HYDROXYPROLINE-BASED DNA MIMICS: A REVIEW ON SYNTHESIS AND PROPERTIES

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This paper is dedicated to Professor Antonín Holý on the occasion of his 70th birthday.

With the aim to improve physicochemical and biological properties of natural oligonucleotides, many types of DNA analogues and mimics are designed on the basis of hydroxyproline and its derivatives, and their properties are evaluated. Among them, two types of DNA mimics representing hetero-oligomers constructed from alternating monomers of phosphono peptide nucleic acids and monomers on the base of *trans*-1-acetyl-4-hydroxy-L-proline (HypNA-pPNAs) and oligomers constructed from monomers containing (2*S*,4*R*)-1-acetyl-4-hydroxypyrrolidine-2-phosphonic acid backbone (pHypNAs) are of particular interest. In a set of *in vitro* and *in vivo* assays, it was shown that HypNA-pPNAs and pHypNAs demonstrated a high potential for the use in nucleic acid based diagnostics, isolation of nucleic acids and antisense experiments. A review with 53 references.

Keywords: PNA; DNA analogues; Phosphono-hydroxyproline; Hybridization; Inhibition of gene expression; Antisense oligonucleotides; Pyrrolidines; Backbone modified oligonucleotides.

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1. INTRODUCTION

In the last years, the search for modified oligonucleotides and analogues with improved hybridization ability and selectivity toward nucleic acid targets attracted increased attention in connection with their application in molecular biology and medicine. A number of nucleic acid mimics have been developed, including successful examples such as charge-neutral peptide nucleic acids (PNAs)[1] and phosphorodiamidate morpholino oligomers (MOs)[2]. These oligonucleotide mimics are unaffected by cellular degradative enzymes, especially by nucleases, and show strong nucleic acids binding. Thus, PNAs demonstrated high affinity to DNA and RNA and stringent mismatch discrimination. However, biological application of classical PNAs *in vivo* is restricted by their low water solubility, tendency to self-aggregation, poor propensity to cross cell membranes and, in part, their inappropriate cellular localization[3–5]. In spite of these facts, the improved delivery of PNAs into cells can be achieved by the use of carrier systems, such as their conjugates with cell-penetrating peptides and some other compounds as well as by the addition of special reagents to the culture medium[5,6]. MOs exhibit high solubility in water despite their lack of charge, due to strong polarity, but they show lower affinity to nucleic acids and mismatch discrimination ability than PNAs [7,8].

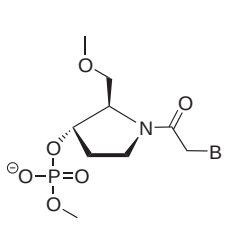
During the past decade, our efforts have been devoted to the design and synthesis of DNA analogues and mimics inducing increased binding affinity to nucleic acids for the use as diagnostic probes and antisense agents. With this aim in view, we obtained PNA-like negatively charged DNA mimics, which contain monomer units on the basis of hydroxyproline connected with phosphonate ester bonds[9–13].

In this work, we review recent developments in the synthesis of hydroxyproline-based DNA analogues and mimics and the progress in the investigation of their physicochemical and biological properties. The potential of the hydroxyproline-based negatively charged PNA analogues as powerful tools for application in molecular biology, particularly in the study of gene expression, is evaluated.

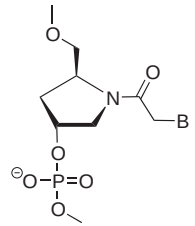
2. PYRROLIDINE-BASED OLIGONUCLEOTIDE ANALOGUES

Some time ago, several research groups were interested in hydroxy-*N*-acetylprolinol derivatives as a sugar substitute in oligonucleotides. Thus, modified oligonucleotides incorporating *trans*-4-hydroxy-*N*-acetyl-L-prolinol (*trans*-4-OH-L-NAP-NA), or its D-analogue, as sugar substitute were synthesized from the corresponding phosphoramidite monomers containing ade-

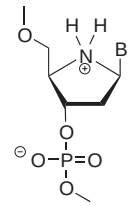
nine (Ade) and thymine (Thy) as nucleobases[14] (Fig. 1). This type of compounds represented an analogue of 2′-5′-connected 3′-deoxyribooligonucleotide with the same number of atoms between their repeating units, and this distance was not respected to the 6-atom spacing found in natural nucleic acids. It was shown that these oligomers display interesting hybridization features with complementary fragments of natural nucleic acids, which preferably hybridize with *trans*-L-hydroxyprolinol-containing oligomers over the oligomers of *trans*-D type. In an attempt to further improve the hybridization efficiency of the prolinol-based oligomers, 3-hydroxy-*N*-acetylprolinol derived nucleotide analogues (*trans*-3-OH-L-NAP-NA) were prepared, where phosphorus spacing was as in DNA molecule[15]. Fully modified oligomers were synthesized from the O-phosphoramidites of the properly protected *trans*- and *cis*-3-hydroxy-*N*-acetylprolinol monomers. The L-*trans* as the D-*trans* homoadenine oligomers of this type were capable of



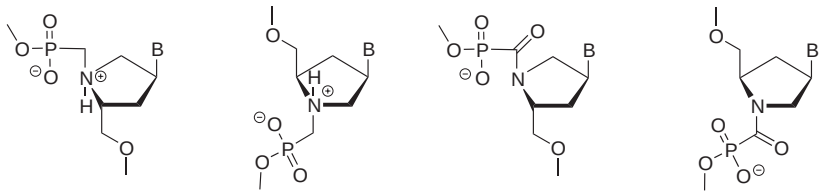
trans-3-HO-L-NAP-NA



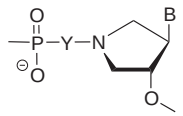
trans-4-HO-L-NAP-NA



pyrro-C-NA



pro-N-NA



Y = CH_2, CH_2CH_2, CH_2CO

pyrro-N-NA

FIG. 1
Pyrrolidine-based analogues of nucleic acids

hybridization with complementary DNA and RNA, whereas no complexation was observed between homothymine oligomers (or the oligomers with the mixed Ade, Thy sequence) and complementary nucleic acid targets. In general, the stability of complexes formed by these oligonucleotide analogues were considerably lower than those detected for 4-hydroxy-*N*-acetylprolinol-based structures.

Starting from *trans*-3-hydroxy-L-proline, the synthesis of the pyrrolidino-*C*-nucleotide analogues (*pyrro*-*C*-NA) was accomplished to obtain oligomers bearing positive charges in the backbone[16,17] (Fig. 1). The synthesis of these oligomers was performed by the standard phosphoramidite method, and the thermal stability of complexes formed by the modified oligomers with complementary targets was measured. It was shown that the stability of these complexes was in general lower than those of the duplexes formed by unmodified oligonucleotides.

