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Terminally L-modified oligonucleotides: pairing, stability and biological properties

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An oligodeoxynucleotide (ODN), capable of reducing the growth of human B lymphocytes carrying the t(14;18) chromosome translocation, was prepared in different 'chemical versions': unmodified phosphodiester, phosphorothioate and phosphodiester capped with L-2'-deoxycytidine. Their binding affinity to the complementary synthetic target was studied by the melting point assay. The ODNs, administered to DOHH2 cells, were compared for stability in the culture medium, cellular uptake, time course of the intact sequence concentration within the cell and ability to inhibit cell growth. The 5',3'-L-capped derivative and the phosphorothioate had comparable potency, superior to that of the unmodified ODN, in agreement with the concentration of undegraded ODNs within the cell.

Key words: Antisense oligodeoxynucleotides, cellular uptake, stability, antitumor activity.

Introduction

Antisense oligonucleotides (aODN) have emerged as a promising new modality to study gene expression[1,2] and are under experimental development for potentially therapeutic purposes.[3–5] These compounds, which can effectively target messenger RNA, have actually shown some favorable properties over more traditional protein-targeting drugs. The greatest advantages derive from the specificity of the Watson–Crick base pairing by which they interact with the target RNA, that offers the ground for a rational design of pharmacological active molecules.

However, for successful outcome *in vivo*, aODN must satisfy a number of pharmacokinetic criteria recently reviewed in some detail.[6] Degradation of unmodified phosphorodiester ODN by soluble and cellular nucleases makes their use in animals and man not easily suitable. Resistance to biological degradation has been addressed mainly by chemical synthesis of ODN analogs with modified internucleotidic linkages such as phosphorothioates (PS) and methylphosphonates,[3] and with modified sugars such as 2'-*O*-alkylnucleotides.[7]

Primarily the PS derivatives are used for the preparation of ODN to be administered *in vivo*, because they are highly resistant to endogenous nucleases and form sequence-specific duplexes with the target sequences, though with reduced target affinity relative to unmodified ODNs. Their main drawback is, however, the tendency to act also by non-antisense mechanisms, like direct interaction with cellular proteins, in both sequence-specific and non-specific ways.[5,8]

Since *in vivo* degradation of ODNs is largely produced by exonucleases, a modality to improve their stability is to cap the sequence with nuclease-resistant structures like hairpin loops [9] or thioated phosphate linkages.[10] Following this approach, the 5' and/or the 3' terminus of an 18 mer ODN under study in our laboratory was capped with an L-2'-deoxynucleoside, and the annealing capacity, stability, cellular uptake and biological activity of the sequences were compared with those of the unmodified and fully phosphorothioate-modified analogs.

Materials and methods

Oligodeoxynucleotides

The following ODNs (5'–3') were used:

PO TCC.CTG.GTT.CCC.CGA.ATA
PS TCC.CTG.GTT.CCC.CGA.ATA
3'-L TCC.CTG.GTT.CCC.CGA.ATA.
5'3'-L *C*.TCC.CTG.GTT.CCC.CGA.ATA. *C*C

Unmodified PO and fully phosphorothioate PS were commercial products whose purity was checked by HPLC (≥90%) and denaturating PAGE. 3'-L and 3',5'-L (*C* means L-2'-deoxycytidine) were prepared

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by automated solid phase synthesis using phosphoramidite chemistry and deprotection by standard procedures. L-2'-deoxycytidine and the corresponding protected phosphoroamidite were synthesized as reported elsewhere,[11] starting from L-(+)-arabinose. Since commercial derivatized supports were used, the two L-modified sequences carry an irrelevant extra D-nucleoside at the 3'-end.[11] The crude oligonucleotides were purified by anion exchange chromatography (DEAE–Sephacel) and converted into sodium salts.

UV melting experiments

Mixtures of equimolar water solutions of each strand with the complementary DNA strand were diluted to 3 μM per strand in the buffer solution (0.1 M Tris–HCl, 0.1 M NaCl corrected to pH 7 with HCl 1 N), heated to 90°C for 15 min, then slowly cooled. During the melting experiments the temperature gradient never exceeded 0.5°C/min, starting from 4°C (moisture condensation on the walls of the cells was prevented by flushing nitrogen inside the cell holder). UV absorbance at 260 nm was recorded at every degree.

Tumor cells

The human follicular B cell lymphoma line DOHH2[12] carrying the t(14;18) chromosome translocation was maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) under standard conditions. The cells were mycoplasma free and viability was controlled by the Trypan blue dye exclusion assay.

