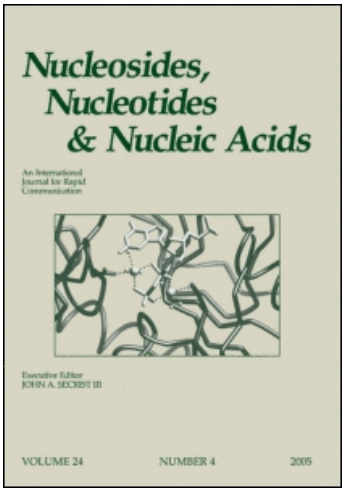

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Phosphono Peptide Nucleic Acids with a Constrained Hydroxyproline-Based Backbone

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ABSTRACT

DNA mimics representing negatively charged analogues of peptide nucleic acids (PNAs), particularly hetero-oligomers constructed from alternating phosphono-PNA residues (pPNA) and monomers on the base of *trans*-4-hydroxy-*L*-proline (HypNA) as well as mimics composed of phosphono-HypNA monomers (pHypNA) were tested in a set of in vitro and in vivo assays, and they demonstrated a high potential for the use in nucleic acid based diagnostic, isolation of nucleic acids and antisense experiments.

Key Words: DNA mimics; Phosphono-PNAs; *trans*-4-Hydroxy-*L*-proline derivatives.

Last years, a large number of nucleic acid analogs have been synthesized, including a such successful example as peptide nucleic acids (for recent reviews see in Ref.[1]). In an attempt to improve the potency of PNAs as diagnostic probes

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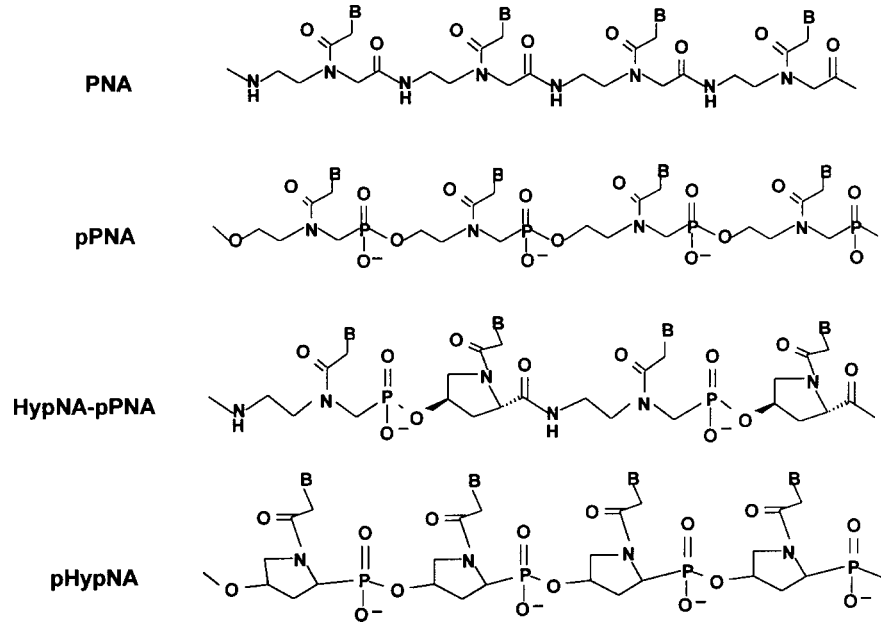
and therapeutics, DNA mimics representing negatively charged analogues of PNAs, in which monomer units are connected with phosphonester bonds, were designed. Thus, the synthesis of phosphono-PNA oligomers (pPNAs) with N-(2-hydroxyethyl)-phosphono glycine, or N-(2-aminoethyl)-phosphono glycine, backbone has been developed using solid phase technique, which was similar to the phosphotriester oligonucleotide synthesis with the O-nucleophilic intra-molecular catalysis.[2–4] Using the same approach, a set of chimeras composed of pPNA and PNA monomers (PNA-pPNAs) was synthesized.[5] The evaluation of properties of these types of DNA mimics revealed that they are fully stable to the action of nucleases and proteases, and the introduction of negative charges to the backbone leads to excellent solubility in water. However, the thermal stability of complexes formed by pPNA and PNA-pPNA mimics with the complementary DNA (or RNA) targets was lower than the stability of corresponding complexes formed by classical PNAs.[2–5]

To continue these investigations, the solid phase synthesis of hetero-oligomers consisting of various amounts of pPNA monomers and PNA-like monomers based on *trans*-4-hydroxy-*L*-proline (HypNA) was accomplished (Fig. 1).[6,7] A HypNA monomer represents conformationally constrained chiral PNA analogue, in which β-C atom of a hydroxyethyl unit and α-C atom of a glycyl unit of the backbone are bridged by methylene group. HypNA-pPNA mimics constructed of alternating pPNA and HypNA residues demonstrated the strong binding to complementary DNA and RNA strands with the stability of complexes very close to that of PNA/DNA(RNA) complexes. Similar to other PNA-related mimics, homo-pyrimidine sequences of these chimeras formed with complementary templates triple helixes, whereas oligomers with mixed sequences gave duplexes.