Recently, a series of prolinol-based modified monomers with phosphonomethyl or phosphonoformyl group attached to the pyrrolidine nitrogen (*pro*-N-NA) was prepared for the use in solid phase synthesis of oligonucleotide mimics[18] (Fig. 1). In these monomers, the 3′- or 4′-carbon atom of ribose was replaced with the nitrogen atom of pyrrolidine, and the oxygen atom of ribose was replaced with methylene group. These protected nucleotide analogues give rise to two types of internucleotide linkages when incorporated into natural oligonucleotides: N–CH_2–P–O–C and N–CO–P–O–C. Each individual pair consisted of two stereoisomers capable of forming the same type of the isosteric internucleotide linkages. Also, several types of nucleoside analogues representing phosphonate derivatives of 3-pyrrolidinol (*pyrro*-N-NA) were obtained and incorporated into oligonucleotide chains. The evaluation of thermal stability of their duplexes with complementary natural oligonucleotides revealed that, in general, oligonucleotides modified with such units exhibited lower affinity to complementary nucleic acids than natural ones[19].

3. CONFORMATIONALLY CONSTRAINED ANALOGUES OF PEPTIDE NUCLEIC ACIDS

In the last years, the efforts of several groups were directed towards improvement of the physicochemical and biological properties of classical PNAs, particularly water solubility, cellular uptake and discrimination ability between DNA and RNA as binding targets. To achieve a success in the latter aim, chirality can be introduced into the PNA molecule, and one of the approaches includes bridging in the backbone to obtain the five-

member cyclic structures, which may contribute towards maintaining the balance between rigidity and flexibility of the PNA molecule. Easily available hydroxyproline is a suitable starting material for the synthesis of conformationally constrained PNA analogues containing a methylene bridge that joins either the aminoethyl-glycyl backbone or the methylene-carbonyl side chain in the molecule. The results obtained in the design, synthesis and evaluation of hybridization properties of different conformationally constrained PNA analogues were recently reviewed[20,21]. A set of chiral PNA analogues was synthesized, and their hybridization properties were investigated. Thus, positive results have been obtained with 4-aminoprolyl-PNA (*apro*PNA) (Fig. 2), which contained a methylene bridge between the β-carbon atom of the aminoethyl segment and the α-carbon atom of the glycyl segment[22]. Although, homo-oligomers prepared from this monomer do not show binding the target DNA or RNA fragments, the introduction of D-*trans* or L-*trans apro*PNA units into the chain of classical PNA oligomer imparts structural pre-organization. The chimeric oligomers with alternating PNA and *ap*-PNA units exhibit high hybridization characteristics together with selectivity upon binding complementary targets[23]. Some success has been obtained with pyrrolidine PNAs (Fig. 2). The (2*R*,4*S*) *pyrro*PNA monomers were obtained from *cis*-4-hydroxy-D-proline, and the homo-Ade oligomer, synthesized from monomers of this type was shown to form stable complexes with both DNA and RNA targets[24]. Also, it was shown that the homo-Thy oligomer, obtained from (2*R*,4*R*) version of monomers of the same type, which were synthesized starting from *trans*-4-hydroxy-L-proline, bound target DNA and RNA with high affinity[25].

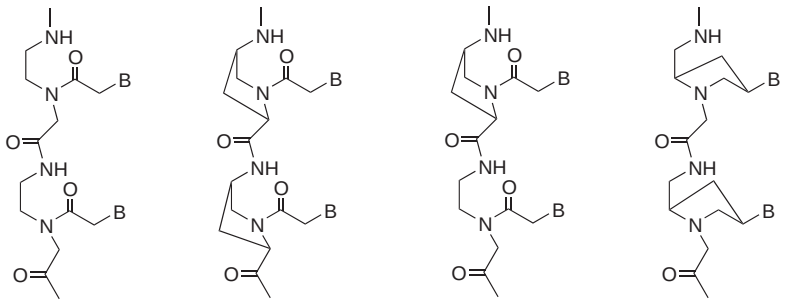


FIG. 2
Conformationally constrained PNA analogues derived from hydroxyproline

It should be noted that several other types of hydroxyproline-based PNA analogues were designed, and these synthetic efforts added several new structures to the growing libraries of pyrrolidine compounds, which may have utility not only in antisense experiments, but in other research areas[20,21]. The next logical step should be the investigation of biological properties of the most successful modifications including cellular uptake, toxicity and *in vitro* and *in vivo* antisense activily.

4. NEGATIVELY CHARGED ANALOGUES OF PEPTIDE NUCLEIC ACIDS: PHOSPHONO-PNAs AND THEIR HETERO-OLIGOMER DERIVATIVES

The historically first type of negatively charged PNA analogues composed of phosphono-PNA monomers (pPNAs) with *N*-(2-hydroxyethyl)phosphonoglycine, or *N*-(2-aminoethyl)phosphonoglycine, backbone was developed by us 10 years ago using solid phase technique, based on the phosphorotriester oligonucleotide synthesis with O-nucleophilic intramolecular catalysis[9]. Then, chimeras composed of pPNA and PNA monomers (PNA-pPNAs) were obtained using the same approach and dimers as building blocks[10]. Later, the solid-phase synthesis of HypNA-pPNA hetero-oligomers, in which PNA monomers were replaced by monomers on the basis of *trans*-4-hydroxy-L-proline (HypNA), was accomplished (Fig. 3)[11,12]. A HypNA monomer represents conformationally constrained chiral PNA analogue, in which the β-C atom of the hydroxyethyl group and the α-C atom of a glycine unit of the backbone are bridged with methylene group. Convenient schemes for the synthesis of the HypNA monomers and HypNA-pPNA dimers were developed[26]. An alternative synthetic scheme for the synthesis of four possible diastereomeric HypNA monomers was published by Verheijen et al.[27] The evaluation of properties of these mimics revealed that they are fully stable to the action of nucleases and proteases, and the introduction of negative charges into the backbone leads to their excellent solubility in water. The most prospective of these three types of mimics were HypNA-pPNA compounds, which demonstrated strong binding to complementary DNA and RNA strands with melting temperatures very close to those of classical PNA/DNA, or PNA/RNA, complexes[11,12]. To continue these investigations, DNA mimics totally constructed from the conformationally constrained chiral pPNA analogues on the basis of 1-acetyl-4-hydroxypyrrolidine-2-phosphonic acid (pHypNAs) were recently designed[11,13].