Stability and cellular uptake assays

ODNs were 5' labeled with [^{32}P]ATP (5000 Ci/mol; Amity, Milan, Italy) using T4 polynucleotide kinase (Perkin Elmer, Norwalk, CT) and purified by 20% PAGE under denaturating conditions, followed by chromatography on a Sep-Pak C18 column and precipitation with ethanol.[13] DOHH2 cell cultures, 10^5/ml, in medium containing 10% FCS heat-inac-

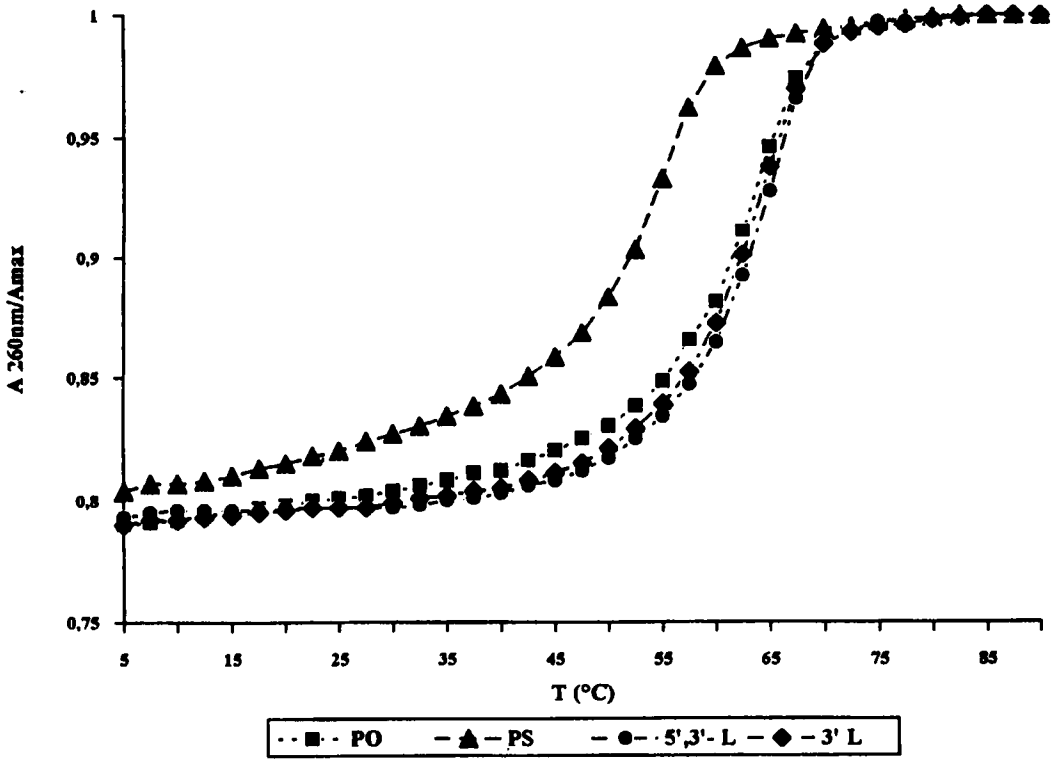

Figure 1. UV melting profiles of equimolar mixtures (3 μM for each strand) of the indicated ODNs with the complementary DNA strand. The $A_{260\ nm}$ values are normalized with respect to the corresponding maximum UV absorbance at 90°C.

tivated for 30 min at 65°C, were incubated at 37°C with ODN 10 μM (1 μM of [^{32}P]ODN plus 9 μM of cold ODN) for the indicated time.

The ODNs in the supernatants were analyzed by 20% PAGE under denaturating conditions, autoradiography was quantified by scanner densitometry and the area was integrated.

Intracellular ODNs were extracted from DOHH2 cells, previously washed in cold medium, by lysing with 2% SDS, 1% β-mercaptoethanol and 7 M urea with phenol–chloroform. They were analyzed by 20% PAGE and intact ODN content was measured as above. The intracellular concentration was calculated assuming a cell volume of 10^{-9} ml. The concentration values indicated are the average of three experiments.

Growth inhibition assay

ODNs, 10 μM, were given daily or in single dose, at the onset of the experiment, immediately after the DOHH2 cells, 10^4/100 μl, were seeded in microplates. Cell growth was evaluated on day 5 by counting viable cells under a microscope by two independent observers and by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay.[14] Assays were run in triplicate and data normalized from three experiments.

Results

An oligodeoxynucleotide sequence (5′-TCC CTG GTT CCC CGA ATA-3′), previously found to exert a specific activity against the growth of the DOHH2 human follicular B cell lymphoma line,[15] was prepared as:

The phosphodiester derivative: **PO**.
The fully phosphorothioate derivative: **PS**.
The 5′,3′-L-dC capped phosphodiester derivative: **5′,3′-L**.
The 3′-L-dC capped phosphodiester derivative: **3′-L**.

