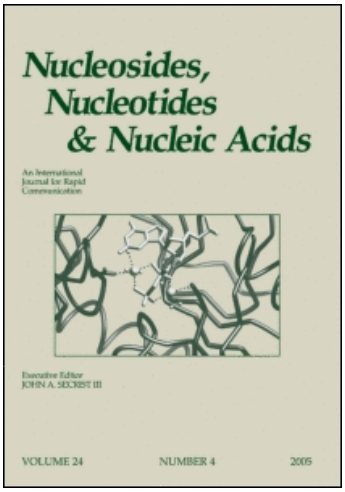

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SYNTHESIS AND APPLICATION OF NEGATIVELY CHARGED PNA ANALOGUES

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□ *Negatively charged DNA mimics containing phosphonate analogues of peptide nucleic acids were designed, and their physicochemical and biological properties were evaluated in the comparison with natural oligonucleotides, classical peptide nucleic acids, and morpholino phosphorodiamidate oligonucleotide analogues. The results obtained revealed a high potential of phosphonate-containing PNA derivatives for a number of biological applications, such as diagnostic, nucleic acids analysis, and inhibition of gene expression.*

Keywords DNA analogues; Phosphono-PNA analogues; Hybridization; Inhibition; Diagnostic; Antisense reagents

INTRODUCTION

Synthetic oligonucleotides, their analogues, and mimics attract a great interest as the potential therapeutic agents and diagnostic tools. Although natural oligonucleotides have many applications based on the hybridization technique, their use is restricted by the biological instability and relatively low thermal stability of their duplexes with complementary nucleic acids. In recent years, a large number of nucleic acid analogues and mimics have been proposed, including such examples as peptide nucleic acids (PNAs)[1,2] and morpholino phosphorodiamidate oligonucleotide analogues (MOs),[3] to improve the oligonucleotides potency in terms

This paper is dedicated to the memory of John A. Montgomery.

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of nuclease resistance and affinity to nucleic acids. In spite of high affinity toward DNA and RNA and stringent mismatch discrimination, the use of PNAs as therapeutics and as sensor molecules in diagnostics is restricted by their poor water-solubility, tendency to self-aggregation in low salt concentrations, and low cellular uptake.[4,5] In their turn, MO oligomers exhibit good hybridization properties, resistance to a broad range of cellular degradative enzymes, and high solubility in water despite their lack of charge, due to their strong polarity. However, they exhibit lower affinity to nucleic acids and mismatch discrimination than PNAs.[3,6,7]

As an alternative to PNAs and MOs, DNA mimics representing negatively charged analogues of PNAs, in which monomer units are connected with phosphono-ester bonds (pPNAs) were designed[8–10] (Figure 1). Later, a set of chimeras composed of pPNA and PNA monomers (PNA-pPNAs) was presented[11,12], as well as HypNA-pPNA hetero-oligomers consisting of alternating pPNA monomers and PNA-like monomers based on *trans*-4-hydroxy-L-proline.[13,14] Recently, conformationally constrained chiral phosphono-PNA analogues on the base of 4-hydroxyproline (pHypNAs) were prepared.[15,16] In this article, we review the methods for the synthesis of these negatively charged types of DNA mimics, their physicochemical and biological properties, and evaluate their potential for application in molecular biology and medicine.

RESULTS AND DISCUSSION

Several years ago, a novel class of DNA mimics representing phosphonate PNA analogues (pPNAs) was designed, and the synthesis of pPNA oligomers containing N-(2-hydroxyethyl)-phosphono glycine (Figure 1), or N-(2-aminoethyl)-phosphono glycine backbone has been accomplished using solid phase and solution techniques.[8–10] The most effective synthesis of pPNA oligomers was achieved using a solid-phase technique, which was similar to the phosphotriester oligonucleotide synthesis with the O-nucleophilic intramolecular catalysis.[8] To construct pPNA oligomers, the procedures to obtain corresponding monomers of types (1) and (2) were developed (Figure 2). These building units have a combination of blocking groups compatible with the technique of the solid-phase phosphotriester synthesis of oligonucleotides, which was applied with some modifications to the construction of pPNA oligomers. Also, a set of optically active and aromatic pPNA analogues was obtained using this technique.[13] The examination of pPNA oligomers properties revealed that they are fully stable to the action of nucleases, and the introduction of negative charges into the PNA backbone led to excellent solubility characteristics. However, the thermal stability of pPNA complexes with complementary DNA (or RNA) targets was lower than the stability of corresponding complexes formed by PNAs.[8]

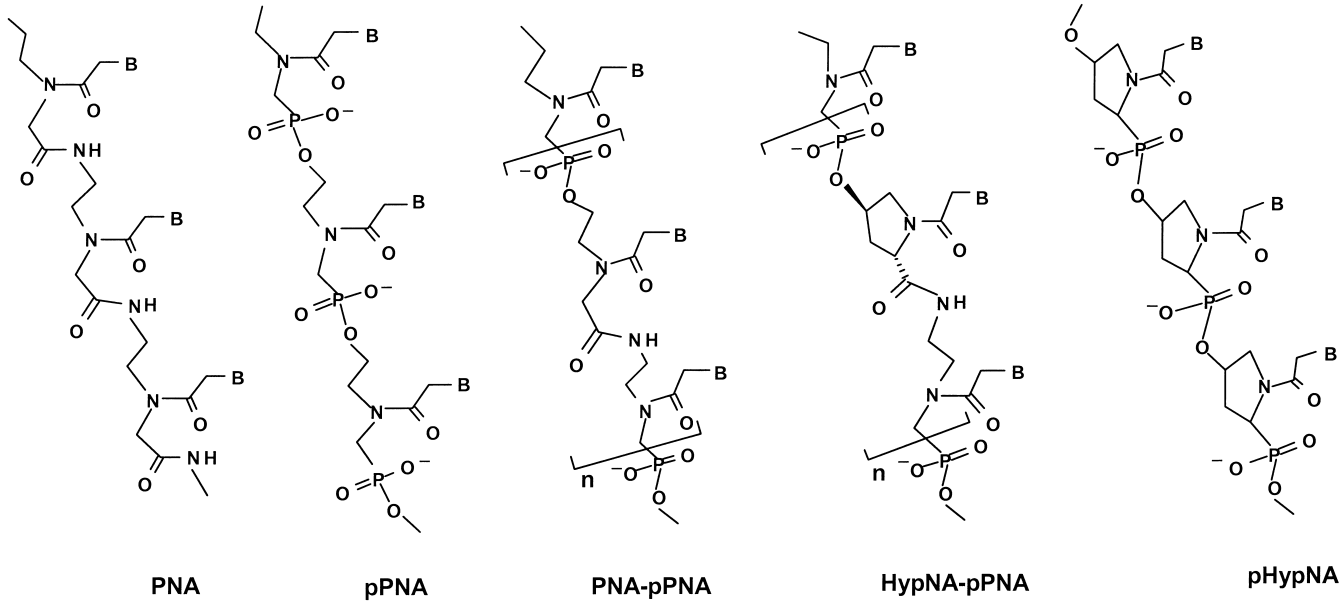


FIGURE 1 General chemical structures of DNA mimics.