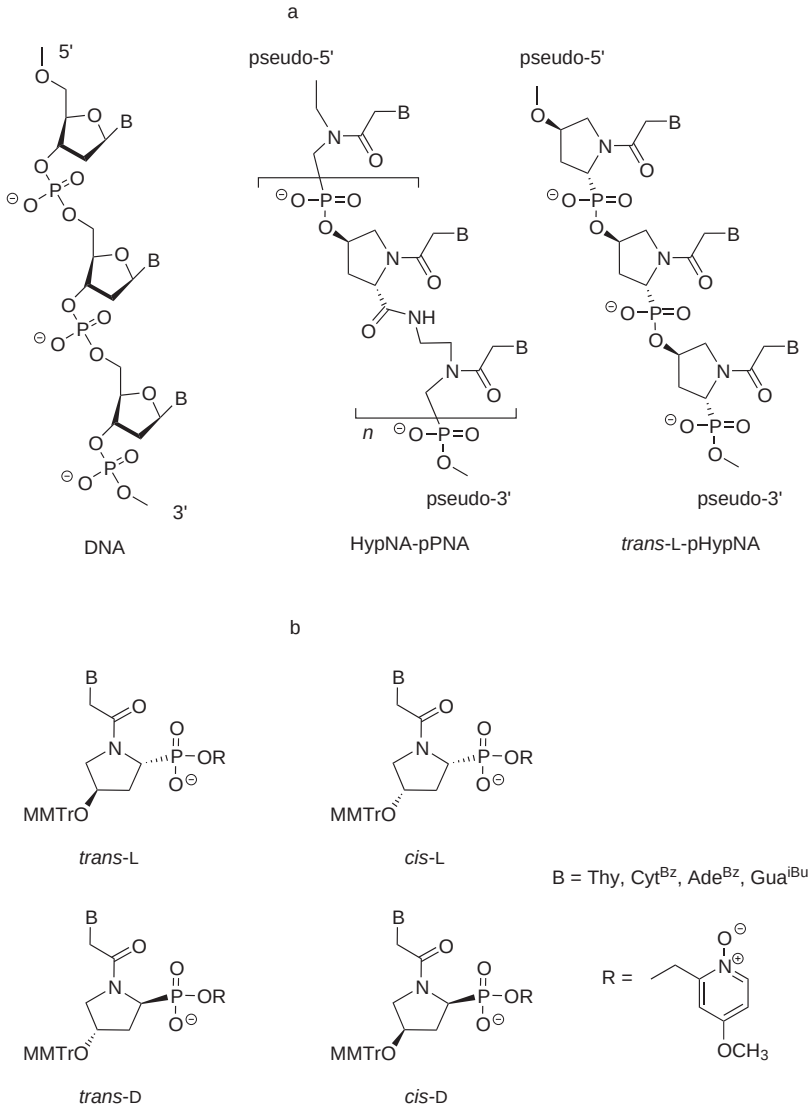


FIG. 3
General chemical structures of DNA mimics: HypNA-pPNA and pHypNA oligomers (a) and chiral pHypNA monomers (b)

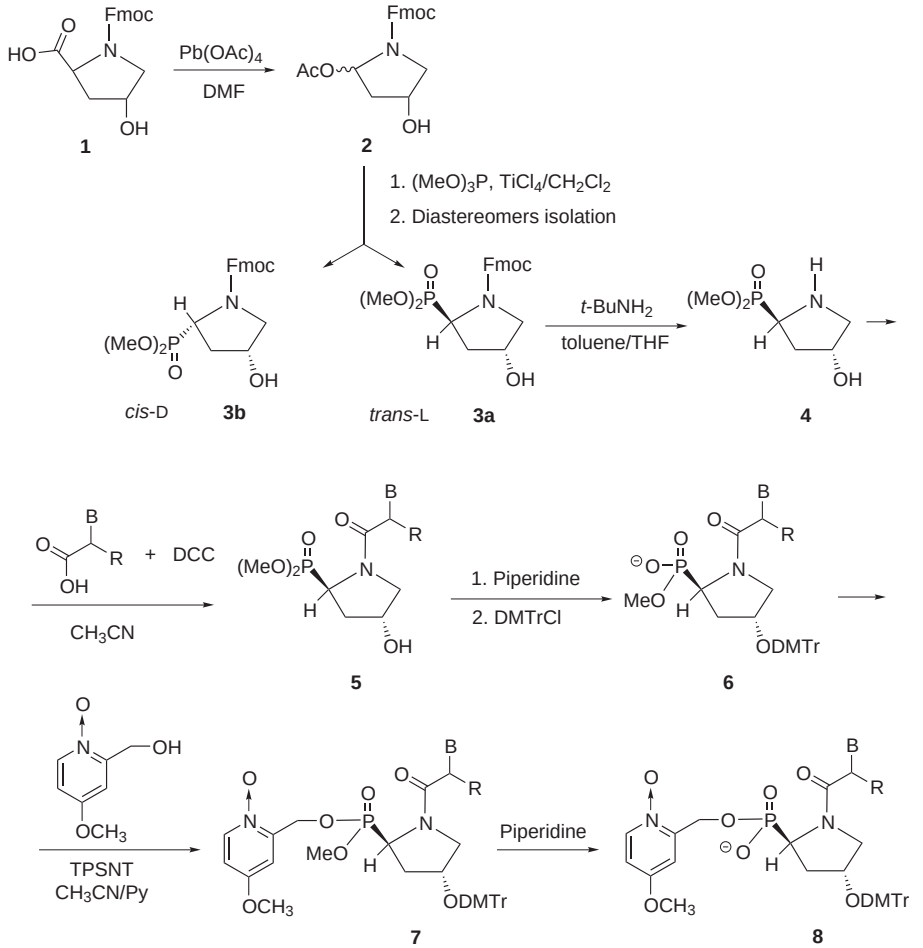
5. LOCKED PHOSPHONO-PNA ANALOGUES ON THE BASIS OF HYDROXYPROLINE (pHypNAs)

5.1. Synthesis of Optically Active pHypNA Monomers and Oligomers

First, the synthesis of four optically active Thy-containing pHypNA monomers was accomplished starting from methyl *trans*-4-hydroxy-L-prolinate as described in ref.[13] The monomers obtained were used in the automated solid-phase synthesis of four chiral homo-Thy pentadecamers with the use of protocols developed by us earlier for the synthesis of pPNA mimics[10]. The investigations of the binding properties of chiral homo-Thy pHypNAs have shown that the oligomers totally constructed from the monomers with *cis*-L or *cis*-D configuration were not able to form stable complexes with the complementary homo-A DNA or RNA targets. The homo-Thy oligomers constructed from *trans*-D or *trans*-L monomers were able to give stable complexes with complementary targets, particularly the oligomer composed of *trans*-L monomers. The latter type of oligomers exhibited strong binding to the complementary DNA/RNA target with high melting temperatures of complexes (up to 75 °C).

On the basis of these data, we extended our investigations to the development of procedures for construction of *trans*-L pHypNA oligomers containing the four natural nucleobases. A convenient general synthetic route was developed to obtain *trans*-L pHypNA monomers containing protective groups compatible with the oligonucleotide synthesis (Scheme 1). The synthesis of monomers employs a common intermediate **4** and allows fast and convenient preparation of Thy, Ade, Gua and Cyt derivatives as well as monomers containing various fluorescent groups, for example pyrene, fluorescein or other reporting groups. The synthesis of the universal intermediate **4** was accomplished starting from *N*-Fmoc-*trans*-4-hydroxy-L-proline. The corresponding protected pyrimidine-1- or purine-9-acetic acid was added to the intermediate **4** to obtain compound **5**. After the removal of one of methyl groups from the phosphonate diester residue by the action of piperidine, 4,4′-dimethoxytrityl (DmTr) protective group was introduced onto the 4-OH function to obtain compound **6**. The catalytic phosphonate protective group was introduced by the action of 4-methoxypyridine-2-methanol 1-oxide in the presence of 4(5)-nitro-1-(2,4,6,-triisopropylbenzene-1-sulfonyl)imidazole (TPSNI). The last synthetic step included the removal of the second methyl protective group from phosphonate function to obtain monomer **8a**. The structures of monomers were confirmed by ^{1}H NMR, ^{31}P NMR and mass-spectrometric analysis. Like for

pPNA mimics, the solid-phase synthesis of pHypNA oligomers was carried out by the phosphorotriester method with O-nucleophilic intramolecular catalysis. Procedures for the synthesis, deblocking and isolation of pHypNA mimics were essentially as described[10]. 2,4,6-Triisopropylbenzene-1-sulfonyl chloride (TPSCl), or 3-nitro-1-(2,4,6-triisopropylbenzene-1-sulfonyl)-1*H*-1,2,4-triazole (TPSNT), were used as condensing agents for the phosphonate



