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Incorporation of a 4-Hydroxy-*N*-acetylprolinol Nucleotide Analogue Improves the 3′-Exonuclease Stability of 2′-5′-Oligoadenylate-Antisense Conjugates

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Abstract—Incorporation of a 4-hydroxy-*N*-acetylprolinol nucleotide analogue at the 3′-terminus of DNA or 2-5A-DNA sequences resulted in a significantly enhanced 3′-exonuclease resistance while the affinity for complementary RNA was only slightly decreased. Furthermore, the binding to and activation of human RNase L by thus modified 2-5A-DNA conjugates was not altered as compared to the parent unmodified 2-5A-DNAs.

Introduction

The selective inhibition of expression of specific genes by oligonucleotides via an antisense strategy[1] provides an attractive and elegant approach to drug discovery. It has been firmly established that the efficacy of antisense agents can be further enhanced by the attachment of 5′-phosphorylated-2′,5′-linked oligoadenylate [(pp)p5′A2′(p5′A2′)$_n$p5′A or 2-5A] to an antisense sequence, to give a 2-5A-DNA hybrid.[2] The presence of 2-5A in the conjugate activates constitutive latent RNase L, which causes selective decay of a target RNA sequence dictated by the complementary DNA antisense domain of the composite oligonucleotide. In cell free systems, 2-5A-DNAs were active against among others modified HIV-TAR-A$_{25}$-*vif* RNA,[2] PKR RNA[3,4] and HIV *gag* RNA.[5]

Despite these positive results, 2-5A-DNA conjugates suffer in more advanced biological systems from rapid degradation by nucleases. Since the hydrolytic cleavage of oligodeoxynucleotides in vivo occurs preponderantly under the action of 3′-exonucleases,[6,7] several strategies to confer 3′-exonuclease stability on antisense probes have been developed.[7–15] For in vivo applications, improved enzymatic stability was imparted to 2-5A-DNAs by introduction of a 3′-3′ phosphodiester linkage at the 3′-terminus[7] or 3′-terminal DNA backbone phosphorothioates.[8] Such stabilized 2-5A-DNAs have effected ablation of PKR mRNA, protein and biological function in intact cells,[4] suppressed cell growth in chronic myelogeneous leukemia cells,[16] blocked human malignant glioma cell growth in nude mice by targeting telomerase RNA[17] and inhibited replication of respiratory syncytial virus (RSV).[18,19] Although these modified 2-5A-DNAs have a longer lifetime in vivo, they are still slowly degraded by cellular 3′-exonucleases. Consequently, the efficacy of 2-5A-DNAs may be enhanced by further improvement of their 3′-exonuclease stability.

In this respect, a novel and promising approach was recently disclosed by Ramasamy and Seifert, indicating that incorporation of L-serinol derived nucleotide analogue **I** (Fig. 1) at the 3′-terminus of an oligonucleotide sequence increased the 3′-exonuclease resistance of the antisense probe.[20,21] Similar results were reported for the introduction of **II** having an additional chiral centre.[22] Unfortunately, the presence of units **I** and **II** at the 3′-terminus of DNA was accompanied by a drop in thermal stability of the corresponding DNA·RNA

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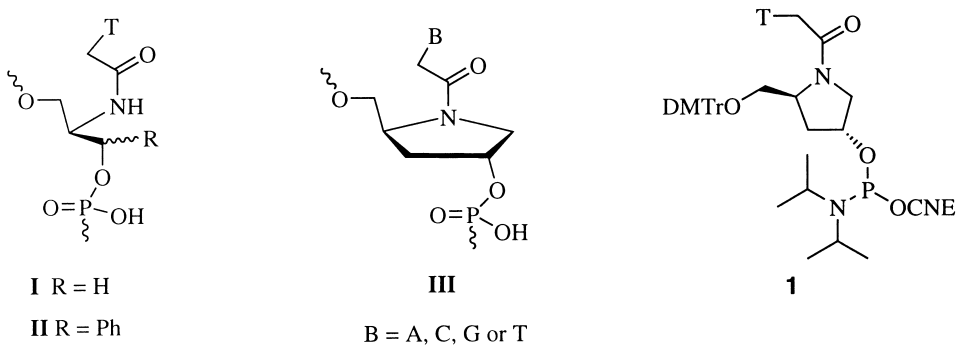


Figure 1.

duplex. Moreover, homooligomers containing **I** or **II** failed to hybridize with complementary RNA. The latter observation may be explained by the large entropy loss upon complexation of the flexible oligomers with RNA, thus disfavoring formation of a double helix. Interestingly, Ceulemans et al. reported that oligomers consisting of the more conformationally restricted *trans*-4-hydroxy-*N*-acetyl-L-prolinol nucleotide analogue (HO-NAP, i.e. type **III**) recognized target RNA, albeit with a rather low affinity and specificity.[23] Furthermore, HO-NAP oligomers survived exposure to the 3′-exonuclease snake venom phosphodiesterase (SVP). Based on these findings, we reasoned that introduction of **III**, instead of **I** or **II**, at the 3′-terminus of a DNA sequence would lead to hybrids which are both highly resistant to exonucleolytic digestion and able to form stable duplexes with a complementary target strand.