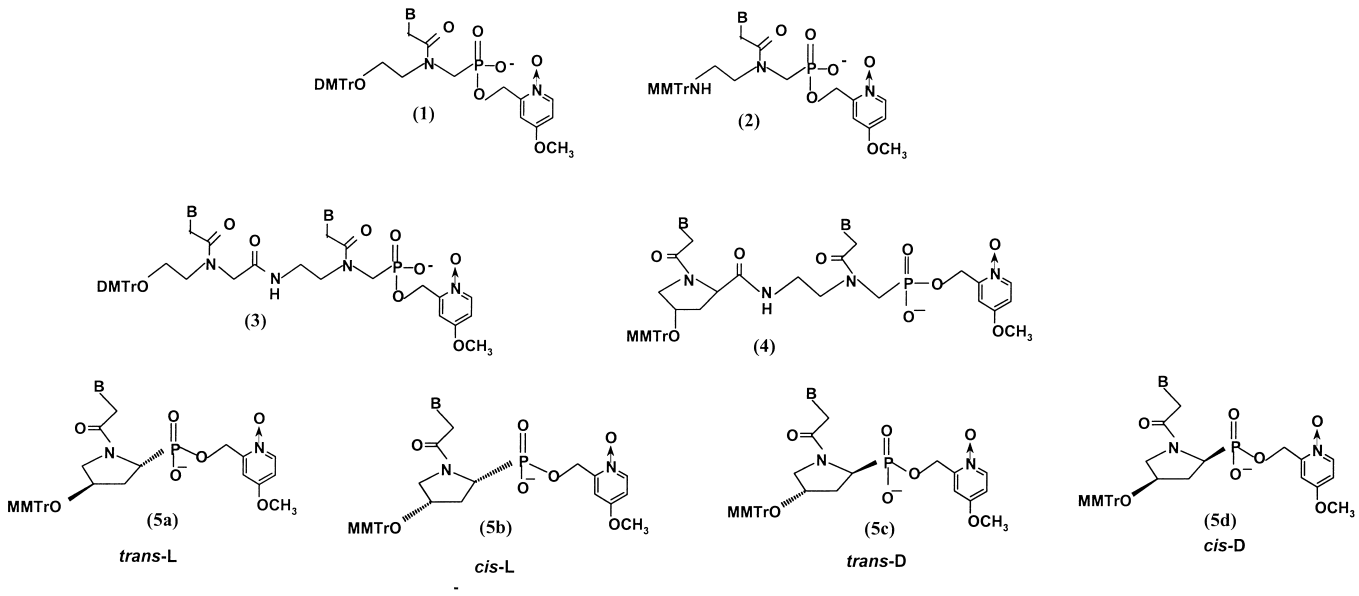


FIGURE 2 Building units for the solid-phase synthesis of negatively charged PNA analogues.

As both PNA and pPNA mimics are isosteric compounds, the chimeras composed of these monomers were obtained similarly to pPNAs on a CPG support using specially synthesized dimer building PNA-pPNA units of type (3) (Figure 2) containing the internal amide bond between the monomer residues.[11] The PNA-pPNA chimeras showed improved hybridization characteristics in comparison with pPNAs and DNA oligomers, which were combined with good water solubility.

To continue these investigations, the synthesis of hetero-oligomers consisting of various amounts of pPNA monomers and PNA-like monomers based on *trans*-4-hydroxy-L-proline (HypNA) (Figure 1) was reported.[17] The general approaches to the synthesis of HypNA monomers and HypNA-pPNA dimers of type (4) (Figure 2) were developed.[17,18] An alternative scheme for the synthesis of four chiral HypNA monomers was proposed by Verheijen et al.[19] A HypNA monomer represents a chiral PNA analogue having the constrained conformation with β-C atom of a hydroxyethyl unit and α-C atom of a glycyl unit of the backbone bridged by methylene group. The HypNA-pPNA hetero-oligomers were constructed on a solid phase from the dimer units (4) similar to PNA-pPNA oligomers. It was shown that the chimeras containing alternating pPNA and HypNA residues demonstrated stronger binding to complementary DNA and RNA strands in comparison with pure pPNAs and equivalent PNA-pPNA chimeras, and the stability of their complexes with complementary nucleic acid targets was very close to that of complexes formed by classical PNAs (Figure 3).[13,14]

More recently, pPNA-like oligomers composed of optically active monomers on the base of 4-hydroxyproline containing phosphonate residues and representing conformationally constrained chiral pPNA analogues were prepared.[15,16] The schemes for the synthesis of optically active pHypNA monomers (5a-d) (Figure 2) were developed. The scheme for preparation of Thy-containing monomers was similar to those developed earlier for pPNA monomers with N-(2-aminoethyl)phosphonoglycine backbone.[15] The alternative scheme was developed for the synthesis of Cyt-, Gua- and Ade-containing pHypNA monomers (V.A. Efimov et al., manuscript in preparation). The investigations of the binding properties of chiral homo-Thy pHypNA oligomers have shown that the oligomers obtained from the monomers of types (5b) and (5d.) with *cis*-configuration are not able to form the stable complexes with the complementary homo-A DNA or RNA oligonucleotides. The oligomer constructed from the units of *trans*-D type (5c) exhibited the properties very close to those of achiral linear pPNAs, whereas the oligomer composed of *trans*-L monomers (5a) exhibited strong binding to the complementary DNA/RNA target with the melting temperatures (T_m) very close to those of the complexes formed by classical PNAs with complementary nucleic acids (Figure 3).[15,16] Similar hybridization properties were exhibited by pHypNA oligomers containing all four nucleobases.

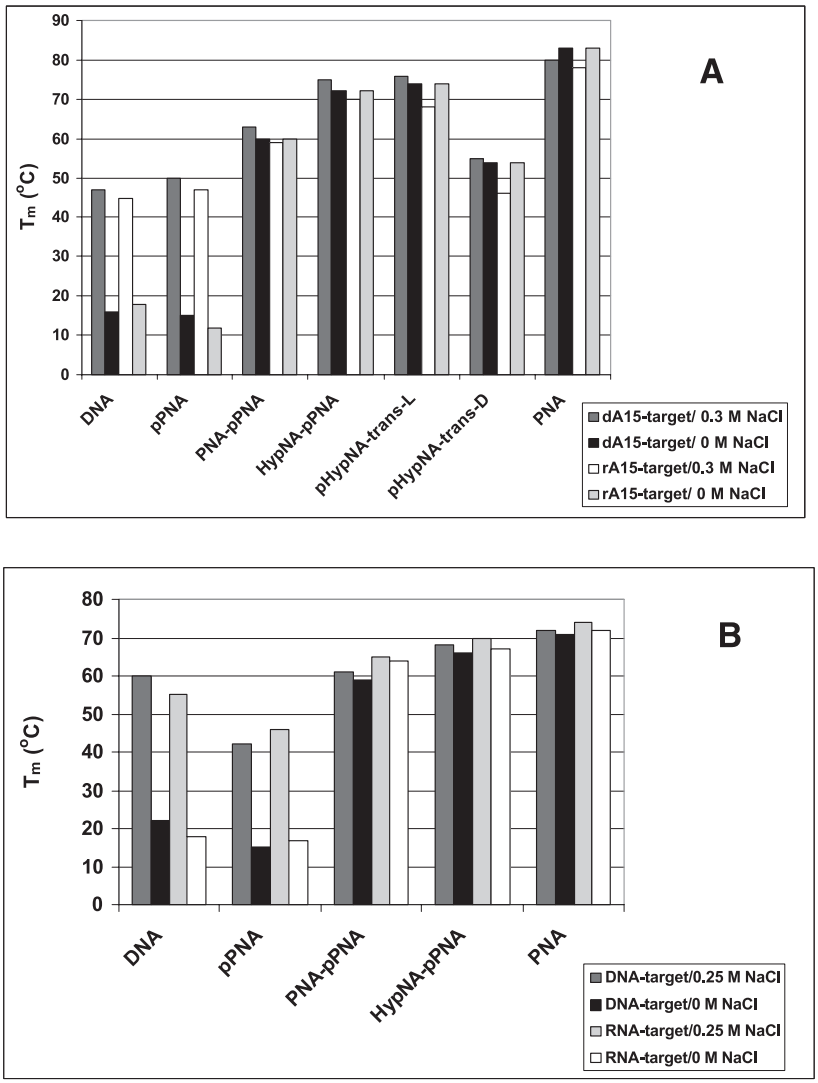


FIGURE 3 Comparison of melting temperatures of complexes formed by PNA-related oligomers with their complementary DNA or RNA targets. **A:** Melting temperatures of 15-mer homo-Thy oligomers complexes. **B:** Stability of complexes formed by 16-mer mimics with a mixed purine-pyrimidine sequence (CTGCAAAGGACACCAT).

