNA conjugates for 72 h at 37 °C (in triplicates in 96-well plates). Viability is shown in comparison to scrambled PNA controls. *P value < 0.05, **P value < 0.001. (b) THSCs cells were treated with 1 μM PNA conjugates for 72 h at 37 °C (in triplicates in 96-well plates). Viability is shown in comparison to scrambled PNA controls. *P value < 0.05.

Figure 3. C9−PNA reduces the colony survival of U87MG glioblastoma cells. Colony formation assay of cells treated with 1.0 μM of either dK4−PNA and C9−PNA or scrambled controls (dK4−PNA-Scr and C9−PNA-Scr). After 2 weeks, the plates were fixed and stained, and the colonies were counted (in triplicates). *P value < 0.05.
PNA−FITC shows a dominant fluorescence in the cytoplasm which is significantly higher than that of dK₄−PNA−FITC. Some punctate green fluorescence is also observed for C₉−PNA−FITC; these foci are inside cells, as verified by the cross-sectional confocal images (Supporting Information, Figure S7). To further quantify the PNA−FITC uptake into U87MG cells, fluorescence-activated cell sorting (FACS) analysis for both PNAs was performed after a 2 h incubation period at the same PNA concentration (0.5 μM). A clear and dominant change in FITC fluorescence is seen for C₉−PNA−FITC (Figure 4B), whereas almost no change in fluorescence is seen for dK₄−PNA−FITC. These results coincide with the confocal images presented in Figure 4A.

The cyclic peptide used in this study was reported by Qian and co-workers¹¹ to enter cells via an energy-dependent endosomal mechanism. It is not clear, a priori, whether or not the introduction of the PNA sequence affects the uptake mechanism of this cyclic peptide. To answer this question, a series of flow cytometry experiments were performed. U87MG cells were incubated for 2 h with 0.5 μM C₉−PNA−FITC in the...
presence of either 10 mM Na,N,/,10 mM 2-deoxy-D-glucose at 37 °C or at 4 °C. Both conditions deplete ATP that is required for endocytosis. As shown in Figure 5, in both conditions, a clear decrease in cellular uptake was observed. These results suggest that the cyclic peptide retains its energy-dependent cellular uptake mechanism even after PNA conjugation.

To further elaborate on the cellular uptake into U87MG cells, we stained the cells with Lyso-Tracker Red (staining lysosomes) and Hoechst (staining nuclei), following a 3 h incubation with C9-PNA−FITC or dK4−PNA−FITC, to follow the cellular localization of FITC-labeled PNAs. As shown in Figure 6, there was a negligible uptake of dK4−PNA−FITC. In contrast, we observed strong punctate green signals of C9−PNA−FITC that are predominantly found in lysosomes and, to a lesser extent, in nuclei.

The cross-sectional confocal images (Supporting Information, Figure S8) further confirm the presence of C9−PNA−FITC inside cells. These results further support the endocytosis-dependent mechanism of uptake for C9−PNA−FITC.

In this study, we have shown efficient blocking of miR-155 by C9−PNA conjugates. We designed and synthesized the C9 peptide from a solid support followed by PNA conjugation. Using this synthetic strategy, one may covalently link the C9 peptide to any PNA sequence, thus providing the PNA−peptide conjugate with high stability and cellular uptake.

### EXPERIMENTAL PROCEDURE

**Materials.** Fmoc-PNA monomers were purchased from PolyOrg, Inc. (USA) and used as received. Amino acids (Fmoc-Lys-(BOC) (K), Fmoc-Lys(MTT)OH (K), Fmoc-d-Arg(pbf)-OH (r), Fmoc-Arg(pbf)-OH (R), Fmoc-i-2-naphthylalanine (γ), Fmoc-d-phenylalanine (f), Fmoc-phenylalanine (F)), and N-9-fluoromethoxycarbonyl chloride were purchased from GL Biochem (Shanghai) Ltd. N-Fmoc-L-glutamic acid 1-allyl ester, N-Fmoc-L-2-naphthylalanine, Fmoc-PNA monomers were purchased from Acros Organics. Tetrakis(triphenylphosphine) was purchased 98%, was purchased from Alfa Aesar. Ahx was purchased from Acros Organics. Tetraakis(triphenylphosphine) was purchased from Strem Chemicals.

**Synthesis of Linear PNAs.** All cell lines (U87MG, NIH08 uterus, and THESCs uterine) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), penicillin, and streptomycin.