The four ODNs were annealed to the complementary DNA sequence and the thermal stability of the resulting duplexes was evaluated by a UV melting experiment. As shown in Figure 1, capping of the natural phosphodiester sequence with L-2′-deoxycytidine at one or both ends did not affect the stability of the duplex (T_m = 65°C), which was instead lowered by about 10°C when PS-ODN was used.

Next, the stability of the radiolabeled ODN derivatives was evaluated in RPMI 1640 culture medium, containing 10% of heat-inactivated FCS, *in the presence of DOHH2 cells*, by PAGE, under denaturating conditions, followed by quantitative densitometry. As shown in Figure 2, the persistence of the starting oligonucleotide was very much dependent upon the chemical structure. After 8 h at 37°C, the concentration of intact PS-ODN and 5′,3′-L-ODN was reduced by 15 and 21%, respectively, while that of 3′ L-ODN and PO-ODN was only 50 and 75%, respectively, of the starting value. After 24 h, the last two derivatives were largely degraded, whereas the concentration of the PS and the 5′,3′-L derivatives was still 82 and 61%, respectively, of the initial value. Thus, under these experimental conditions, the presence of the L-nucleoside, at both ends of the phosphodiester oligonucleotide, greatly enhanced its stability toward nuclease degradation, while protection at just the 3′ terminus was much less efficient.

The time course of intracellular concentration of PO, PS and 5′,3′-L was determined by measuring, with scanning densitometry, the amount of undegraded, radiolabeled oligonucleotide obtained by cells lysis followed by gel electrophoresis and assuming a volume of 10^{-9} ml/cell. The results are presented in Figure 3. All three derivatives are rapidly taken up by the DOHH2 cells, reaching the maximum value at about 2 h. At this time point the concentration of undegraded material inside the cells is about 30 (PO) and 15 (PS and 5′,3′-L) times higher than in the medium. After 8 h, these values were practically unchanged for both modified deri-

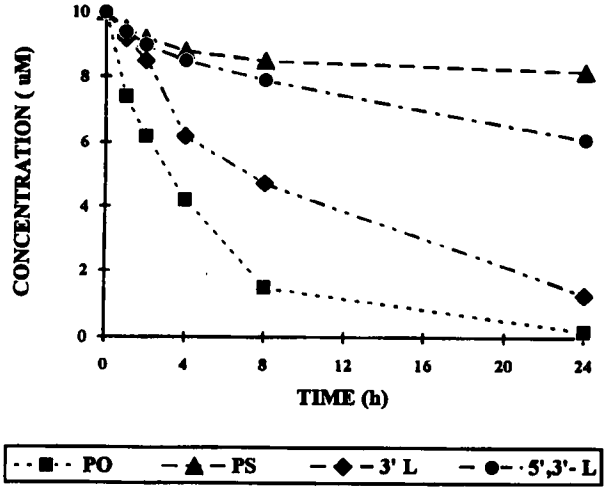

Figure 2. Concentration (μM) of undegraded ODN in the supernatant of DOHH2 cell culture.

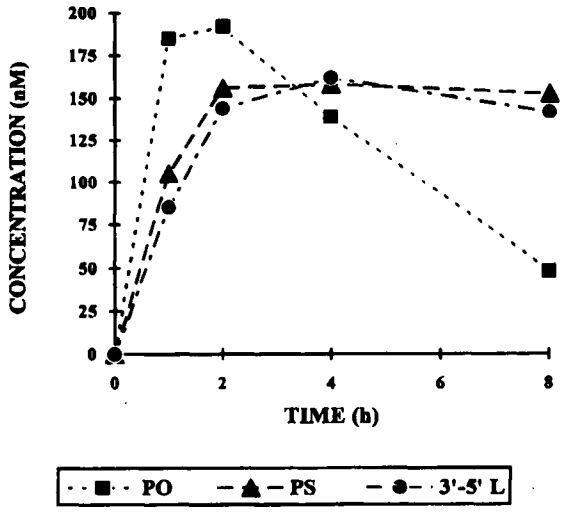

Figure 3. Intracellular concentration (nM) of undegraded ODN.

vatives; the natural PO derivative, instead, was reduced to 25% of its peak level. After 24 h (data not shown), more than 50% of integral PS and L-capped sequences was found; the PO-ODN instead was undetectable.