In general, the evaluation of physicochemical and biological properties of negatively charged PNA derivatives revealed that they have excellent solubility in water and are fully stable to the action of nucleases and proteases. In contrast to natural oligonucleotides and their phosphorothioate analogues, negatively charged PNA-relative mimics as well as classical PNAs do not activate RNase H upon binding to complementary RNA (Figure 4). From the titration data and the electrophoretic behavior, it was concluded that,

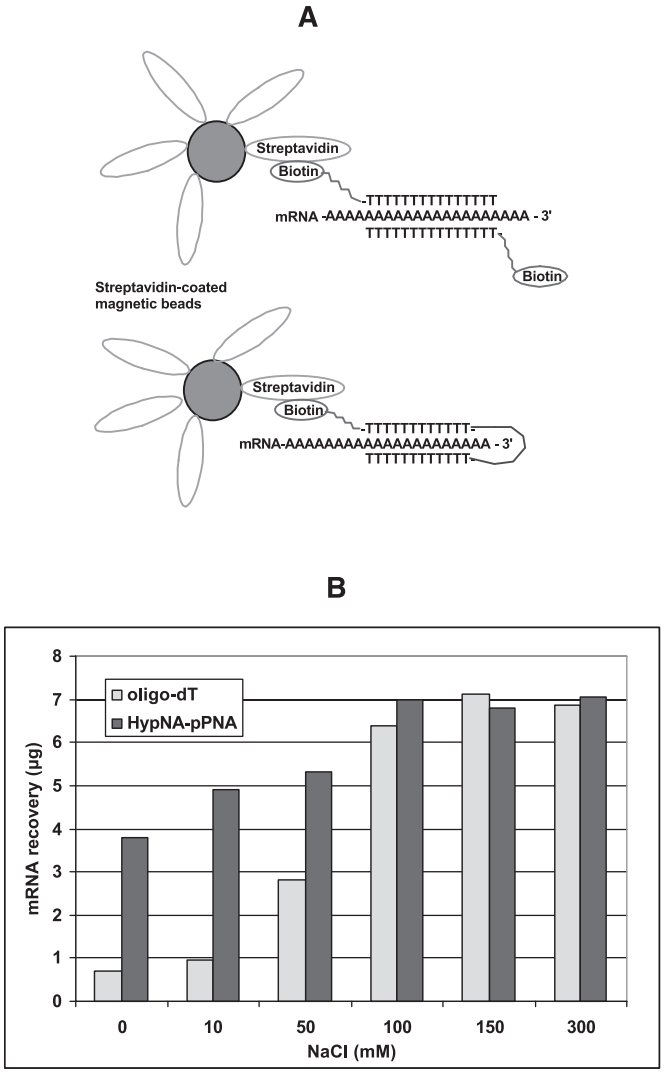


FIGURE 4 Isolation of poly-A mRNA using a biotinilated mimic oligomer and streptavidin-coated magnetic beads. **A:** Schematic representation of poly-A^+ mRNA complexes with oligo-Thy linear and clamping mimics. **B:** The influence of a salt concentration on the recovery of poly-A^+ mRNA. Aliquots (400 μg) of RNA were captured at increasing NaCl concentrations (0–300 mM) in binding buffer containing 20 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ using biotinilated oligo-dT_{18} or homo-Thy 14-mer HypNA-pPNA (1:1) mimic probe and streptavidin-coated magnetic beads. After washing and elution from the beads, the recovery of isolated poly-A^+ mRNA was calculated by image analysis of agarose gels.

similar to PNAs, homo-pyrimidine sequences of all phosphonate containing mimics formed with complementary DNA (or RNA) targets triple helixes, whereas oligomers with mixed nucleobase sequences formed duplexes with nucleic acid targets.[11,13,16] The direct comparison of melting temperatures of HypNA-pPNA and MO duplexes with complementary synthetic oligoribonucleotides has shown that the T_m values of 18-b.p. HypNA-pPNA/RNA duplexes (76–88° C) are practically equivalent to those of 25-b.p. MO/RNA duplexes.[20,21]

The examination of a salt concentration influence on melting temperatures of duplexes and triplexes formed by PNA-related mimics with complementary DNA and RNA targets revealed that, similar to classic PNAs, the stability of complexes between nucleic acid targets and PNA relative mimics, particularly pHypNAs and hetero-oligomers composed of alternating PNA-pPNA or HypNA-pPNA residues, are not dependent on ionic strength. The decrease of a salt concentration has practically no effect on duplexes and triplexes formed by these mimics (Figure 3). Nevertheless, the hybridization properties of pure pPNA oligomers were similar to those of natural oligonucleotides, and the decreasing T_m values of pPNA/DNA(RNA) complexes was observed in low salt concentrations.[15,16]

Biomolecular Probes

Oligonucleotide hybridization technique is widely used in molecular biology studies including the detection of a target DNA and RNA and their sequencing. In particular, it becomes a very attractive technique for diagnostics and for discovery of novel therapeutic agents. Excellent hybridization properties of negatively charged PNA analogues stimulated the investigation on their effectiveness as biomolecular probes in diagnostic and molecular biology experiments.

Thus, the binding selectivity of these mimics was investigated. It was shown that the hybridization of the mimic oligomers with DNA and RNA targets occurred in a sequence-specific manner,[14,15] and the formation of complexes between mimic probes and non-complementary targets was not detected. Some results obtained in melting experiments are shown in Table 1. The introduction of one mismatch in the center of a 16-18-mer sequence gave a significant drop (17–23°C) in the melting temperature of a duplex depending on base mismatch position in the sequence. Oligomers with two separately situated mismatches were not able to form stable complexes with the targets. In general, PNA-pPNA and HypNA-pPNA chimeras showed in ~1.5 times better mismatch discrimination factors than natural oligonucleotides, and their characteristics were close to those for PNAs.[14]

Also, some mimics were tested as capture and detection probes for the construction of arrays for nucleic acid hybridization analysis, and their high

TABLE 1 Stability of Complexes Formed by HypNA-pPNA Oligomers and Natural Oligonucleotides Having Matched (or Mismatched) Sequences with DNA or RNA Targets[a]

Probe sequence[b]	Target[b]	Oligonucleotide		HypNA-pPNA	
		T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
TGGTCTCAAGTCAGTGT	d(CACTGACTTGAGACCA)	64		58	
TGGTCTCAAGTCAGTGT	d(CACTGAgTTGAGACCA)	57	7	41	17
TGGTCTCAAGTCAGTGT	d(CACTGAgTgGAGACCA)	47	17	<20	
TGGTCTCAAGTCAGTGT	d(CACTGACTTGAGtCCA)	54	10	47	12
TGGTCTCAAGTCAGTGT	d(CACTGACTTGAGACgA)	62	2	55	3
TGGTCTCAAGTCAGTGT	d(CACTGACaTGAGACCA)	53	11	43	16
CTGCAAAGGACACCATGA	d(TCATGGTGTCCTTTGCAG)	54		69	
CTGCAAAGcACACCATGA	d(TCATGGTGTCCTTTGCAG)	42	12	49	20
TTTTTTTTTTTTTTT	d(AAAAAAAAAAAAAAA)	36		81	
TTTTcTTTTTcTTTT	d(AAAAAAAAAAAAAAA)	<10	>26	24	57
TTTTTTTcTTTTTTT	d(AAAAAAAAAAAAAAA)	24	12	59	22
TGAGGCAGACATATTTCC	*ntl*-RNA			78	
TGAGGCAGgCATATTTCC	*ntl-RNA*			63	15
AACTGTCCTTATCCATCA	*uroD*-RNA			74	
AACTGaCCTTtTCCATCA	*uroD*-RNA			35	39
GCAGCCCCTCCATCATCC	*chordin*-RNA			88	
GCAGCCgCTCCtTCAT	*chordin*-RNA			69	19
CAAGTGTAGGGGTGCC	*dharma*-RNA			84	

[a]An oligomer was mixed with the equimolar amount of its DNA or RNA target in 10 mM Tris-HCl (pH 7.5)/0.5 M NaCl/10 mM $MgCl_2$ to create the corresponding complex. After the annealing at 90°C for 3 min, the mixture was slowly cooled to 10°C. The changes in absorbance at 260 nm versus temperature were measured with a heating rate 0.5°C/min from 10–95°C. Melting temperatures (T_m) were taken to be the temperature of half-dissociation and were obtained from a plot of the derivative of $1/T$ vs. absorbance at 260 nm.