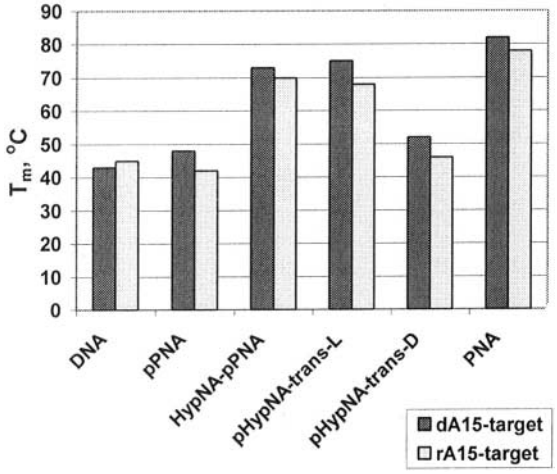
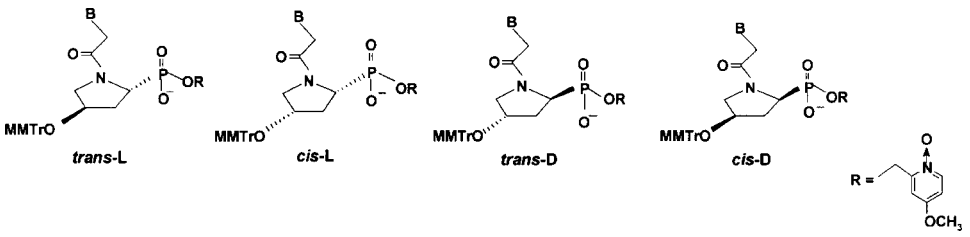


Figure 1. The hybridization properties of homo-Thy pHypNA oligomers. Comparison of melting temperatures of complexes formed by 15-mer mimics with the complementary DNA(RNA) targets in 0.1 M NaCl/0.02 M Tris-HCl (pH 7)/0.01 M $MgCl_2$.

Excellent hybridization properties of HypNA-pPNA hetero-oligomers stimulated us to undertake the synthesis of DNA mimics totally constructed from the conformationally constrained chiral pPNA analogues on the base of 4-hydroxy-N-acetyl-pyrrolidine-2-phosphonic acid (pHypNAs).[6] We hoped that such a ring constraint in the backbone could lead to the pre-organization of the pPNA structure and thus impose entropic advantages for binding complementary target in comparison with linear pPNA molecules. The synthesis of four optically active pHypNA monomers was accomplished starting from methyl ester of *trans*-4-hydroxy-*L*-proline as described in Ref.[8]. The Thy-containing monomers were used in the automated synthesis of homo-Thy 15-mers on the standard CPG support and protocols developed by us earlier for pPNAs synthesis.[2,5]



Investigations of the binding properties of chiral pHypNAs have shown that the oligomers totally constructed from the monomers of with *cis-L*, or *cis-D,* configuration were not able to form the stable complexes with the complementary homo-A DNA or RNA targets. The oligomer constructed from the *trans-D* monomers

exhibited the properties very close to those of achiral linear pPNAs, whereas the oligomer composed of *trans-L* monomers exhibited strong binding to the complementary DNA/RNA target with the melting temperatures of complexes very close to those formed by classical PNAs with complementary nucleic acids (Fig. 1).[8] From the data of titration experiments and the electrophoretic behavior, it was concluded that homo-pyrimidine pHypNAs similar to other PNA-related oligomers form with complementary DNA/RNA templates triple helixes. Moreover, it was found that in contrast to natural oligonucleotides and pPNAs, but similar to classical PNAs and alternating HypNA-pPNA chimeras, *trans-L* and *trans-D* pHypNAs can effectively hybridize to complementary targets in low salt concentrations, and T_m values of their complexes with DNA (or RNA) targets are not dependent on ionic strength.[8] The synthesis of pHypNAs containing all four nucleobases and investigation of their properties are in progress.

The evaluation of the effectiveness of negatively charged PNA-related mimics in assays based on the hybridization technique revealed a high potential of these mimics as bio-molecular probes for the solution and solid phase analysis.[7,9] It was shown that 16–18-mer HypNA-pPNA probes can effectively discriminate between single base mismatches in the target sequence, and their use gives rise to the detection sensitivity as compared to that of natural oligonucleotides. Thus, the introduction of one mismatch in the center of a sequence gives a drop in the melting temperature of 17–23°C, whereas oligomers with two separately situated mismatches are not able to form stable complexes with the targets. No increase in background was observed for mimic probes if compared to DNA probes.[7] At the same time, the shelf-life of mimics is considerably longer than oligonucleotides.