We here present the incorporation of thyminyl HO-NAP units in DNAs and 2-5A-DNAs targeted at RSV RNA as well as an evaluation of the enzymatic and thermal stability of the thus obtained antisense oligomers. In addition, it will be demonstrated that the resulting antisense probes retain their ability to activate RNase L mediated hydrolysis of target mRNA and to inhibit viral replication.

Results and Discussion

Initially, the influence of the incorporation of a growing number of HO-NAP building blocks at the 3′-terminus of DNA on the enzymatic and thermal stability was investigated. To this end, mixed pentadecamers **3**–**5** ($T_{(14-n)}t_nT$ where "T" stands for thymidine, "t" for a thymine HO-NAP moiety and $n = 1$, 2 or 3, Table 1) as well as the natural DNA fragment **2** (T_{15}) were assembled on a fully automated DNA synthesizer using a standard phosphoramidite synthesis protocol. Thus, stepwise elongation of thymidine, immobilized to CPG beads via a succinyl linker, was executed using HO-NAP phosphoramidite[24] **1** and standard thymidine phosphoramidite to give oligomers **2**–**5**. The enzymatic stability of **2**–**5** was analyzed by monitoring the hyperchromicity[25] upon addition of one unit of SVP to a buffered solution of the oligomer. The results of the enzymatic degradation experiments are summarized in Table 1. Incorporation of one or more HO-NAP monomers at the 3′-terminus of the DNA sequence (i.e. **3**, **4**, **5**) resulted in a significant enhancement of the enzymatic stability. The native DNA pentadecamer **2** was completely hydrolyzed after 10 min under the reaction conditions (entry 1). In contrast, the half-life ($t_{1/2}$) increased more than 35-fold upon introduction of one HO-NAP moiety at the 3′-end of the adduct (cf. **3**, entry 2). Further analysis of the reaction mixtures by HPLC revealed that introduction of a growing number of HO-NAPs was accompanied by a proportional increment in stability (data not shown). In all cases, considerable amounts of intact starting material could still be detected after 15 h under the reaction conditions.

Prompted by the favorable outcome of the enzymatic degradation studies, the influence of incorporation of a growing number of HO-NAP moieties in DNA on the hybridization properties with poly(rA) was investigated. It was established that incorporation of modified building unit **1** is accompanied by a proportional drop in thermal stability of about 3–4 °C per modification (Table 1, entries 2–4). Although these studies are limited to homosequences, it is evident that the presence of a single HO-NAP modification will not inhibit duplexation with complementary RNA.

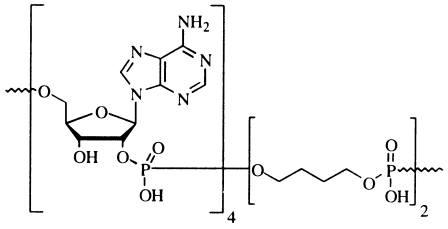
To investigate the feasibility of 2-5A-DNAs containing a HO-NAP moiety at the 3′-terminus as antisense agents, 2-5A-DNAs **6**–**8** (Table 2) were synthesized by modification of earlier reported procedures.[2–5] The sequence of the antisense domain in **6**–**8** is complementary to a consensus sequence in the RSV genome, which was shown to be a suitable target for inhibition of viral replication by 2-5A-DNAs.[19] The 3′-exonuclease stability of 2-5A-DNAs **6**–**8** was determined as before.

Table 1. Enzymatic degradation by SVP and duplex stability with poly(rA) of **2**–**5**

Entry	Sequence[a]	$t_{1/2}$ (min)	T_m (°C)[b]	ΔT_m/modification (°C)
1	T_{15} (**2**)	3.5	38.1	—
2	$T_{13}tT$ (**3**)	>150	35.0	−3.1
3	$T_{12}t_2T$ (**4**)	>150	30.1	−4.0
4	$T_{11}t_3T$ (**5**)	>150	27.0	−3.7

[a]Capital and small letters stand for DNA and HO–NAP, respectively. [b]Hybridization studies were performed with poly(rA) in a phosphate buffered solution (10 mM) at pH 7, with a NaCl concentration of 100 mM.

Table 2. Sequences of 2-5A-DNAs **6–8**



Structure of 2-5A-bu_2

Entry	Compound	Sequence[a]
1	**6**	ps2-5A-bu_2-AAA.AAT.GGG.GCA.AAT.A
2	**7**	p2-5A-bu_2-AAA.AAT.GGG.GCA.AAt.A
3	**8**	ps2-5A-bu_2-AAA.AAT.GGG.GCA.AAt.A

[a]"p" denotes a terminal phosphate, whereas "ps" stands for a terminal phosphorothioate, "bu" indicates a butyl spacer and "t" indicates a thymine HO–NAP moiety.