[b]The probe sequence is shown from 5′- to 3′-end for oligonucleotides or from pseudo 5′ to pseudo 3′-end for mimics. Lowercase base in the sequence represents a mismatch position.

potential was demonstrated.[14,22,23] Thus, the examination of mimics as capture probes in solid-phase hybridization assays revealed that PNA-pPNA and HypNA-pPNA probes worked well in the same way but giving increased signal intensities compared to natural oligonucleotides due to their higher binding affinity. In solid-phase experiments, mimic capture probes showed better mismatch discrimination properties than oligonucleotide probes.[14] Also, these mimics were tested for the construction of soluble branched detection probes for signal amplification in sandwich hybridization assays.[14,22] Assay formats for sandwich hybridization consisted of a comb-type polyacrylamide-oligomer capture probe immobilized on a glass surface and a comb-type polyacrylamide-based detection-amplification probe. In general, this assay system with PNA-pPNA and HypNA-pPNA probes was capable to detect 1–5 amol of a target with the use of radioactively labeled amplifier probes that is suitable for a variety of biology testing applications. The other variant of the detection probe for sandwich hybridization was a mimic conjugate with the double-stranded DNA fragment of ~1000 bp-length obtained by PCR. This detection format showed the detection limit in the range of 1–100 amol.[23]

The application of negatively charged single-stranded PNA-relative mimics as capture probes in nucleic acids analysis is stimulated by their unique property do not interact with some intercalating dyes, particularly ethidium bromide, ethidium homodimer, homodimeric oxazole yellow (YOYO), and thiazole orange (TOTO) dyes. At the same time, the duplexes and triplexes of these mimics with captured DNA(RNA) targets exhibit a fluorescence under UV-light after the staining.[23] The application of a such visualization method in conjunction with these mimics is promising for the analysis of long nucleic acid targets, because it does not require a preliminary radioactive or fluorescent target labeling.

The selection of intact polyadenylated mRNA from cells and tissues is an essential step for many functional genomic applications, such as construction of cDNA libraries and microarrays, Northern and dot-blot analyses, and quantitative real-time PCR. So, further extending the range of negatively charged PNA-analogues application was connected with the development of highly efficient procedure for isolation of intact 3′ poly(A)-tailed mRNA from cells and tissues using homo-Thy affinity ligands.[24] Triplex forming homo-Thy pPNA, PNA-pPNA, and HypNA-pPNA oligomers of "linear" and "clamping" types conjugated with biotin residue were tested The clamping molecules represented *bis*-oligomers composed of two linear homo-Thy chains connected with a neutral flexible linker (Figure 4a). The biotin residue allowed the conjugation to a streptavidin-coated surface, particularly magnetic beads, to isolate mimic/mRNA complexes. It was demonstrated that mimic oligomers hybridized to poly-A mRNA tails with a high degree of specificity and give very low background binding of the probe

to unwanted rRNA. Exceptionally high affinity for DNA and RNA targets, good water solubility, and nuclease resistance of these mimics allowed one to improve the mRNA isolation procedure giving rise to a several-fold increase of mRNA yield compared with oligo-dT selected samples, and the application of mimic oligomers allowed for the isolation of a representative mRNA population including mRNA with short poly-A tracks.[24] It should be noted that the use of PNA-pPNA and HypNA-pPNA oligo-Thy probes enables efficient isolation of poly-A^+ mRNA from extracted total RNA samples in buffers with a low-salt concentration (Figure 4b), and this decreases the variety of non-polyadenylated RNA co-purification due to the enhanced destabilization of mRNA secondary structure in low salt concentration. Additionally, their advantages over natural oligonucleotides include reduced DNA contamination that is a consequence of possibility of DNase treatment during the mRNA preparation.

Among the technologies currently under the development for nucleic acids detection and quantification, one of the most promising is molecular beacons.[25] Classical molecular beacons are hairpin-shaped oligodeoxyribonucleotide probes, containing a fluorophore attached to one end and a quencher attached to the other end. These molecules consist of the loop portion representing a probe sequence complementary to the target and the stem formed by short complementary sequences. Beacons can report the presence of complementary nucleic acids targets without having to separate probe-target hybrids from the excess of a probe in hybridization assays. In the absence of a target, the fluorophore is held close to the quencher and fluorescence cannot occur. When the probe sequence hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, restoring fluorescence (Figure 5a). The experiments on the construction of novel types of chimeric molecular beacons including the beacons with PNA segments were reported.[26–28] Also, the synthesis of chimeric beacons composed of DNA, RNA, and phosphono-PNA mimic segments as well as fully composed of the HypNA-pPNA residues was accomplished.[16] The evaluation of properties of these compounds in model assays with the complementary and mismatched synthetic DNA and RNA targets was carried out. The results obtained on the effectiveness of the mimic chimeric beacons shown that these molecules were capable of detecting targets in solution starting from 1 pmol (Figure 5b). In comparison, the oligodeoxyribonucleotide beacons of the same sequence were in 10 times less sensitive in the same conditions, and negative controls containing mismatched or irrelevant molecules, did not generate a signal. Molecular beacons are widely used for in vitro RNA and DNA monitoring, in biosensor applications and for gene monitoring in living systems.[29] Thus, the rapid and sensitive detection of RNA in living cells using peptide-linked molecular beacons that possess self-delivery,

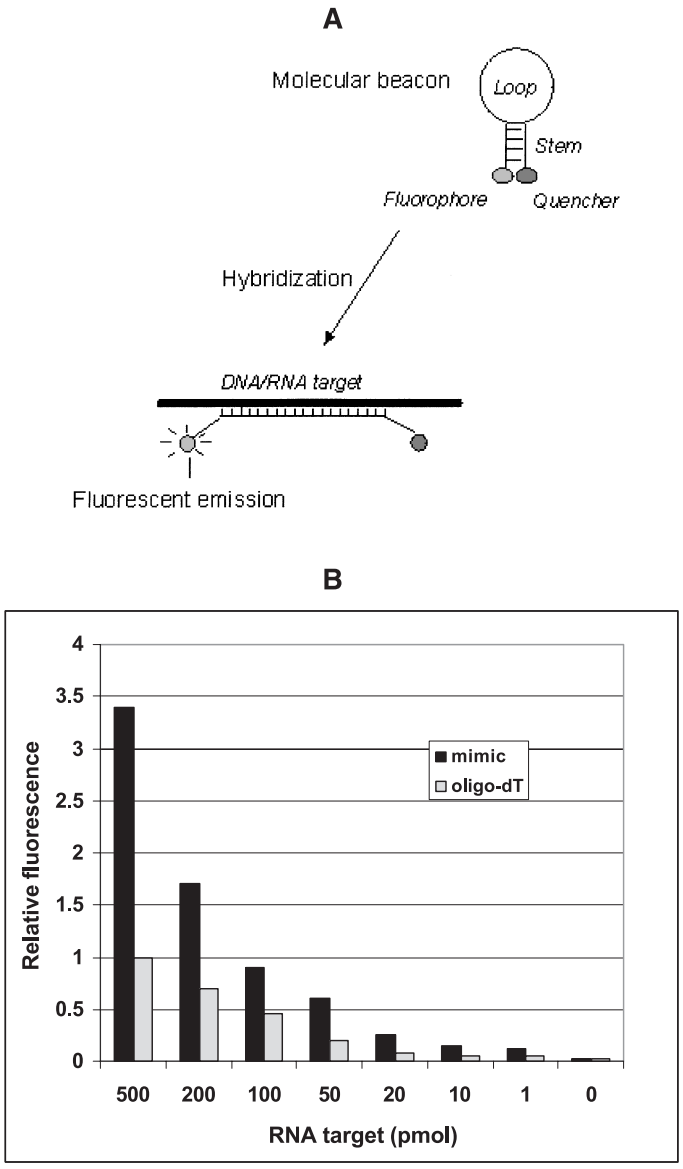


FIGURE 5 A: Fluorescent analysis of a target single-stranded RNA (DNA) using molecular beacons. **B:** The comparison of the effectiveness of molecular beacons in solution. The molecular beacon 5′-Flu-d(CAGAGC)-(ACACTTACACTTTTACAC)-d(GCTCTG)-Dab composed of DNA stem part and HypNA-pPNA loop part (underlined sequence) (beacon 1), or fully representing natural oligodeoxyribonucleotide (beacon 2) (500 pmol) was mixed with the decreasing amounts of the 18-mer RNA target complementary to the loop part of a beacon in 200 μL of 0.1 M NaCl/0.02 M Tris-HCl (pH 7.5)/ 0.01 M $MgCl_2$ at 50°C.

targeting, and reporting functions was demonstrated.[31,32] In this connection, the mimic beacons can be very useful for in vivo applications in view of higher biological stability of mimic oligomers in the comparison with natural oligonucleotides. Moreover, they show an improved discriminatory power and increased affinity for nucleic acid targets compared to DNA probes. Together with the insensitivity of PNA-relative beacons to the presence of salt and DNA-binding/processing proteins, their potential as robust tools for in vivo recognition of specific sequences can be estimated as very high. In the perspective, the investigations on the ability of negatively charged PNA-relative oligomers to detect and quantify the expression of specific endogenous mRNAs in living cells and tissues in real time can give rise to novel opportunities for biological and disease studies and can significantly impact drug discovery and medical diagnostic.