B = Thy, Cyt^{Bz}, Ade^{Bz}, Gua^{iBu}, or pyrene
R = H (**a**) or CH_3 (**b**)

SCHEME 1

diester bond formation. Comparing the synthesis of HypNA-pPNA oligomers, the time of the condensation reaction was reduced from 6 to 3 min. Steps and conditions in a typical elongation cycle are summarized in Table I. After completion of the synthesis, the removal of catalytic P-protective group from the inter-phosphonate bonds of oligomers was carried out by the action of benzenethiol–triethylamine–dioxane (1:2:2, v/v/v) at room temperature for 3–4 h. Then, oligomers were removed from the support with parallel removal of N-protecting groups from heterocycles by the action of concentrated aqueous ammonia and isolated by gel filtration on Pharmacia NAP-10 columns. Purification of mimics was achieved by anion-exchange FPLC, or denaturing gel electrophoresis. The identity of oligomers was confirmed by mass spectrometry (MALDI-TOF).

Another type of mimic monomers obtained according the procedure shown in Scheme 1 was *trans*-L pHypNA-Me **8b**, in which the residue of (thymin-1-yl)acetic acid was replaced by the residue of (*R*)-2-(thymin-1-yl)- or (*S*)-2-(thymin-1-yl)propanoic acid. This type of molecules had an additional chiral methyl group in the position close to the heterocycle. Two diastereoisomeric monomers **8b** were used for the automated synthesis of the corresponding homo-Thy pHypNA-Me oligomers. The experiments on the hybridization of these stereo-specific oligomers with complementary DNA and RNA fragments revealed dramatic loss of the hybridization ability of pHypNA-Me oligomers in comparison with *trans*-L pHypNA oligomers in both cases (data not shown). Earlier, it was shown that the distance be-

TABLE I
Elongation cycle for the solid phase synthesis of pHypNA oligomers

Step	Solvents and reagents	Time, min
1. Detritylation	3 % dichloroacetic acid in dichloromethane	1.5
2. Wash	acetonitrile	1
3. Wash	acetonitrile–pyridine (3:1, v/v)	1.0
4. Coupling	0.05 M P-component; 0.12 M TPSCl (or TPSNT) in acetonitrile–pyridine (3:1, v/v)	3.0
5. Wash	acetonitrile–pyridine	0.5
6. Capping	acetic anhydride–1-methylimidazole–acetonitrile (1:1:8, v/v/v)	0.5
7. Wash	acetonitrile	1.5

tween the nucleobases in PNA oligomers is of great importance for the stability of their complexes with the complementary nucleic acid targets[28]. Thus, the extension of a linker to the nucleobase from methylenecarbonyl to ethylenecarbonyl in the classical PNA molecule had a negative influence on hybridization properties of PNAs, and the thermal stability of the complexes formed from these modified PNA oligomers and the complementary DNA was significantly lower than that of the corresponding complexes involving unmodified PNA. In our experiments we did not change the length of this linker; however, the replacement of H atom in position 2 of (thymine-1-yl)acetic acid with methyl group also had a negative influence on hybridization properties of pHypNA oligomers.

*5.2. Physicochemical Properties of trans-*L *pHypNA Mimics*

Based on the results obtained, the following investigations on the properties and potential applications of pHypNAs were carried out using oligomers constructed from *trans*-L pHypNA type of monomers. The investigation of hybridization properties of *trans*-L pHypNAs with mixed nucleobase sequences revealed that, similar to HypNA-pPNAs, they are able to form very stable duplexes with complementary DNA and RNA targets (Table II). A comparison of melting temperature (T_m) of HypNA-pPNA and MO duplexes with complementary synthetic oligonucleotides has shown that the T_m values of eighteen bp HypNA-pPNA/RNA duplexes (62–77 °C) are close to those of twenty five bp MOs/RNA duplexes[12,29,30]. We have found that the incorporation of pHypNA monomers into HypNA-pPNA oligomer chains gives chimeras with strong affinity to DNA and RNA targets. Also, these mimics are able to hybridize with each other giving stable pHypNA/HypNA-pPNA and pHypNA/pHypNA complexes with complementary chains (data not shown). The results obtained prove that these two types of mimics are fully compatible. At the same time, chimeras with alternating pHypNA and DNA monomers did not show any hybridization activity with complementary targets. From the titration data and electrophoretic behavior, it was concluded that, similarly to PNAs and other PNA-like mimics containing phosphonate ester bonds, homopyrimidine pHypNA sequences form with complementary DNA (or RNA) targets triple helixes, whereas oligomers with mixed sequences formed duplexes[29,31].

The evaluation of the effectiveness of HypNA-pPNA and *trans*-L pHypNA mimics in assays based on the hybridization technique revealed their high potential as biomolecular probes for the solution and solid phase analysis. Thus, the hybridization of the mimic oligomers with DNA and RNA targets

TABLE II
Stability of complexes formed by DNA mimics and natural oligonucleotides with complementary DNA or RNA targets[a]

Probe sequence[b]	Target[b]	Oligonucleotide		HypNA-pPNA		pHypNA	
		T_m, °C	ΔT_m, °C	T_m, °C	ΔT_m, °C	T_m, °C	ΔT_m, °C
TTTTTTTTTTTTTT	d(AAAAAAAAAAAAAA)	37		78		76	
TTTTgTTTTTcTTT	d(AAAAAAAAAAAAAA)	<10	>25	20	58	29	42
TTTTTTTaTTTTTT	d(AAAAAAAAAAAAAA)	23	14	55	23	48	23
TTTTTTTTTTTTTT	r(AAAAAAAAAAAAAA)	35	–	–	–	64	–
AAAAAAAAAAAAAAA	d(TTTTTTTTTTTTTTT)	37	–	–	–	54	–
TGGTCTCAAGTCAGTG	d(CACTGACTTGAGACCA)	64	–	58	–	61	–
TGGTCTCAAGTCAGTG	d(CACTGAgTTGAGACCA)	57	7	41	17	45	16
TGGTCTCAAGTCAGTG	d(CACTGACTTGAGtCCA)	54	10	47	11	49	12
TCACTCAACACTCAC	d(GTGAGTGTTGAGTGA)	57	–	58	–	55	–
TCACTCAACACTCAC	d(GTGAGTGgTGAGTGA)	48	9	38	20	38	17
TCACTCAACACTCAC	d(GTGAG-GTTGAGTGA)	47	10	39	19	37	18
Pyr_2TCACTCAACACTCAC	d(GTGAGTGTTGAGTGA)	–	–	–	–	56	–
Pyr_2TCACTCAACACTCAC	d(GTGAGTGTTGAGTGACTATTT)	–	–	–	–	59	–
TCACTCAACA Pyr_2CAC	d(GTGAGTGTTGAGTGA)	–	–	–	–	42	13
CTGCAAAGGACACCATGA	d(TCATGGTGTCCTTTGCAG)	54	–	69	–	67	–

TABLE II
(*Continued*)