HPLC analysis revealed that unmodified 2-5A-DNA **6** was completely degraded within the hour by snake venom phosphodiesterase (Fig. 2(a)). In sharp contrast, HO-NAP containing 2-5A-DNAs **7** and **8** were inert towards digestion by SVP. Thus, after prolonged incubation (48 h) under the same conditions, no trace of degradation products could be detected (Fig. 2(b)).

In order to determine the biological activity, we employed two different methodologies.[26,27] The first provided a measure of the ability of the modified 2-5A-DNA to bind to human RNase L as determined by competition of the analogue 2-5A-DNA with p5′A2′p5′A2′p5′A2′p5′A3′[^{32}P]p5′C3′p.[26] In this assay, all three analogues (**6**, **7**, **8**) displayed identical binding affinities for RNase L (Table 3). The second assay[27] addressed the relative ability of the analogues to activate RNase L to cleave poly(U)p[^{32}P]Cp. It is apparent from the data that introduction of the HO-NAP residue did not cause any diminution in the ability of the 2-5A-DNA chimera to activate human RNase L. The EC_{50}'s for **6** and **8** were the same. When the 5′-terminal monophosphorothioate of compound **8** was converted to a simple 5′-monophosphate, there resulted a marginal (3-fold) increase in activation ability which was not related to a parallel increase in binding, thereby suggesting that the small potency difference was related to post-binding events. In vivo this may be offset by an increased susceptibility of the simple phosphate to phosphatase action. This differential behavior of 2-5A 5′-monophosphates as compared to 5′-monophosphorothioates has not been observed previously. In addition to these findings, preliminary results indicated that all three 2-5A-DNAs (**6–8**) inhibited the replication of RSV in primary cotton rat cells with similar efficiency.

Table 3. RNase L binding and activation by 2-5A-DNAs **6–8**

Entry	Compound	IC_{50} (nM)[a]	EC_{50} (nM)[b]
1	**6**	57 ± 16	27 ± 7
2	**7**	60 ± 12	7.3 ± 1.7
3	**8**	62 ± 5	23 ± 4

[a]Radiobinding assays were performed in human RNase L-containing CEM cell lysate ($n=4$). IC_{50} is the concentration of compound required to displace 50% of ^{32}P-labeled 2-5A probe.
[b]RNase L activation assays were performed in vitro with a poly(U) substrate ($n=3$–4). EC_{50} is the concentration of compound required to induce RNase L cleavage of 50% of the substrate.

Conclusions

The results presented in this paper show that the anchoring of a 4-hydroxy-*N*-acetylprolinol unit at the 3′-end of an oligodeoxynucleotide is completely compatible with the 2-5A-antisense strategy. Introduction of one HO-NAP residue allows full activation potency of the 2-5A-DNA chimeras as compared to parent unmodified 2-5A-DNA constructs, minimally diminishes affinity for target mRNA, and at the same time imparts substantial stability to exonuclease degradation. Insofar as the HO-NAP modification should not endow an oligonucleotide backbone with many of the non-specific biological effects that characterize phosphorothioate nucleic acids,[28] it may be a good companion for further application to the 2-5A-antisense strategy of RNA ablation.

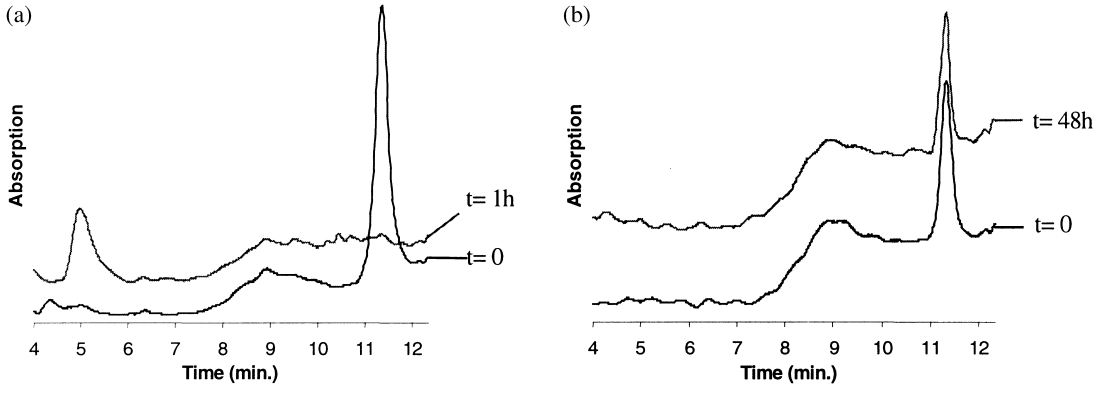


Figure 2. Upon addition of 1 unit of SVP to 0.6 OD of the oligomer in 2.0 mL Tris–HCl buffer (100 mM, pH 8.6, containing 100 mM NaCl and 14 mM $MgCl_2$), RP-HPLC was performed on a Biocad Vision reversed phase HPLC system equipped with a Platinum C-18 column applying a gradient of acetonitrile (0–20%) in triethylammonium acetate buffers (50 mM, pH 7). HPLC pattern of digestion of (a) **6** and (b) **8** by snake venom phosphodiesterase.

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