Delivery of Mimics into Living Cells

Among a new generation of DNA analogues and mimics, PNAs and their derivatives constitute very promising tools for antisense technology.[33] Classical PNAs allow specific gene targeting, they are resistant to nucleases and proteases, and bind RNA and DNA targets in a sequence-specific manner with high affinity. However, they are inefficient in vivo, which is, in part, the consequence of their poor propensity to cross cell membranes and/or to their inappropriate cellular localization. Moreover, their poor solubility and tendency to self-aggregation are important factors restricted their biological use.[33,34] Nevertheless, in the number of studies the improved delivery of PNAs into cells has been achieved by the use of carrier systems, such as their conjugates with cell-penetrating peptides and some other compounds.[33,35 36]

The experiments on the cell delivery of negatively charged PNA-related mimics, particularly PNA-pPNAs and HypNA-pPNAs, into bacterial and eukaryotic cells were also initiated. It was shown that free PNA-pPNA and HypNA-pPNA chimeric oligomers are able to penetrate into the living cells and distribute in the cytoplasm. In contrast to antisense oligonucleotides, which are effectively delivered to eukaryotic cells via cationic liposome complexation, the delivery of negatively charged mimics via this route did not gave the distinguished positive results.[16,36] It should be noted that HypNA-pPNA oligomers have no toxic effect on cells grows in the concentration up to 5–7 μM. For example, the density of *E. coli* cells in the samples treated with these oligomers was the same or even 10% higher than in control samples, which were not treated. Some results on the delivery of HypNA-pPNA oligomer into *HeLa* cells are shown in Figure 6.

Later, it was revealed that addition of a peptide having the sequence (K E T W F E T W F T E W S Q P K K K R K V) (non-covalent

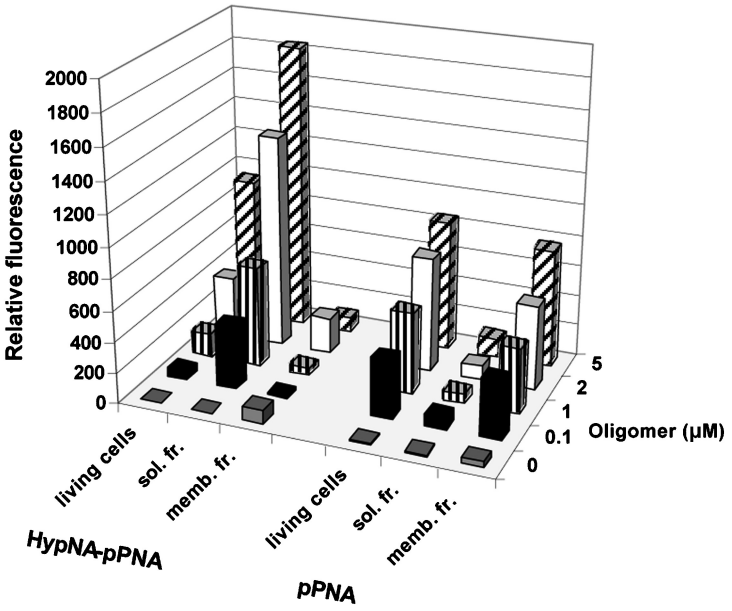


FIGURE 6 Delivery of a fluorescein labeled HypNA-pPNA and pPNA 20-mers into *HeLa* cells. Oligomer in 0.1–5 μM concentrations was incubated with the cells for 24 h at 37°C. Then, cells were washed, and fluorescence was measured in living cells as well as in the soluble and membrane fractions of cells homogenate. The average result of the four parallel experiments is shown.

peptide-based delivery system Pep-2) improves the delivery of negatively charged PNA analogues into living cells.[37] Thus, PNA and HypNA-pPNA oligomers as well as classical phosphorothioate oligonucleotide with the same 18-mer sequence (CATCGGGCTTGGAGGGAT), which specifically targeted first codons of the open reading frame of the cyclin B1 gene, were tested. Cultured *HS-68* or *HeLa* cells were overlaid with the preliminary prepared mixtures of fluorescein-labeled oligomers with a 25-fold molar excess of Pep-2 in the presence of serum and examined by confocal microscopy. Experiments were performed on both fixed and living cells. In the absence of a carrier system, mimic oligomers barely entered cells, whereas associated with Pep-2, they were rapidly delivered into more than 90% of cells and localized mainly in the cytoplasm with some staining in the nucleus. Maximal uptake was achieved in 2 h.[36]

Downregulation of Proteins

In the first experiments, the ability of mimic oligomers for green fluorescent protein (GFP) suppression in *E. coli* cells containing a vector super productive for GFP was tested using an antisense HypNA-pPNA 19-mer (ATCATGGTCATAGCTGTTT). As negative controls, the oligomers

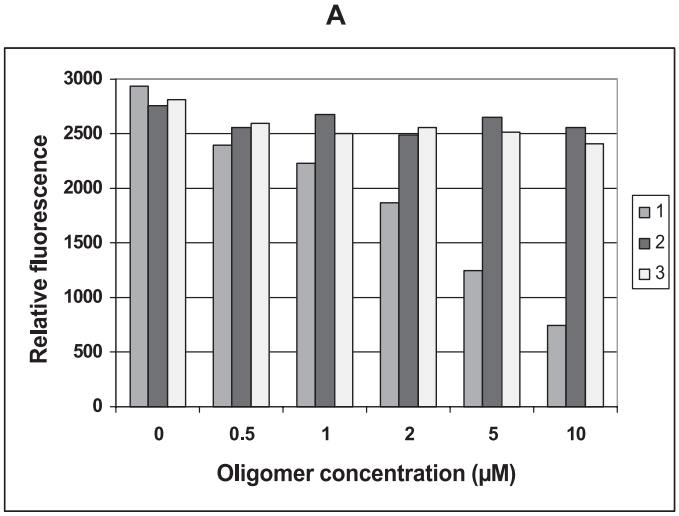
with scrambled and mismatched sequences were used. As it follows from Figure 7a, the antisense mimic oligomer was able to inhibit the protein production in three times, whereas the control oligomers had no effect on the GFP production.

Also, a HypNA-pPNA 19-mer (CTGCGATATTTTCTTGTAC) was tested in the suppression of AChE-R production in rat brain cell culture in the comparison with a phosphorothioate oligonucleotide of the same sequence. It was found that the HypNA-pPNA antisense oligomer specifically inhibited the production of AChE-R when supplement in the cell culture medium in 0.5 μM concentration and its effect was comparable with the effect of the control thio-oligonucleotide (Figure 7b).

Then, it was examined whether the levels of cyclin B1, a protein that is essential for cell cycle progression, were downregulated by the delivery of HypNA-pPNA 18-mer sequence (CATCGGGCTTGGAGGGAT), and to what extent, compared to classical PNAs and antisense phosphorothioate oligonucleotides with the same sequence.[37] The oligomers with scrambled (TAGGGAGGTTCGGGCTAC) and mismatched sequences (CATCaGGCTT aGAGGGAT) were used as the controls. The effect was analyzed in several cell lines, particularly *HeLa* cells, human fibroblasts (*HS 68*), and *293* cells. Cyclin B1 protein levels were significantly reduced with 50 nM antisense HypNA-pPNA and completely abolished with 100 nM HypNA-pPNA in the presence of Pep-2. In contrast, cyclin B1 protein levels were not affected by either 2 mM scrambled or mismatched HypNA-pPNA/Pep-2. Cationic lipid-mediated delivery of HypNA-pPNA was less efficient, and a 500-nM concentration of HypNA-pPNA oligomer was required to achieve significant reduction of cyclin B1 protein. In these experiments, a potent antisense effect of HypNA-pPNA oligomer was estimated after the quantification of cyclin B1 protein levels as in 8.5-fold and 25-fold higher than that of PNA oligomer and phosphorothioate oligonucleotide, respectively.[35]

It was also revealed that the antisense HypNA-pPNA oligomer designed to target cyclin B1 not only induced a dramatic decrease in protein levels, but also affects cyclin B1 mRNA.[35] In contrast to natural oligonucleotides and their analogues, PNAs and their derivatives do not able to activate RNase-H upon binding to RNA, so their ability to affect mRNAs is more likely to be directly associated with steric hindrances. Nevertheless, the mechanism through which these compounds contribute to downregulation of gene expression is unknown, and further studies will be necessary for its elucidation.