The other advantage of negatively charged PNA-relative mimics is their inability to interact with intercalating dyes, particularly ethidium bromide, ethidium homodimer, homodimeric oxazole yellow and thiazole orange dyes. At the same time, the duplexes and triplexes of these mimics with DNA(or RNA) targets exhibit fluorescence under UV-light after the staining with these dyes.[9] This property can be especially useful for the application of PNA-related mimics as capture probes in micro chip analysis and nucleic acids isolation, because a visualization of their complexes with nucleic acid targets does not require the preliminary radioactive or fluorescent target labeling.

Recently, we have undertaken the experiments on the construction of novel types of chimeric molecular beacons, particularly the molecules containing RNA and mimic segments.[10] Molecular beacons are hairpin-shaped oligonucleotide probes, containing a fluorophor attached to one end and a quencher attached to the other end.[11] They can report the presence of complementary nucleic acid targets in hybridization assays without having to separate probe-target hybrids from the excess of a probe. We have synthesized molecular beacons containing a HypNA-pPNA mimic segment in the loop part as well as fully composed of mimic residues and evaluated their properties in model assays with the complementary and mismatched oligonucleotide targets. The results on the interaction of chimeric beacons with the target in solution shown that the mimic probes were capable of detecting target starting from 1 pmol. In comparison, oligodeoxyribonucleotide beacon of the same sequence was in 10 times less sensitive (Fig. 2). The negative controls, which contained irrelevant molecules, did not generate a signal (data not shown).

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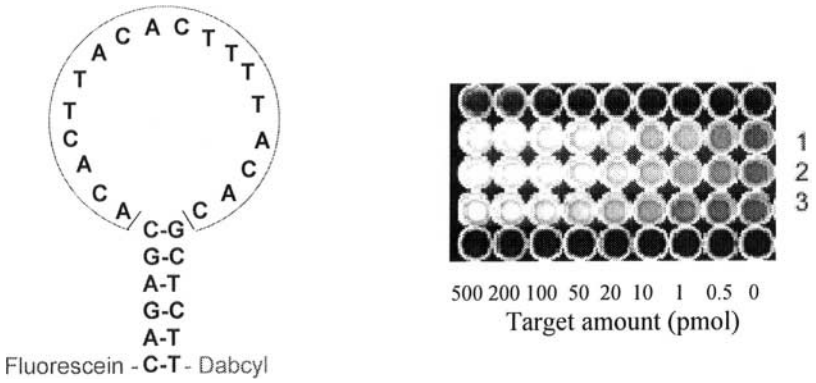

Figure 2. The sequence of chimeric beacons composed of DNA stem part and HypNA-pPNA loop (1), fully composed of HypNA-pPNA dimers (2) or fully composed of DNA monomers (3), and a photo-image of the solution fluorescent analysis with these molecular beacons (500 pmol) and decreasing amounts of a complementary DNA target in 200 μL of 0.1 M NaCl/0.02 M Tris-HCl (pH 7.5)/0.01 M $MgCl_2$ at 55°C.

The experiments with other types of mimic containing molecular beacons are in progress.

The experiments on the cell delivery of PNA-related mimics into HeLa cells were initiated using and a 20-mer fluorescein labeled oligomers. It was shown that HypNA-pPNA chimeric oligomers are able to penetrate into the living cells and distribute in the cytoplasm (Fig. 3), while the analogous pPNA oligomer was accumulated in cell membranes (data not shown).

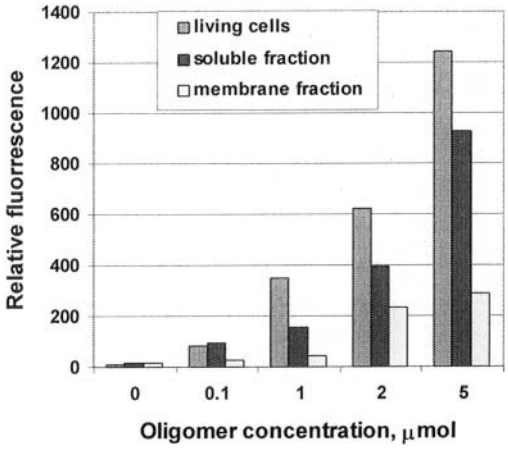

Figure 3. Delivery of a fluorescein labeled HypNA-pPNA 20-mer into HeLa cells. Oligomer in 0.1–5 μM concentrations was incubated with the cells for 24 h at 37°C without an effector, cells were washed, and a fluorescence was measured in living cells as well as in the soluble and membrane fractions of cell lysate.

Very recently, HypNA-pPNA oligomers were tested as in vivo translation inhibitors in zebrafish embryos (K. Urtishak, M. Choob, E. Wickstrom, S. Farber, manuscript in preparation). It was shown, that antisense 18-mer HypNA-pPNAs sequences effectively target four developmental genes of zebrafish particularly *chordin, uroD, notail* and *dharma,* without nonspecific effects. In these experiments, HypNA-pPNAs appear more specific and effective antisense agents than morpholino phosphorodiamidate oligonucleotide analogues, which were recently used for this purpose by S. Ekker and co-workers,[12] and PNAs, and it can be concluded that they are a powerful addition to the list of potential anti-sense and anti-gene therapeutics.

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