Probe sequence[b]	Target[b]	Oligonucleotide		HypNA-pPNA		pHypNA	
		T_m, °C	ΔT_m, °C	T_m, °C	ΔT_m, °C	T_m, °C	ΔT_m, °C
CTGCAAAGcACACCATGA	d(TCATGGTGTCCTTTGCAG)	42	12	49	20	52	15
ATCATGGTCATAGCTGTT	d(AACAGCTATGACCATGAT)	61	–	58	–	63	–
ACACTTACACTTACAC	d(GTGTAAGTGTAAGTGT)	57	–	42	–	45	–
ACACTTACACTTACAC	r(GUGUAAGUGUAAGUGU)	52	–	57	–	62	–
CCCTATAGTGAGTGTCGT	d(ACGACACTCACTATAGGG)	61	–	60	–	63	–
CCCTATAGTGAGTGTCGT	r(ACGACACUCACUAUAGGG)	65	–	72	–	69	–
GCTCTCGTCGCTCTCCAT	d(CATGGAGAGCGACGAGAGC)	69	–	65	–	63	–
GCTCTCGTCGCTCTCCAT	r(CAUGGAGAGCGACGAGAGC)dTT	68	–	76	–	75	–
GCTCTCGTCaCTCTCCAT	d(CATGGAGAGCGACGAGAGC)	58	11	45	20	42	21
GCTCTCGTCaCTCTCCAT	r(CAUGGAGAGCGACGAGAGC)dTT	58	10	59	17	56	19
r(GCUCUCGUCGCUCUCCAUG)dTT	r(CAUGGAGAGCGACGAGAGC)dTT	86	–	–	–	–	–

[a] An oligomer (5–10 μM) was mixed with an 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 20 °C. The changes in absorbance at 260 nm versus temperature were measured at a heating rate 0.5 °C/min from 15 to 95 °C. The temperature of a half-dissociation was taken as melting temperature (T_m). It was obtained from a plot of the derivative of $1/T$ vs absorbance at 260 nm. [b] The probe sequence is shown from the 5′- to 3′-end for oligonucleotides or from pseudo-5′- to pseudo-3′-end for mimics. Lower-case base in the sequence represents a mismatch position.

occurred in a sequence-specific manner, and the formation of complexes between mimic probes and non-complementary targets was not detected. It was shown that similarly to HypNA-pPNAs, 16-18-mer pHypNA probes can effectively discriminate between single base mismatches or deletions in the target sequence. The introduction of one mismatch in the center of a sequence gives a drop in the melting temperature 12–23 °C depending on the length of oligomer, base mismatch position and the sequence (Table II). In contrast to natural oligonucleotides, HypNA-pPNA and pHypNA oligomers can effectively hybridize with complementary targets at low salt concentrations, and T_m values of their complexes with DNA/RNA targets are not dependent on ionic strength[13,29].

The sequence-specific recognition of double-stranded DNA (dsDNA) is a topic of considerable interest in the development of tools for molecular biology, diagnostics, therapeutics and bionanotechnology. A remarkable feature of thymine-rich PNAs is their ability to recognize complementary sequences within duplex DNA by strand invasion that results in local strand displacement (formation of a D-loop)[32] (Fig. 4). Similarly to PNAs, negatively charged analogues demonstrate the effect of strand invasion in homopyrimidine DNA regions due to triplex formation. The melting experiments and the gel mobility shift assay for the interaction of a hexadeca polypurine/polypyrimidine DNA duplex with the corresponding polypyrimidine pHypNA dodecamer have shown that under the addition of a mimic oligomer in various molar ratios to the duplex, the DNA strand containing a polypyrimidine sequence was displaced from the duplex by the competing homopyrimidine mimic chain giving the invasion complex of the triplex type. Thus, two transitions (T_m = 58 and 75 °C) were detected for a complex of the mimic oligomer with dsDNA in the thermal denaturation experiment, which corresponded to duplex and triplex meltings, respectively. Some results in mobility shift analysis of the complex formation and melting experiments are shown in Figs **4b**, **4c**).

In last years, the development of homogeneous fluorescence assays for nucleic acids detection based on the introduction of a single kind of fluorophores to oligonucleotides and their mimics attracts rising attention[33,34]. Usually, such assays are exploiting a modulation of fluorescence intensity, which occurs during the hybridization process. We investigated fluorescence properties of pHypNA oligomers containing one or more pyrene-modified *trans*-L monomers. The effect on duplex stability upon incorporation of a pyrene-containing monomer into pHypNA chains was evaluated by UV thermal denaturation experiments and compared with the corresponding unmodified reference duplexes (Table II). Thus, the pHypNA

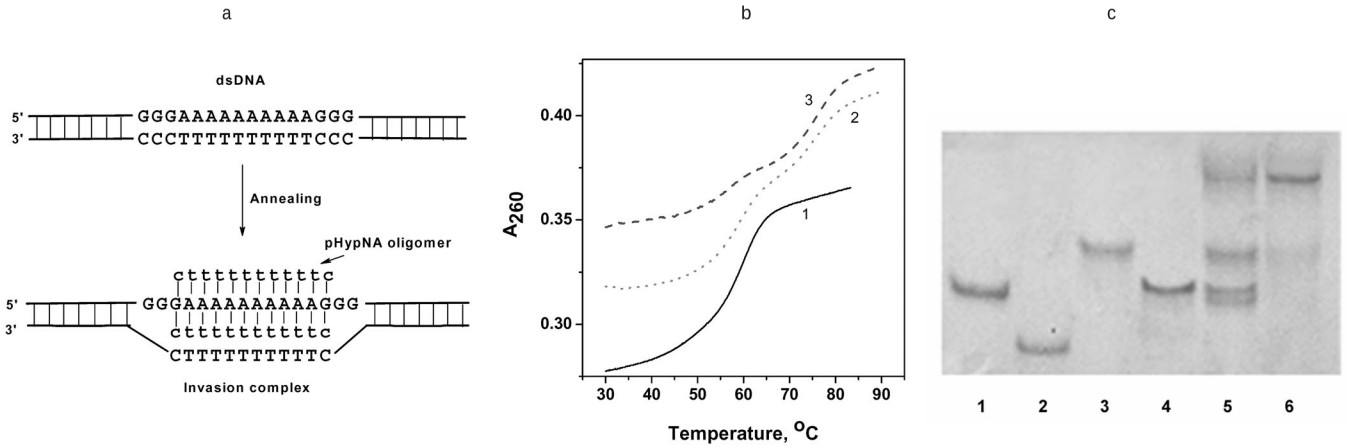


FIG. 4

Strand invasion of the16-bp double stranded DNA by homopyrimidine pHypNA oligomer (CTTTTTTTTTTC). a Schematic representation of strand invasion of dsDNA by PNA-related oligomer; b melting curves of dsDNA (*1*) and its complexes with pHypNA oligomer in 1:1 (*2*) and 1:1.5 (*3*) molar ratio obtained in 10 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/0.1 M NaCl; c mobility shift assay of a complex of dsDNA and pHypNA oligomer in 1:1 molar ratio in a 15% native polyacrylamide gel: d(CCCTTTTTTTTTTCCC) (1), complementary strand d(GGGAAAAAAAAAAGGG) (2), pHypNA oligomer (CTTTTTTTTTTC) (3), the duplex formed by the complementary DNA strands (4), a mixture formed after incubation of the DNA duplex with pHypNA oligomer at 20 °C for 5 h (5), the triplex formed by pHypNA oligomer with DNA d(GGGAAAAAAAAAAGGG) in 2:1 molar ratio (6)