Finally, it was shown that antisense cyclin B1 HypNA-pPNA oligomer is able to block cancer cell proliferation.[37] In the presence of Pep-2, low concentrations (0.5 and 1 μM) of HypNA-pPNA consistently reduced proliferation of the human breast cancer cells MCF-7 by 70 and 92%, respectively. For comparison, classical PNA oligomer affected cell proliferation only by



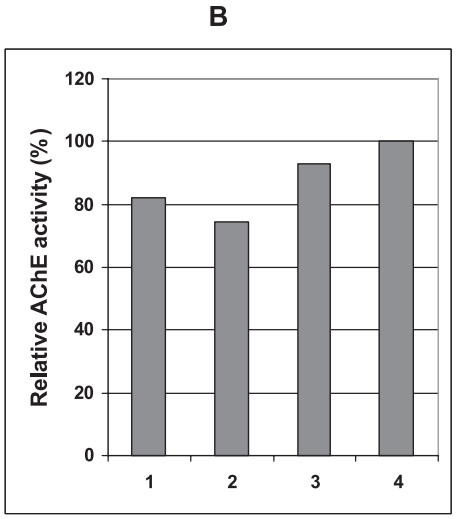


FIGURE 7 A: Inhibition of green fluorescent protein (GFP) production in bacterial cells by a 19-mer HypNA-pPNA oligomer (ATCATGGTCATAGCTGTTT). (1) Competent DH5α/pHAT-GFPuv cells (100 μL) were treated with the oligomer in a 1–10 μM concentration for 2 h at 37°C. Then, the Amp-containing (50 mg/Ll) growth medium (900 μL) was added, and the cell culture was incubated at 37°C. The fluorescence was measured after 18 h. The cells with the addition of HypNA-pPNAs with scrambled (ACTTACACTTACACTTACC) (2) and mismatched (ATCATGcTCATtGCTGTTT) (3) sequences were used as the controls. The average result of the three parallel experiments is shown. **B:** Suppression of AChE-R production in rat brain cell culture. The cells in the growth medium supplemented with 10% FCG and 2 mM L-Glu were treated for 24 h at 37°C with a solution of 19-mer HypNA-pPNA oligomer (CTGCGATATTTTCTTGTAC) (1) solution to obtain the final concentration of 0.5 μM. As controls, the cells treated with phosphorothioate oligonucleotide with the same sequence (2) cells treated with HypNA-pPNA oligomer with a scrambled sequence (ACTTACACTTACACTTACC) (3) and cells without the treatment by oligomers (4) were analyzed. The AchE activity assays were performed in triplicates as described.[30] The average data on the relative AChE activity of three parallel analysis is shown.

TABLE 2 Comparison of the Inhibition of Cancer Cell Proliferation by Cyclin B1 Antisense PNA and HypNA-pPNA Oligomers (CATCGGGCTTGGAGGGAT)[a]

Oligomer (μM)	Proliferation (%)			
	HypNA-pPNA	HypNA-pPNA/Pep-2	mHypNA-pPNA/Pep-2[b]	PNA/Pep-2
0.5	100	30	97	95
1	98	8	90	65
2	69	5	86	36

[a]Exponentially growing MCF-7 cells were incubated with increasing concentrations of oligomers in the presence or in the absence of Pep-2. Cells were counted after 8 days of incubation. The values represent the average of four separate experiments. The values are given relative to the data in control experiments obtained without the addition of oligomers.
[b]Oligomer with mismatched sequence (CATCaGGCTTaGAGGGAT).

35% for 1 μM and by 64% for 2 μM in the same conditions. Without the addition of Pep-2, free HypNA-pPNA oligomer also exhibited a slight decrease in cell proliferation at 2 μM concentrations (Table 2).

In two recent reports, HypNA-pPNA oligomers were successfully used for the investigation of the mechanism of Mallory body (MB) formation in the liver cells of chronic liver diseases.[38,39] Gene-specific mimic antisense oligomers designed to inhibit the expression of p62 (a scaffolding protein that binds to polyubiquitin) and valosin-containing protein (VCP) were added to the medium of the primary mouse cell cultures. Chariot II was used as vehicle for transfecting the oligomers into the cells. The transfection efficiency was nearly 100%. The results obtained with HypNA-pPNAs indicate that p62 is involved in the mechanism of MB formation,[38] while VCP is located in MBs in mouse and human livers and plays an important role in inducing MB formation.[39]

Targeted Gene Knockdown in vivo

Last years, DNA mimics, particularly MOs, were successfully utilized in the model vertebrate zebrafish (*Danio rerio*) for genome-wide, sequence-based, reverse genetic screens during embryonic development.[40] Recently, negatively charged PNA analogues were evaluated as an alternative to MOs for oligonucleotide inhibition of gene expression in zebrafish embryos, and it was shown that HypNA-pPNA chimeric oligomers, similarly to MOs, are effective and specific in vivo translational inhibitors in zebrafish.[20,21,41] Thus, gene knockdown experiments targeting the zebrafish *Egfl7* were performed using two different sets of antisense oligomers, which represent both MOs and HypNA-pPNAs. One set (AS_{-47}) hybridized to the 5′-untranslated region (UTR) and blocks translation, and the other (AS_{195}) hybridized with an exon-intron junction, resulting in intron retention and hence premature translation termination. Both types of oligomers gave

identical results, and their microinjections in the amount of 4 ng per embryo caused specific vascular defects with minimal non-specific effects.[41]

Moreover, in zebrafish injection experiments, antisense HypNA-pPNAs produced strong loss-of-function phenotypes for four targeted genes (*chordin*, *notail*, *uroD*, and *dharma*), which encode a variety of different products (two transcription factors, a secreted growth factor antagonist, and a biosynthetic enzyme).[20,21] In all cases, injection of HypNA–pPNAs caused null or near-null phenotypes in 96–99% of the embryos (Figure 8). These genes act over stages ranging from the blastula through the pharyngula, and *uroD* gene is first expressed at 30 hpf. Also, zebrafish embryos were injected with MO and HypNApPNA antisense oligomers against a tumor suppressor gene, whose mechanism of action remains unclear. First pass bioinformat-

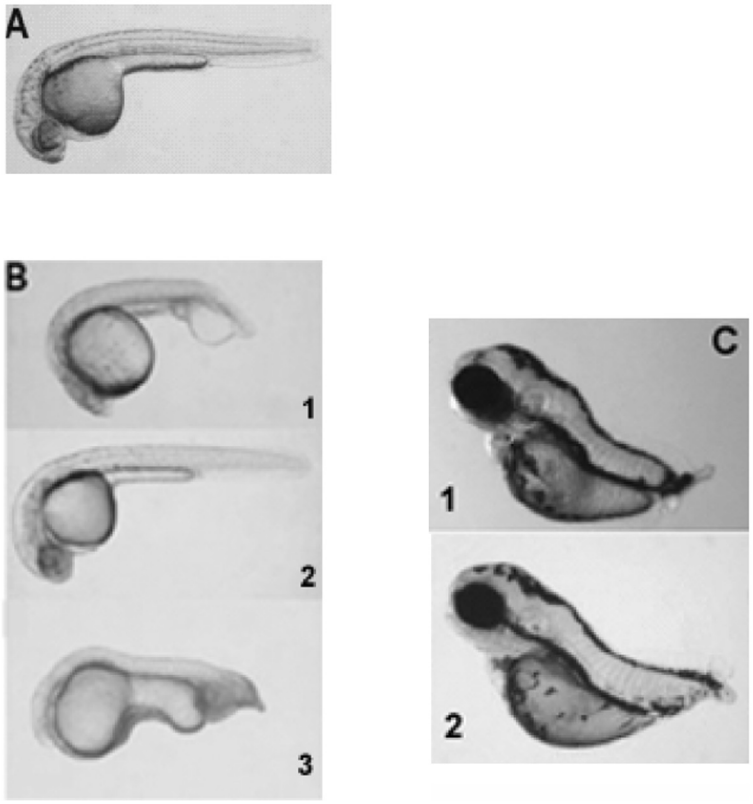


FIGURE 8 Inhibition of gene expression in zebrafish embryos by antisense HypNA-pPNA oligomers. **A:** Uninjected embryos displayed wild-type morphology at 24 h postfertilization. **B:** 1. Embryos injected with *chordin* HypNA-pPNA oligomer phenocopy the null *chordin*$^{-/-}$ mutation. 2. Embryos injected with *chordin* HypNA-pPNA oligomer having 2-base mismatches appear wild-type. 3. Phenotype of *chordin* null$^{-/-}$ mutant. **C:** 1. Phenotype of *notail* null (*ntl*$^{-/-}$) mutant. 2. *notail* null (*ntl*$^{-/-}$) mutant was phenocopied by the injection of HypNA-pPNA oligomer targeted *notail* 5′ untranslated region sequence (78-95). Embryos were observed at 72 h postfertilization.

