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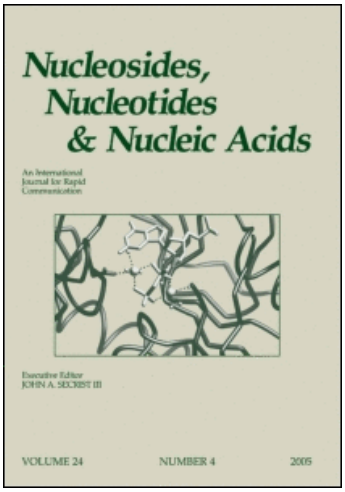

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OLIGONUCLEOTIDE CONJUGATED TO LINEAR AND BRANCHED HIGH MOLECULAR WEIGHT POLYETHYLENE GLYCOL AS SUBSTRATES FOR RNase H.

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ABSTRACT: Two conjugates of an anti-HIV oligonucleotide (ODN) with different high molecular weight monomethoxy polyethylene glycols (MPEGs) have been tested for their activity as substrate towards RNase H. The MPEG does not impede the formation of the regular hybrid duplex with the target RNA sequence as pointed out by the persistence of the RNase H activity; thus, these derivatives stimulate the hydrolysis of RNA by the enzyme at the same site and with the same extent of cleavage as the native sequence.

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Introduction.

It is an unquestionable fact that an oligodeoxynucleotide complementary to an mRNA sequence can inhibit gene expression when taken up by mammalian cells.[1,2] This could be accomplished through steric hindrance or degradation by RNase H, an ubiquitary ribonuclease able to interact with DNA.RNA hybrids.[3] Evidence for RNase H dependent mode of action has been presented for mRNA translational inhibition by complementary ODNs in cell-free systems.[4] It is known that RNase H specifically and endonucleolytically degrades the phosphodiester bond of the RNA strand of a DNA.RNA hybrid, and produces 5'-phosphate and 3'-hydroxyl termini.[5]

As a consequence of their easy *in vivo* degradation and difficulties in targeting and cell penetration, various modifications of the ODNs have been extensively investigated with the objective of allowing the hybridization with the RNA target on the

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one hand, and introducing more favorable pharmacological properties on the other .[6] To this purpose the conjugation of ODNs with different molecules have been investigated; among few polymeric units, high molecular mass polyethylene glycol (PEG) received much attention due to absence of toxicity and the profitable amphiphilicity.[7]

Recently, we have synthesized an antisense oligonucleotide conjugated to different high molecular mass MPEGs.[8] The melting behavior and nuclease stability of these derivatives have been investigated,[9] and their antisense activity against HIV-1 infection have been measured.[10] In this paper the ability of these MPEG-conjugated to form hybrid duplexes with the complementary RNA sequence and to act as substrates toward an RNase H from E. Coli is reported.

Results

In the previous paper[9] the biological features of an antisense oligonucleotide conjugated with the linear MPEG, as well as the branched $(MPEG)_2$, have been investigated and compared with those of the native molecule. From these studies it has been ascertained that the high-molecular mass conjugated polymers do not modify the thermal stability of the duplex formed with its complementary sequence, whilst they are responsible for an effective protection of the supported ODNs from biodegradation. These effects are almost identical for the two MPEGs employed. On the other hand, only in case of the linear MPEG-conjugated derivative has an antisense activity been observed.

To further delineate the behavior of the MPEG-conjugated 12mers, their ability to complex the complementary RNA sequence and, consequently, to be a substrate for the RNase H from E. Coli have been tested, and compared to that of the native, unmodified sequence. In fact, the capacity of ODNs to form hybrids with mRNA that are substrates for RNase H is considered an important feature in the design of any nucleic acid derivative to be proposed as real antisense agents.[11] The target sequence and the structure of the investigated compounds are reported in FIG.1

Kinetic experiments show that the hydrolytic reactions are completed within one hour at 20 °C, and that, in the presence of any of the investigated 12mers, the cleavage proceeds at the two sites U^{14} and A^{17}, as reported in FIG. 2. The effect of the RNase H on the hydrolysis of RNA in the presence of different conjugates, as well as on the

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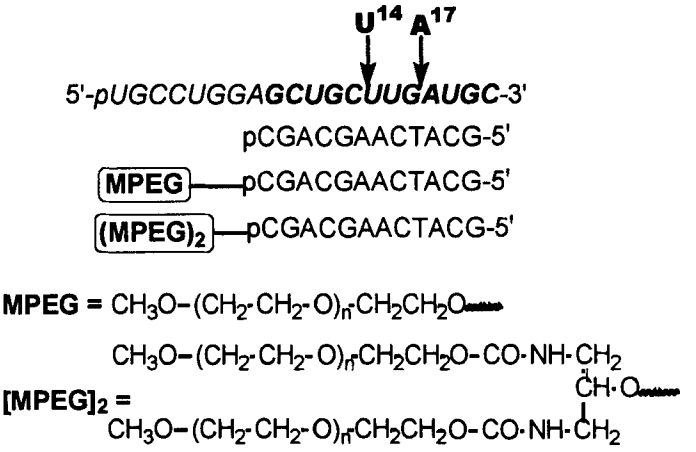

Figure 1. Sequences of the RNA target (italic) and of the investigated ODNs. The arrows indicate the sites of cleavage. The general structures of the conjugated MPEGs are indicated.