oligomers terminally modified with one or two pyrene-functionalized monomers display some increase in thermal stability of complexes formed with DNA (RNA) targets compared with complexes of unmodified oligomers of the same sequence, whereas the introduction of one or two pyrene residues into internal positions of a mimic chain had a negative influence on the melting temperatures of the corresponding duplexes (ΔT_m = 9–14 °C). However, the difference in melting temperatures of the duplex formed by the unmodified oligomer and that with the internal insertion of the pyrene-containing monomer was lower than in the case of the introduction of a mismatched position to oligomer sequence (Table II). This effect can be explained by stacking interactions between pyrene residues and nucleobases, which are usually accompanied by quenching of fluorescence. For single-stranded mimics containing a cluster of two pyrene units, an excimer emission with additive increase in fluorescence intensity was observed, whereas probes containing one pyrene unit exhibited a monomer emission. The excimer emission of duplexes between pHypNA probes with an internal insertion of two pyrene residues as bulged next-nearest neighbors was quenched under hybridization with DNA/RNA complements (Fig. 5). The same fluorescent properties were shown by duplexes formed by doubly pyrene-modified at pseudo 5′-end mimic oligomers and nucleic acid targets with protruding 3′-ends. As it can be expected, a significant increase in excimer fluorescence brightness was observed upon the hybridization of mimic oligomers terminally labeled with two pyrene residues to DNA (RNA) targets, if pyrene residues were extruded to the outside of the duplex and not involved in the duplex formation with nucleobases of the complementary DNA strand (Fig. 5). This indicates that two pyrenyls in the same strand are in close contact after formation of the duplex (or triplex) between single-stranded DNA (or RNA) and the mimic chain. The emission from duplexes formed by such multiple-modified probes at 0.5 μM and lower concentrations can be easily seen by the naked eye using standard UV trans-illuminators. This property of oligomers containing two terminal pyrene residues was utilized by us to obtain the evidence of the ability of pHypNA oligomers with mixed purine–pyrimidine sequences to displace the isosequential strand from the natural oligodeoxyribonucleotide duplex. The single-stranded pHypNA oligomer (Pyr_2TCACTCAACACTCAC) containing two terminal pyrene residues displayed a weak excimer emission as a major fluorescence. The enhancement of excimer emission was observed when this oligomer was mixed with the corresponding DNA duplex containing the DNA strand complementary to pHypNA oligomer. This effect confirmed that the pyrene-containing oligomer was involved in the duplex

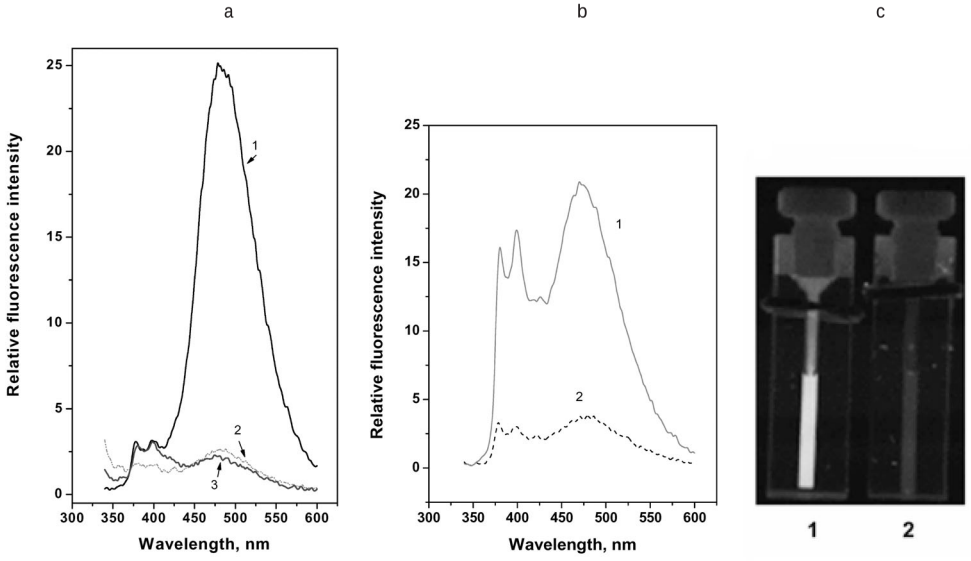


FIG. 5

Fluorescent spectra and images of bis-pyrene modified pHypNA oligomers. a Fluorescence spectra of a duplex formed by the oligomer (Pyr_2TCACTCAACACTCAC) with two terminal pyrene monomers and the complementary target d(GTGAGTGTTGAGTGA) (*1*), a single-stranded oligomer (Pyr_2TCACTCAACACTCAC) (*2*) and its duplex with the target d(GTGAGTGTTGAGTGACTATTT) with protruding 3′-end (excitation wavelength 330 nm) (*3*). b Fluorescence spectra of a pHypNA oligomer (TCACTCAACA Pyr_2CAC) containing internal insertions of pyrene residues (*1*) and its duplex with complementary target d(GTGAGTGTTGAGTGA) (*2*). c Fluorescence image of an 0.05 μM solution of pHypNA oligomer (Pyr_2TCACTCAACACTCAC) hybridized to the complementary d(GTGAGTGTTGAGTGA) target (1) and non-complementary oligonucleotide d(CACTGACTTGAGACCA) (*2*) (sample solutions were illuminated with a UV trans-illuminator at 302 nm)

formation with the complementary DNA strand from dsDNA, and the pyrene moieties not involved in duplex formation were in sufficient proximity. The additional data on the pHypNA oligomer interaction with dsDNA were obtained from a gel electrophoresis assay (Fig. 6). In the control experiments, no increase in excimer emission was observed, when oligomer (Pyr_2TCACTCAACACTCAC) was added to double-stranded DNA with a scrambled sequence.

6. POTENTIAL OF HypNA-pPNA AND pHypNA MIMICS IN NUCLEIC ACIDS DETECTION AND ISOLATION

The binding selectivity of negatively charged mimics gave rise to investigation of their properties as capture and detection probes for the construction of arrays for nucleic acid hybridization analysis, and their high potential was demonstrated[35]. The application of negatively charged single-stranded PNA-related mimics as capture probes in nucleic acids analysis is stimulated by their unique property not to 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 or RNA exhibit fluorescence under UV light after staining[35]. This property was fully preserved by pHypNA oligomers, and this visualization method in conjunction with mimic probes is promising for the analysis of long nucleic acid targets, because it does not require a preliminary radioactive or fluorescent target labeling.