ics analysis of the transcription profiles from RNA samples extracted from treated and control embryos identified up- and downregulated genes, of which one clear example was p53. Both MOs and HypNA-pPNAs against a tumor suppressor gene induced comparable upregulation of p53, illustrating similar effects on transcription profiles.[42] The mimics were also used for the investigation of lipid methabolism and the development of vertebrate central nervous system in zebrafish.[43,44] It was found that HypNA-pPNA-based transient gene silencing is feasible in zebrafish embryos and provides a valuable reverse genetic screening strategy.

The results obtained clearly show that HypNA-pPNAs are stable in vivo and suggest that, similar to MOs, these compounds can act as effective antisense inhibitors over a range of developmental stages. Moreover, it was observed that HypNA-pPNA 18-mers displayed comparable potency to MO 25-mers as gene knockdown agents with greater mismatch stringency than MOs. Thus, the evaluation of the action of chordin HypNA-pPNAs and MOs containing 2 and 4 mismatches revealed that mismatched HypNA-pPNA displayed no activity, while the MOs with 2 mismatches induced frequent *chordin*$^{-/-}$ phenotypes, and the MOs with 4 mismatches induced a nonspecific phenotype. Also, the evidence for sequence-specificity of HypNA-pPNAs action derived from the analysis of *ntl* gene (Figure 8). A single-base mismatch in the *ntl* HypNA-pPNA oligomer induced the *ntl*$^{-/-}$ phenotype in only 30% of the injected larvae, while the fully complementary HypNA-pPNA induced the *ntl*$^{-/-}$ phenotype in virtually all embryos injected. In contrast, a 25-mer MO with the same single-base mismatch produced the *ntl* null phenotype in 98% of injected embryos. These results demonstrate that 18-mer HypNA-pPNA oligomers display higher sequence specificity in comparison to MO 25-mers.[20,21]

After finding that HypNA-pPNAs can effectively knockdown early acting genes, the determination of later acting genes targeting was carried out. For this test, *uroD*, which encodes uroporphyrinogen decarboxylase, was selected. Similarly to other genes, the specificity was observed for the action of *uroD* oligomers, and an injection of a HypNA-pPNA oligomer with two mismatches in the sequence gave a strong phenotype in less than 30% of embryos in comparison to 100% for the perfect match HypNA-pPNA oligomer injection.[20,21]

Another key advantage of HypNA-pPNA for gene knockdown studies is the lack of nonspecific effects. For all four genes that were targeted, doses of HypNA-pPNA, which are high enough to produce strong loss-of-function phenotypes, produced few or no non-specific effects. The lack of non-specific effects was especially evident in the case of the HypNA-pPNA 18-mer targeted *dharma* gene. It elicited the *dharma* (*bozozok*)$^{-/-}$ phenotype in zebrafish embryos in contrast to analogous MO 25-mer. The latter produced a weak phenocopy at low doses and a severe non-specific

effect, widespread degeneration, at high doses. Injection of HypNA-pPNA 18-mer produced a specific *dharma* mutant phenocopy with very little cell death and an extremely strong loss-of-function phenotype in up to 84% of the injected embryos.[20,21] It is evident that further studies are required to determine whether the lack of non-specific effects results from the relatively short sequences that can be used effectively in HypNA-pPNAs in contrast to relatively long MOs, or from an inherent advantage of the HypNA-pPNAs structure relative to MOs. In any case, these results demonstrated that HypNA-pPNA antisense reagents can effectively and specifically target sequences that lead to non-specific effects with MO and indicate that HypNA-pPNAs can act as specific antisense inhibitors of gene function in zebrafish embryos and might be of use in other organisms.

CONCLUSIONS

The analysis of the properties of PNA-relative mimics bearing negative charges allows to conclude that these DNA mimics are very promising tools for the application as specific probes for nucleic acid detection, isolation, and analysis as well as potential anti-sense and anti-gene therapeutics. These compounds combine high hybridization and discrimination characteristics with good water solubility and biological stability as well as the ability to penetrate cell membranes. Their antisense effect lasts over a period of several days, due to the high stability of the chimera, and this represents a very potent technology for administering antisense-based drugs in the context of therapeutic applications. The results obtained in gene knockdown experiments in vivo validate HypNA-pPNAs as an alternative to MO oligomers for reverse genetic studies. The stronger hybridization and greater specificity of HypNA-pPNAs enable knockdown of mRNAs unaffected by MO oligomers. Moreover, the ability of these mimics efficiently blocks cancer cell proliferation at very low concentrations suggests that these compounds are good candidates for the development of a potential anticancer drug. In general, the examination of the properties of PNA-related mimics clearly demonstrated their high potential for the use as biomolecular probes and antisense reagents.

REFERENCES

1. Nielsen, P.; Egholm, M.; Berg, R.; Buchardt, O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science **1991**, 254, 1497–1500.
2. Nielsen, P.; Egholm, M. (Eds.). *Peptide Nucleic Acids, Protocols and Applications*. Horizon Scientific Press: Norfolk, England, 1999.
3. Summerton, J.; Weller, D. Morpholino antisense oligomers: Design, preparation, and properties. Antisense Nucleic Acid Drug Dev. **1997**, 7, 187–195.
4. Weiler, J.; Gausephohl. H.; Hauser, N.; Jensen, O.; Hoheisel, J. Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays. Nucleic Acids Res. **1997**, 25, 2792–2799.