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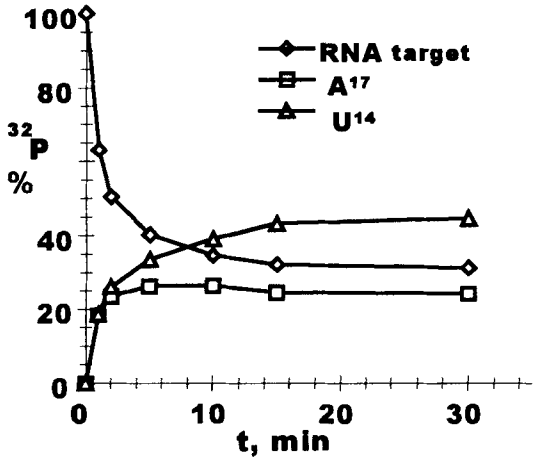

Figure 2. Dependence of the hydrolysis by Rnase H of the RNA component in the DNA-RNA hybrids as a function of the reaction time. Curves designated as U^{14} and A^{17} correspond to the target hydrolyzed at the phosphodiester bond between U^{13} and U^{14}, and A^{17} and U^{18} respectively.

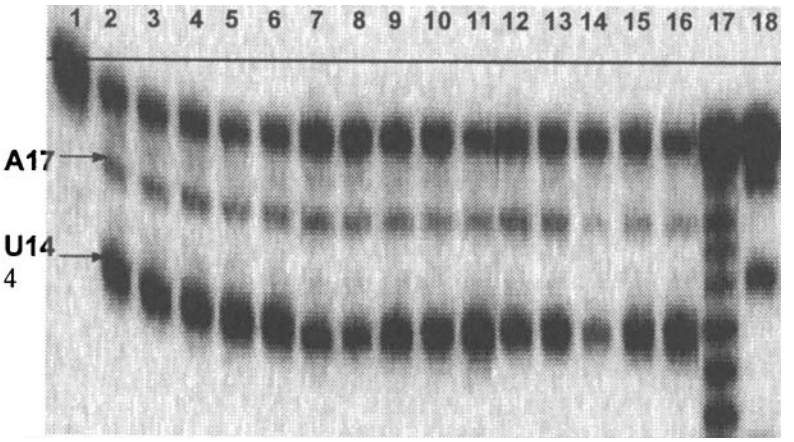

Figure 3. Autoradiogram of the sequencing gel showing the RNA target cleavage by Rnase H in presence of the different 12mers. Lanes: 1, target RNA in the reaction conditions; 2-6, target degradation in the presence of the native 12mer; 7-11, target degradation in presence of the MPEG-conjugated 12mer 12-16, target degradation in presence of the $(MPEG)_2$-conjugated 12mer [the concentrations of the ODN range from 10^{-6} to 2 x 10^{-5} M]; 17, incomplete sodium bicarbonate hydrolysis of the target RNA; 18, incomplete T_1 ribonuclease hydrolysis of the target RNA.

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native sequence, is shown in FIG. 3. The limiting extent of the cleavage depends on the concentration of the added 12mer and of its MPEG-conjugates. Its value achieved 55-72 %, depending on the concentration of the 12mer probe. The maximum extent of cleavage for all the derivatives has been attained at the probe concentration of about 10^{-6} M.

Conclusions.

Under the reported conditions, the MPEG-conjugation does not affect the structure of the hybrid duplex nor the consequent RNase H activity; thus, these derivatives stimulate the hydrolysis of the RNA by the enzyme at the same site and with the same extent of cleavage as the native sequence. From these data it is not possible to recognize any significant difference between the linear MPEG and branched $(MPEG)_2$ moieties conjugated to the same ODN. In conclusion both the high-molecular mass MPEGs do not impede the formation of the regular hybrid duplex with the target RNA

sequence of the conjugated oligonucleotides, acting as regular substrates for RNase H. Hence, the absence of antisense activity of the same branched conjugate, as previously reported[10], can not be charged to inefficacy of interaction of this DNA.RNA hybrid with RNase H.

Experimental

Oligonucleotides : MPEG-conjugated 12mers has been synthesized in a 0.1 mmolar scale following the published procedures.[8] Native 12mer was acquired from Genset-France as HPLC purified product and used as such. 20mer RNA for RNase H assay was kindly provided by Dr. A. G. Venyaminova, and Dr. M. N. Repkova, (Novosibirsk Institute of Bioorganic Chemistry, Russia). Other oligonucleotides were synthesized in Zarytova's laboratory following standard procedures, and modified at their extremities as reported.[12] The concentrations of the oligonucleotides in the reported experiments were determined spectophotometrically, by using a molar extinction coefficient at 260 nm of 117,300 M^{-1} x l x cm for each 12mer and 185,600 for the target RNA.

RNA labeling :_The target 20mer RNA was ^{32}P-labelled with [γ-^{32}P] ATP, 0.1 mCi in 0.015 ml of a mixture containing 50 mM TrisHCl, 10 mM $MgCl_2$, 0.1 mM spermidine, 0.1 mM EDTA, 5 mM dithiothreitol (DTT) , pH 7.6, containing 5 U of T4 polynucleotide kinase (SibEnzym). Labeled RNA was purified by 20% PAGE, and eluted from the gel in a 0.25 M NH_4COOH, 0.5 mM EDTA, 0.05 M Na dodecylsulfate, and precipitated with ethanol.

RNase H assay :_The experiments were carried out in a 20 mM HEPES buffer, pH 8.0, containing 50 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT. The RNA concentration was 10^{-7} M, while ODNs concentration ranged from 10^{-7} up to $2x10^{-6}$ M. The reaction mixtures (10 µl) were incubated at 20 °C for 15 min, then 0.15 U of RNase H (Promega) were added. Digestion was carried out for 1 h at 20 °C, then 1 µl of a 0.1 γ/ µl of a polyuridine solution was added to each probe. Reaction mixtures were precipitated by a 2% $LiClO_4$ in acetone, dissolved in 2 µl of a urea-dyes solution and loaded on to 20% polyacrylamide gel. The kinetic experiments were performed under the same conditions.

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