**miR-155 Reverse Transcription Quantitative Real-Time PCR.** Total microRNA was isolated from cells using the mirPremier microRNA Isolation Kit from Sigma, and cDNA was synthesized from 1 μg RNA using the qScript microRNA cDNA Synthesis Kit in a final volume of 20 μL according to the manufacturer’s instructions (Quantabio). Real-time PCR was performed on a real-time PCR Connect ST System BioRad, and PCR parameters were adjusted as recommended by the manufacturer (PerfeCTa SYBR Green SuperMix, Quantabio). Primers for miR-155 were purchased from Hylabs (See Supporting Information, Table S1).

**Cell Proliferation Assay.** Cells [U87MG glioblastoma cells, THESCs uterine cells, and NIH08 uterus cells (3 × 105)] were seeded in a 96-well plate and incubated for 24 h for cell attachment. Next, dK4−PNA (and its scrambled control) and C9−PNA (and its scrambled control) were added after medium replacement and incubated (37 °C, humidified atmosphere containing 5% CO2) for 72 h. Cell proliferation was then measured using the XTT (sodium 3-[1(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) proliferation assay, according to the manufacturer’s instructions (Biological Industries, Israel).

**Clonogenic Assay.** After 24 h of transfection, 1000 and 500 U87MG cells were seeded in triplicates in six-well plates with 2 mL of medium (DMEM, 10% FCS). After 14 days, the cells were
fixed with 2.5% glutaraldehyde solution for 10 min, stained with 1% methylene blue solution, and counted.

**Live Cell Imaging.** Twelve hours prior to FITC-labeled PNA conjugate addition, U87MG cells were plated on an eight-well chamber removable microscope-sterilized glass slide (NBT New Bio Technology Ltd.). The cells were grown until reaching 70–80% confluence. Before adding the PNAs, the medium was replaced and the cells were incubated (37 °C, humidified atmosphere containing 5% CO₂) with 0.5 μM of dC₉–PNA–FITC or C₉–PNA–FITC in complete medium, over a period of 3 h, followed by two washings with phosphate-buffered saline (PBS). For negative control, untreated cells were used. Cell fluorescence analysis was performed using a confocal laser scanning microscope, Olympus FV300.

**Cell Staining for Colocalization Studies.** The initial procedure was carried out as described for live cell imaging. For lysosome staining, a solution of 50 nM Lyso-Tracker Red (Molecular Probes) was added to the cells and incubated at 37 °C for 20–30 min. To achieve nuclear staining, the cells were washed twice with PBS and incubated with 0.01 mM Hoechst (bis-benzimide H 33342 trihydrochloride, Merck) at 37 °C for 15 min. Thereafter, the cells were fixed with 4% formaldehyde (Biolab, Israel) and washed three times with PBS prior to imaging. Cell fluorescence analysis was performed using a Zeiss LSM 710 confocal laser scanning system (Carl Zeiss Micro Imaging GmbH, Jena, Germany).

**FACS Analysis of FITC-Labeled PNA Conjugates.** Flow cytometry studies were performed by plating 2.0 × 10⁵ U87MG cells/well prepared in six-well plates, and the cells were allowed to adhere overnight under normal culture conditions. The medium (DMEM, 10% FCS) was replaced and the cells were incubated (37 °C, humidified atmosphere containing 5% CO₂) with 0.5 μM of dC₉–PNA–FITC or C₉–PNA–FITC in a complete medium, over 2 h. The medium with the PNA conjugate was removed, and the cells were washed with PBS. Then, the cells were detached from the wells with 400 μL of 0.25% trypsin (PBS). For negative control, untreated cells were used. Cell cytometry studies were performed by plating 2.0 × 10⁵ U87MG cells prepared in a cell culture medium and was added to the cells and incubated at 37 °C for 20–30 min. To achieve nuclear staining, the cells were washed twice with PBS and incubated with 0.01 mM Hoechst (bis-benzimide H 33342 trihydrochloride, Merck) at 37 °C for 15 min. Thereafter, the cells were fixed with 4% formaldehyde (Biolab, Israel) and washed three times with PBS prior to imaging. Cell fluorescence analysis was performed using a Zeiss LSM 710 confocal laser scanning system (Carl Zeiss Micro Imaging GmbH, Jena, Germany).

**HPLC chromatograms, mass spectra, primer sequences, cross-sectional confocal images, and XTT assay (PDF)**

### Associated Content

**Supporting Information**
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### Notes
The authors declare no competing financial interest.

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### ABBREVIATIONS
PNA, peptide nucleic acid; CPP, cell-penetrating peptide; WT, wild type; SCR, scrambled; C₉, cyclic-(F-Φ-P-R-R-R-yΦ)K; PMO, phosphorodiamidate morpholino oligomer; DMD, Duchenne muscular dystrophy; PEG, polyethylene glycol; pHLP, pH low insertion peptide

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