The biological activity of the modified ODNs was studied by the DOHH2 cell growth-inhibition assay. The ODN sequence specificity and the DOHH2 restricted activity has been reported.[15] Based on the observed stability and intracellular concentration, two schedules were used: a single dose at the time of cell seeding or daily treatments. The cell cultures were terminated on day 5. The PS-ODN was given once and the PO-ODN was administered daily. Both schedules were used to evaluate the activity of the L-capped ODNs. The DOHH2 cell number was reduced by 50% by a single dose, on day 0, of both PS-ODN and 5',3'-L-ODN. No additional specific activity was obtained by a daily treatment. In contrast, DOHH2 cell number was not reduced by a single treatment with PO-ODN (not shown) or 3'-L-ODN, while growth inhibition of 50% was achieved by daily treatment with both compounds (Table 1).

Table 1. Inhibition of growth of DOHH2 cells in the presence of the indicated ODNs

ODN	Cells ($\times 10^4$/ml ± SE)	MTT (OD[a] $\times 10^3$ ± SE)
–	135 ± 10	860 ± 78
PO[b]	72 ± 6[d]	444 ± 35
PS[c]	74 ± 5[d]	463 ± 39
3'-L[b]	78 ± 3[d]	432 ± 41
3'-L[c]	132 ± 14	793 ± 67
5',3'-L[c]	81 ± 6[d]	473 ± 29

[a]Optical density.
[b]Daily administration.
[c]Single dose administration.
[d]$p \leq 0.05$

Discussion

Oligonucleotides have great promise as agents for blocking gene expression in a specific way, thus they may be very useful as tools in molecular and cell biology and as potential therapeutic agents. One of the many hurdles to overcome towards these aims is the sensitivity of the natural phosphodiester bond to the degradation by serum and intracellular nucleases. Several modifications of the phosphodiester backbone have resulted in improved stability; among the many ODN analogs which have been developed, the most widely used are the phosphorothioates (PS-ODN), where one of the non-bridging oxygens in each phosphate moiety is replaced by a sulfur atom. They hybridize to the target sequence with high specificity, but, in general, with slightly reduced affinity than the corresponding phosphodiester ODNs. Their major drawback is, however, a propensity to interact with cellular proteins by both sequence-specific and non-specific mechanisms.[5,8,16–18] This finding has lead to the synthesis and use of ODN analogs with the lowest possible degree of chemical modification compatible with good stability, like PO-ODN with PS linkage caps at the sequence termini,[10] and to structural modifications that can serve the same purpose, like flanking an active PO sequence with hairpin structures endowed with nucleolytic resistance.[9,19,20] We have already investigated the use, as potential antimessenger oligonucleotides, of L-DNAs, the enantiomers of the natural sequences, which are extremely resistant to nucleolytic enzymes;[21] unfortunately they fail to bind to complementary, single-stranded, natural sequences.[11] Since, however, it was known that the covalent attachment of L-2'-deoxycytidine (L-dC) at the ends of an unmodified oligomer provides good stability toward exonuclease hydrolysis,[22] this type of capping strategy was used to prepare the sequences 3'-L-ODN, with L-dC at the 3' end and 5',3'-L-ODN, with L-dC at both termini. The behavior of these modified oligonucleotides was compared to that of the unmodified PO-ODN and of fully phosphorothioate PS-ODN. As expected, the terminally modified oligomers had the same hybridization ability of the natural

sequence, quite superior to that of the PS derivative. The stability in culture medium, *in the presence of DOHH2 cells*, of the 5′,3′-L-ODN was comparable to that of the fully thioated derivative, while that of the 3′-L-ODN was far lower, though still larger than that of the unmodified sequence. This result is quite surprising, since it is generally assumed that degradation, both in the medium and within cells, is mostly due to the activity of 3′ exonuclease. Inside cells, the concentration of undegraded PS and 5′,3′-L oligomers reached a maximum at 2 h and, most interestingly, remained at about this level for several hours with both compounds, while that of the natural ODN decreased rapidly, in agreement with the observations made at the extracellular level. Thus, within DOHH2 cells under the present experimental conditions, the activity of endonucleases must be rather low, since, in contrast to the transformation of all phosphodiester linkages into phosphorothioates, end-capping offers no obvious protection toward this type of nucleolytic enzymes. The pattern of the biological activity of the different oligonucleotide derivatives parallels rather well that of their concentration and persistence, as full length sequence, within the cells. The sizeable increase in stability and biological activity achieved in the present case, by just capping a natural phosphodiester oligomer with L-dC, cannot yet be considered as a strategy to improve the performance of natural ODN, in general. For example, the resistance to nucleolytic degradation of other modified oligonucleotides depends upon the specific sequence, not just the type of chemical modification.[23] Most important, the uptake, stability and intracellular localization of modified as well as natural oligonucleotides is very much dependent upon the cell type and the modality of ODN treatment.[5,24,25].

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