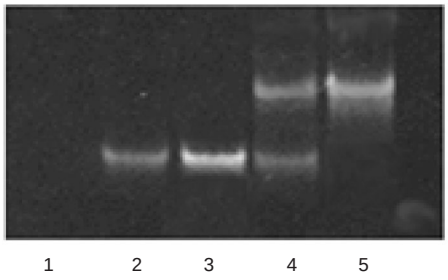


FIG. 6
Analysis of the strand displacement in double-stranded DNA by pyrene-labeled pHypNA oligomer (Pyr_2TCACTCAACACTCAC) with the use of electrophoresis in 15% native polyacrylamide gel. Before loading onto a gel, duplexes were mixed with equimolecular amounts of pHypNA oligomer in 0.1 M NaCl/0.02 M Tris-HCl (pH 7.5)/0.01 M $MgCl_2$ and incubated at room temperature for 3 h. The image was obtained under illumination at 302 nm

The phosphonate-containing PNA-like mimics, pPNAs, PNA-pPNAs and HypNA-pPNAs, were shown to be very useful for the isolation of intact RNAs, particularly polyadenylated mRNA, from cells and tissues, which is an essential step for many functional genomic applications. A highly efficient procedure for isolation of intact 3′ poly(A)-tailed mRNA from cells and tissues using homo-Thy affinity ligands based on triplex forming homo-Thy mimic oligomers conjugated with biotin residue was developed[36]. The biotin residue allowed the conjugation to a streptavidin-coated surface, particularly magnetic beads, to isolate mimic/mRNA complexes. Mimic oligomers of the pHypNA type can be successfully applied to this procedure: they are able to hybridize to poly-A mRNA tails with a high degree of specificity and give very low background binding of the probe to unwanted rRNA (data not shown). An exceptionally high affinity to RNA targets, good water solubility and nuclease resistance of the negatively charged PNA-related mimics gave rise to a several-fold increase in mRNA yield, compared with oligo-dT selected samples, together with isolation of a representative mRNA population including mRNA with short poly-A tracks. Similar procedures can be developed for isolation of specific native RNAs from living organisms. It should be noted that the application of HypNA-pPNA and pHypNA probes enables efficient isolation of RNA from extracted total RNA samples in buffers with low salt concentrations due to the enhanced destabilization of mRNA secondary structure under these conditions. Another of their advantages over natural oligonucleotides is reduced DNA contamination of RNA samples that is a consequence of possibility of DNase treatment during RNA preparation.

Another promising application of HypNA-pPNA and pHypNA mimic oligomers is molecular beacons. Homogeneous fluorescence assays for nucleic acids detection and quantification are widely used in molecular biology, and molecular beacons rely on distance-dependent fluorescence quenching for such assays. These molecules can report the presence of complementary nucleic acid targets without having to separate probe-target hybrids from the excess of a probe in hybridization assays[37]. Molecular beacons are used for *in vitro* RNA and DNA monitoring, in biosensor applications and for gene monitoring in living systems[38,39]. The construction of novel types of chimeric molecular beacons including the beacons with classical PNA segments[40] as well as chimeric beacons composed of DNA, RNA and negatively charged PNA analogues were reported[41]. The evaluation of properties of these compounds in model assays with the complementary and mismatched synthetic DNA and RNA targets has shown that mimic chimeric beacons are about 10 times more sensitive in the detection of nucleic acid

targets in solution than oligodeoxyribonucleotide beacons of the same sequence[40]. As the rapid and sensitive detection of RNA in living cells using modified molecular beacons that possess self-delivery, targeting and reporting functions was demonstrated[41], the mimic beacons can be very useful for *in vivo* applications in view of their higher biological stability, an improved discriminatory power and increased affinity for nucleic acid targets in the comparison with natural oligonucleotide probes. Together with the insensitivity of PNA-related beacons to the presence of a salt and the DNA-binding/processing proteins, their potential as robust tools for *in vivo* recognition of specific sequences can be estimated as very high. The investigations of the ability of pHypNA beacons to detect and quantify the expression of specific endogenous mRNAs in living cells and tissues in real time are now in progress.

7. BIOLOGICAL PROPERTIES OF HypNA-pPNA AND pHypNA MIMICS

7.1. Stability and Cell Delivery

All negatively charged PNA-like mimics show excellent solubility in water and stability to the action of nucleases and proteases. In contrast to natural oligonucleotides and their phosphorothioate analogues, these mimics as well as classical PNAs do not activate RNase H upon binding to complementary RNA [42]. Earlier investigations of the cell delivery of negatively charged PNA-related mimics, particularly HypNA-pPNAs, revealed that free oligomers can slowly penetrate into bacterial and eukaryotic living cells and be distributed in the cytoplasm[31], and the addition of lipofectin or oligofectamine for delivering into eukaryotic cells via cationic liposome complexation did not show the pronounced positive results[42]. Also, it was shown that the addition of a non-covalent peptide delivery system (based on a peptide having the sequence KETWFETWFTEWSQPKKKRKV) considerably speeds up the delivery of HypNA-pPNAs into more than 90% of living cells with localization in the cytoplasm and nucleus, and the maximal uptake can be achieved in 2 h. Now, we have found that pHypNA oligomers preserve the penetrating ability of HypNA-pPNAs. Free pHypNA oligomers are able to penetrate into eukaryotic cells, but the addition of Lipofectamine-2000 can significantly improve the delivery of pHypNAs, as well as HypNA-pPNAs into eukaryotic cells (Fig. 7)[43]. Similarly to HypNA-pPNAs, pHypNA oligomers have no toxic effect on cell growth in the concentration up to 5–7 μM.

7.2. Down-Regulation of Protein Biosynthesis

In first experiments on the down-regulation of protein production, the ability of negatively charged hydroxyproline-based mimic oligomers to suppress expression of green fluorescent protein in *E. coli* cells was tested[29,31]. An antisense HypNA-pPNA oligomer demonstrated ~70% inhibition of GFP production, whereas control oligomers with scrambled and mismatched sequences had no effect. Also, HypNA-pPNAs were tested in the suppression of AChE-R production in rat brain cell culture in 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 supplemented to the cell culture medium in 0.5 μM concentration and its effect was comparable with that of the control phosphorothioate oligonucleotide[29,31]. The down-regulation of the levels of cyclin B1 (a protein essential for cell cycle progression) production was demonstrated for antisense HypNA-pPNA octadecamer in 50–100 nM concentrations[42]. This effect was analyzed in several cell lines, particularly HeLa cells, human fibroblasts (*HS 68*) and *293* cells in comparison with the effect of classical PNAs and antisense phosphorothioate oligonucleotides with the same sequence. In these experiments, an antisense effect of HypNA-pPNA oligomer was estimated after the quantification of cyclin B1

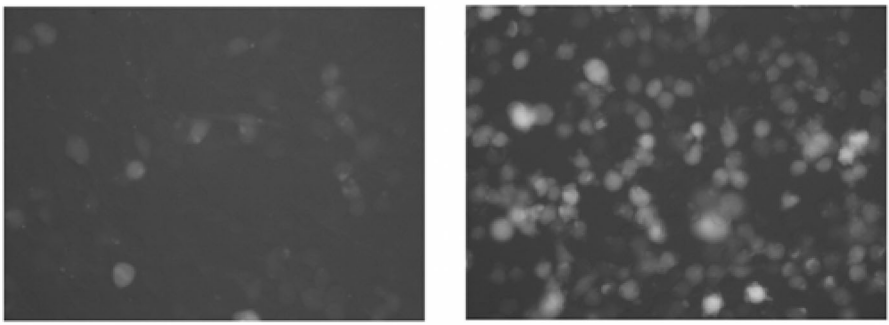


FIG. 7
Delivery of a HypNA oligomer (Flu-ACACTTACACTTACAC) into eukaryotic cells. The fluorescein labeled oligomer in 0.5 μM concentration was incubated with the unfixed Phoenix Eco cells at 37 °C for 16 h in culture medium. Then, the cells were washed, and microscopy images were taken with an Olympus CK40 inverted microscope and DP50 Olympus CD camera. A green filter was used to view the fluorescein fluorescence. a Incubation in the absence of a cell-penetrating agent; b incubation in the presence of Lipofectamine-2000 (1 μl per 0.1 nmol of the oligomer)

protein levels 8.5-fold and 25-fold higher than that of PNA oligomer and phosphorothioate oligonucleotide, respectively[42]. Also, it was shown that antisense cyclin B1 HypNA-pPNA oligomer is able to reduce proliferation of human breast cancer cells MCF-7 in the 0.5–1 μM concentration by 70–92%, whereas classical PNA oligomer reduced cell proliferation only by 35% for 1 μM and by 64% for 2 μM under the same conditions. In 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[43] as well as for down-regulation of various genes.