5. Bergman, F.; Bannwarth, W.; Tam, S. Solid phase synthesis of directly linked PNA-DNA-hybrids. Tetrahedron Lett. **1995**, 36, 6823–6826.
6. Stein, D.; Foster, E.; Huang, S.; Weller, D.; Summerton, J. A specificity comparison of four antisense types: Morpholino, 2′-*O*-methyl RNA, DNA, and phosphorothioate DNA. Antisense Nucleic Acid Drug Dev. **1997**, 7, 151–157.
7. Summerton, J.; Stein, D.; Huang, S.; Matthews, P.; Weller. D; Partridge, M. Morpholino and phosphorothioate antisense oligomers compared in cellfree and in-cell systems. Antisense Nucleic Acid Drug Dev. **1997**, 7, 63–70.
8. Van der Laan, A.; Stromberg, R.; van Boom, J.; Kuyl-Yeheskiely, E.; Efimov, V.; Chakhmakhcheva, O. An approach towards the synthesis of oligomers containing a N-2-hydroxyethyl-aminomethyl phosphonate backbone: A novel PNA analogue. Tetrahedron Lett. **1996**, 37, 7857–7860.
9. Peyman, A.; Uhlmann, E.; Wagner, K.; Augustin, S.; Breipohl, G.; Will, D.; Schafer, A.; Wallmeier, H. Phosphonic ester nucleic acids (PHONAs): Oligonucleotide analogues with an achiral phosphonic acid ester backbone. Angew. Chem. Int. Ed. Engl. **1996**, 35, 2636–2638.
10. Kehler, J.; Henriksen, U.; Vejbjerg, H.; Dahl, O. Synthesis and hybridization properties of anacyclic achiral phosphonate DNA analogue. Bioorg. Med. Chem. **1998**, 6, 315–322.
11. Efimov, V.; Choob, M.; Buryakova, A.; Kalinkina, A.; Chakhmakhcheva, O. Synthesis and evaluation of some properties of chimeric oligomers containing PNA and phosphono-PNA residues. Nucleic Acids Res. **1998**, 26, 566–575.
12. Peyman, A.; Uhlmann, E.; Wagner, K.; Augustin, S.; Weiser, C.; Will, D.; Breipohl, G. PHONA-PNA co-oligomers: Nucleic acid mimetics with interesting properties. Angew. Chem. Int. Ed. Engl. **1997**, 36, 2809–2812.
13. Efimov, A.; Buryakova, A.; Choob, M.; Chakhmakhcheva, O. Phosphonate analogues of peptide nucleic acids and related compounds: Synthesis and hybridization properties. Nucleosides & Nucleotides **1999**, 18, 1393–1396.
14. Efimov, V.; Buryakova, A.; Chakhmakhcheva, O. Synthesis of polyacrylamides N-substituted with PNA-like oligonucleotide mimics for molecular diagnostic applications. Nucleic Acids Res. **1999**, 27, 4416–4426.
15. Efimov, V.; Chakhmakhcheva, O. Phosphono-PNAs: Synthesis, properties and applications. Collection Symp. Series **2002**, 5, 136–144.
16. Efimov, V.; Klykov, V.; Chakhmakhcheva, O. Phosphono peptide nucleic acids with a constrained hydroxyproline-based backbone. Nucleosides, Nucleotides & Nucleic Acids **2003**, 22, 593–599.
17. Efimov, V.; Buryakova, A.; Choob, M.; Chakhmakhcheva, O. Peptide nucleic acids and their phosphonate analogues. II. Synthesis and physico-chemical properties of hybrids containing serine and 4-hydroxyproline residues. Bioorgan. Khim. **1999**, 25, 611–622.
18. Efimov, V.; Chakhmakhcheva, O. Synthesis of DNA mimics representing HypNA-pPNA hetero-oligomers. Meth. Mol. Biol. **2004**, 288, 147–163.
19. Verheijen, J.; van Roon, A.-M.; van der Laan, A.; van der Marel, G.; van Boom, J. Synthesis of DNA-(3′)-PNA chimeras with conformationally restricted linkers based on 4-hydroxyproline. Nucleosides & Nucleotides **1999**, 18, 493–508.
20. Urtishak, K.; Choob, M.; Tian, X.; Sternheim, N.; Talbot, W.; Wickstrom, E.; Farber, S. Targeted gene knockdown in zebrafish using negatively charged peptide nucleic acid mimics. Developmental Dynamics **2003**, 228, 405–413.
21. Wickstrom, E.; Choob, M.; Urtishak, K.; Tian, X.; Sternheim, N.; Talbot, S.; Archdeacon, J.; Efimov, V.; Farber, S. Sequence specificity of alternating hydroxyprolyl/phosphono peptide nucleic acids against zebrafish embryo mRNAs. Journal of Drug Targeting **2004**, 12, 363–372.
22. Efimov, V.; Choob, M.; Buryakova, A.; Phelan, D.; Chakhmakhcheva, O. PNA-related oligonucleotide mimics and their evaluation for nucleic acid hybridization studies and analysis. Nucleosides, Nucleotides & Nucleic Acids **2000**, 20, 419–428.
23. Efimov, V.; Chakhmakhcheva, O. Solid phase synthesis of PNA-like oligonucleotide mimics and their use for polyacrylamide-based molecular diagnostic assays. In *Solid Phase Synthesis and Combinatorial Libraries*; Empton, R., Ed.; Mayflower Worldwide: Kingswinford, UK, 2001; 75–78.
24. Phelan, D.; Hondorp, K.; Choob, M.; Efimov, V.; Fernandez, J. Messenger RNA isolation using novel PNA analogues. Nucleosides, Nucleotides & Nucleic Acids **2001**, 20, 1107–1111.
25. Tyagi, S.; Kramer, F. Molecular beacons: Probes that fluoresce upon hybridization. Nature Biotechnology **1996**, 14, 303–308.

26. Efimov, V.; Chakhmakhcheva, O.; Choob, M.; Archdeacon, D. Using chimeric DNA/RNA molecular beacons for target-specific signal amplification. Collection Symp. Series **2002**, 5, 308–311.
27. Kuhn, H.; Demidov, V.; Coull, J.; Fiandaca M.; Gildea, B.; Frank-Kamenetskii; M. Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. J. Am. Chem. Soc. **2002**, 124, 1097–1103.
28. Ortiz, E.; Estrada, G.; Lizardi, P. PNA molecular beacons for rapid detection of PCR amplicons. Molecular and Cellular Probes **1998**, 12, 219–226.
29. Tan, V.; Wang, K.; Drake, J. Molecular beacons. Current Opinion in Chemical Biology **2004**, 8, 547–553.
30. Birikh, K.; Sklan, E.; Shoham, S.; Soreq, H. Interaction of "readthrough" acetylcholinesterase with RACK1 and PKSβII correlates with intensified fear-induced conflict behavior. Proc. Natl. Acad. Sci. USA **2003**, 100, 283–288.
31. Nitin, N.; Santangelo, P.; Kim, G.; Nie, S.; Bao, G. Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells. Nucleic Acids Res. **2004**, 32, e58.
32. Bratu, D.; Cha, B.-J.; Mhlanga, M.; Kramer, F.; Tyagi, S. Visualizing the distribution and transport of mRNAs in living cells. Proc. Natl. Acad. Sci. USA **2003**, 100, 13308–13313.
33. Shiraishi, T.; Nielsen, P. Down-regulation of MDM2 and activation of p53 in human cancer cells by antisense 9-aminoacridine-PNA (peptide nucleic acid) conjugates. Nucleic Acids Res. **2004**, 32, 4893–4902.
34. Hyrup, B.; Nielsen, P. Peptide nucleic acids (PNA): Synthesis, properties and potential applications. Bioorg. Med. Chem. **1996**, 4, 5–23.
35. Cutrona, G.; Carpaneto, E.; Ulivi, M.; Roncella, S.; Landt, O.; Ferrarini, M.; Boffa, L. Effects in live cells of a c-myc anti-gene PNA linked to a nuclear localization signal. Nat. Biotechnol. **2000**, 18, 300–303.
36. Koppelhus, U.; Nielsen, P. Cellular delivery of peptide nucleic acid. Adv. Drug Delivery Rev. **2003**, 55, 267–280.
37. Morris, M.; Chaloin, L.; Choob, M.; Archdeacon, J.; Heitz, F.; Divita, G. Combination of a new generation of PNAs with a peptide-based carrier enables efficient targeting of cell cycle progression. Gene Therapy **2004**, 11, 757–764.
38. Nan, L.; Wu, Y.; Bardag-Gorce, F.; Li, J.; French, B.A.; Fu, A.N.; Francis, T.; Vu, J.; French S.W. p62 is involved in the mechanism of Mallory body formation. Exp. Mol. Pathol. **2004**, 77, 168–175.
39. Nan, L.; Wu, Y.; Bardag-Gorce, F.; Li, J.; French, B.A.; Wilson, L.T.; Nguyen, S.K.; French S.W. RNA interference of VCP/p97 increases Mallory body formation. Exp. Mol. Pathol. **2005**, 78, 1–9.
40. Nasevicius, A.; Ekker, S. Effective targeted gene "knockdown" in zebrafish. Nature Genetics **2000**, 26, 216–220.
41. Parker, L.; Schmidt, M.; Jin, S.-W.; Gray, A.; Beis, D.; Pham, T.; Frantz, G.; Palmieri, S.; Hillan, K.; Stainier, Y.; de Sauvage, F.; Ye, W. The endothelial-cell-derived secreted factor *Egfl7* regulates vascular tube formation. Nature **2004**, 428, 754–758.
42. Wickstrom, E.; Urtishak, K.A.; Choob, M.; Tian, X.; Sternheim, N.; Cross, L.M.; Rubinstein, A.; Farber, S.A. Downregulation of gene expression with negatively charged peptide nucleic acids (PNAs) in zebrafish embryos. Methods Cell Biol. **2004**, 77, 137–158.
43. Ho, S.Y.; Thorpe, J.L.; Deng, Y.; Santana, E.; DeRose, R.A.; Farber, S.A. Lipid methabolism in zebrafish. Methods Cell Biol. **2004**, 76, 87–108.
44. Ninkovic, J.; Tallafuss, A.; Leucht, C.; Topczewski, J.; Tannhauser, B.; Solnica-Krezel, L.; Bally-Cuif, L. Inhibition of neurogenesis at the zebrafish midbrain-hindbrain boundary by the combined and dose-dependent activity of a new hairylE(spl) gene pair. Development **2005**, 132, 75–88.