Recently, we have found that pHypNA oligomers are also highly effective in gene silencing experiments in eukaryotic cells[44]. To illustrate this, a novel dual-color vector, p2FP-RNAi (Evrogen, Russia), was used to test functionality of pHypNAs as antisense agents. This plasmid is a mammalian expression vector encoding two fluorescent reporters: red fluorescent protein, JRed, which functions as a positive transfection marker, and green fluorescent protein, TurboGFP, which stands as an indicator of siRNA or antisense oligomer efficiency. TurboGFP is an improved variant of the green fluorescent protein CopGFP cloned from copepoda *Pontellina plumata*[45]. It reveals bright green fluorescence and fast maturation when expressed in eukaryotic cells and does not form aggregates in long-term cultures. Synthetic octadecamer pHypNA and HypNA-pPNA oligomers directed against TurboGFP sequence as well as the corresponding short double-stranded RNA (dsRNA) were synthesized. Phoenix Eco cells were transfected with the p2FP-RNAi vector mixed with one of these types of oligomers in the presence of Lipofectamine-2000 reagent. The microscopic fluorescent analysis of cells performed 24 h after delivery clearly indicates that TurboGFP has been more efficiently silenced by pHypNA antisense oligomer in comparison with the corresponding HypNA-pPNA oligomer, and its efficiency was close to that of dsRNA targeted on the same sequence (Fig. 8)[44]. In control experiments, pHypNA and HypNA-pPNA oligomers with scrambled or mismatched sequences practically did not influence the production of the green fluorescent protein.

In 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[46–51]. Negatively charged HypNA-pPNAs were evaluated as an alternative to MOs for oligonucleotide inhibition of gene expression in zebrafish embryos. It was shown that HypNA-pPNA chimeric oligomers, similarly to MOs, are effective and specific *in vivo* translational inhibitors in zebrafish and, in an amount of 4 ng per embryo, caused specific vascular defects with minimal

non-specific effects[50,51]. It was found that HypNA-pPNA-based transient gene silencing is feasible in zebrafish embryos and provides a valuable reverse genetic screening strategy. Similarly to MOs, these compounds can act as effective antisense inhibitors in living organisms over a range of developmental stages. It was observed that HypNA-pPNA octadecamers displayed potency comparable with MO pentacosamers as gene knockdown agents with greater mismatch stringency than MOs. Thus, 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 pentadecamer MO with the same single-base mismatch produced the *ntl* null phenotype in 98% of injected embryos. These results demonstrate that octadecamer HypNA-pPNAs display higher sequence specificity in comparison with MO pentadecamers[50,51]. Another key advantage of HypNA-pPNA for gene knockdown studies is the lack of nonspecific effects; HypNA-pPNA doses, which are high enough to produce strong loss-of-

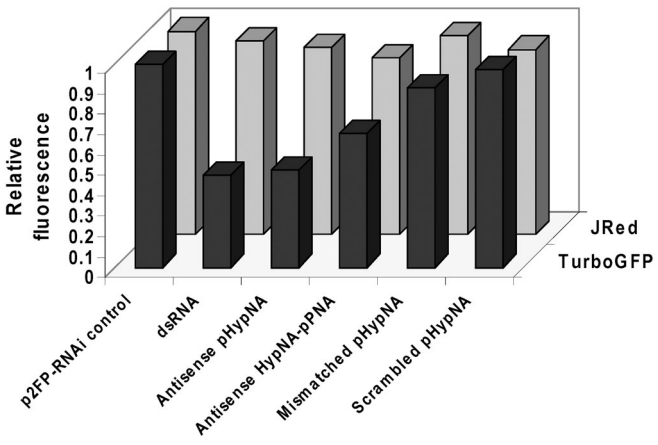


FIG. 8

Suppression of TurboGFP production in eukaryotic cells. The cells transfected with p2FP-RNAi dual-color vector were treated in the growth medium at 37 °C for 24 h with 0.5 μM solutions of the pHypNA or HypNA-pPNA antisense octadecamers with the sequence GCTCTCGTCGCTCTCCAT designed to target the start codon region of TurboGFP mRNA. As controls, the cells treated with the corresponding dsRNA targeting the same sequence, cells treated with pHypNA oligomers with mismatched (GCTCTCGTCaCTCTCCAT) and scrambled (ACTTACACTTACACTTAC) sequences, and cells untreated with antisense oligomers (p2FP-RNAi control) were analyzed. The average fluorescence data for three separate experiments are shown

function phenotypes, produced few or no nonspecific effects. Experiments on the examination of pHypNAs antisense properties in zebrafish embryos are currently in progress.

Recently, the gene for a novel protein Ras-like GTPase Ras-dva, which is expressed during *Xenopus laevis* neurulation in a very restricted area surrounding the anterior margin of the neural plate was identified[52], and it was reported that Ras-dva and its homologues in other species constitute a novel family of Ras-like small GTPases[53]. The down-regulation of the Ras-dva functioning by the MO antisense oligomer results in head development abnormalities, which include reduction of the forebrain, olfactory pits, otic vesicles, branchial arches and malformations of the head cartilages. Similar abnormalities were observed on 60–85% of developing tadpoles after microinjecting antisense pHypNA or HypNA-pPNA oligomers[44]. These results clearly demonstrate that these negatively charged mimics can act as specific antisense inhibitors of gene function in zebrafish and *Xenopus* embryos, and might be of use in other living organisms.

8. CONCLUSIONS AND OUTLOOK

This review presents some efforts initiated to design DNA analogues and mimics on the basis of pyrrolidine and hydroxyproline with the aim in view to obtain a compound possessing optimal physicochemical and biological properties for their use in recognition of nucleic acid targets *in vitro* and *in vivo*. In general, the results obtained demonstrated a high potential of conformationally constrained PNA-related mimics, which makes them very promising for further evaluation as potential compounds for diagnostic and therapeutic applications. Thus, the analysis of properties of HypNA-pPNA and *trans*-L pHypNA oligomers allows one to conclude that these DNA mimics are effective tools for the application as specific probes for nucleic acid detection, isolation and analysis as well as potential antisense and antigene 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. As their effect lasts over a period of several days, due to their high stability in living cells, it represents a very potent technology for administering antisense-based drugs for future therapeutic applications. The results obtained in gene knockdown experiments *in vivo* validate HypNA-pPNAs and pHypNAs as useful tools for reverse genetic studies.

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