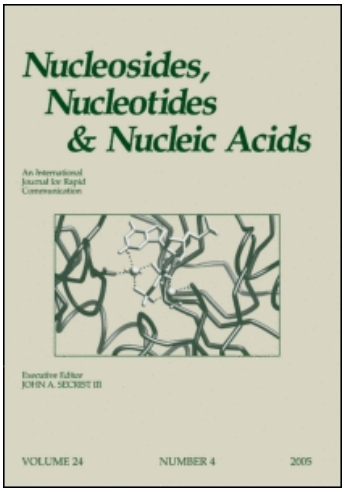

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Ötvös, L. , Sági, J. , Sági, Gy. and Szemz, A.(1999) 'Base Modified Oligodeoxynucleotides. II. Increase of Stability to Nucleases by 5-Alkyl-, 5-(1-Alkenyl)-, and 5-(1-Alkynyl)-pyrimidines', Nucleosides, Nucleotides and Nucleic Acids, 18: 9, 1929 — 1933
To link to this Article: DOI: 10.1080/07328319908044854
URL: http://dx.doi.org/10.1080/07328319908044854

NUCLEOSIDES & NUCLEOTIDES, 18(9), 1929–1933 (1999)

BASE MODIFIED OLIGODEOXYNUCLEOTIDES. II[1]. INCREASE OF STABILITY TO NUCLEASES BY 5-ALKYL-, 5-(1-ALKENYL)- AND 5-(1-ALKYNYL)-PYRIMIDINES

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ABSTRACT. The effect of pyrimidine base substitution on the sensitivity of oligonucleotides to nucleases has been studied with two series of self complementary deoxyoligonucleotides containing n-alkyl, n-(1-alkenyl) or n-(1-alkynyl) groups at C5 of pyrimidines, $(dA\text{-}r^5dU)_{10}$ and $(dG\text{-}r^5dC)_6$. The rate of hydrolysis by snake venom phosphodiesterase and in human serum decreased with increasing length and unsaturation of the substituent.

INTRODUCTION. The protection of antisense oligonucleotides from the degradation of nucleases is one of the major tasks currently facing the successful development of antisense therapeutics. Numerous nucleases exist and have been shown to degrade oligonucleotides. In serum the dominant nuclease activity is 3'-exonuclease. In cells and other bodily fluids both 3'- and 5'-exonucleases and endonucleases are present. A wide range of modifications have been used to enhance the stability of oligonucleotides against nucleases (2-5). C5 substitution of pyrimidines offers major groove modification without affecting the hybridization properties of antisense oligonucleotides (6). Moreover, increased duplex stability was found for self-complementary oligonucleotides, $(dA\text{-}r^5dU)_{10}$ and $(dG\text{-}r^5dC)_6$ derivatized by 1-alkynyl groups at C5 of pyrimidines compared to $(dA\text{-}dT)_{10}$ and $(dG\text{-}dC)_6$, respectively (7). Self-complementary polynucleotides, poly $(dG\text{-}r^5dU)$ containing n-alkyl groups longer than methyl at C5 of uracil were compared with poly (dA-dT) and showed enhanced stability toward pancreatic DNAse, spleen DNAse, snake venom phosphodiesterase and micrococcal

nuclease (8). Enzymic hydrolysis studies have been extended to the two series of self-complementary oligonucleotide derivatives $(dA\text{-}r^5dU)_{10}$ and $(dG\text{-}r^5dC)_6$ mentioned above. Self complementary oligonucleotides were used to slow down the hydrolysis rate to a measurable level due to hybridized duplexes. This paper summarizes the results of investigations with snake venom phosphodiesterase and human serum.

RESULTS. Initial rates for the enzymic hydrolysis by snake venom phosphodiesterase, (PDE) and human serum of two series of self-complementary oligonucleotides derivatized by n-alkyl, n-(1-alkenyl) or n-(1-alkynyl) groups at C5 of pyrimidines, $(dA\text{-}r^5dU)_{10}$ and $(dG\text{-}r^5dC)_6$, were determined and compared with those found for the natural compounds $(dA\text{-}dT)_{10}$ and $(dG\text{-}dC)_6$, respectively. Results obtained with PDE are summarized in Table 1. For oligos derivatized by an n-alkyl group, the initial rate of hydrolysis was continuously decreased with increasing length of the substituent in both series. Members of the $(dG\text{-}r^5dC)_6$ series proved to be more resistant than those of the $(dA\text{-}r^5dU)_{10}$ series. No degradation was observed with oligos containing a substituent longer than five carbon atoms during 30 min. Oligos having alkenyl or alkynyl groups showed a sharper rate decrease with chain length.

Initial rates of degradation of oligos in human serum are given in Table 2. A comparison of the data in Tables 1 and 2 indicates similar trends for the hydrolysis by PDE and the degradation in human serum. However, substitution caused greater increase of resistance to the degradation in human serum than to the hydrolysis by PDE.

In a separate experiment, the half life of $(dA\text{-}^{hexynyl5}dU)_{10}$ was determined in human serum and found to be 330 min at 37°C. The natural 20-mer $(dA\text{-}dT)_{10}$ had a half life of 4.7 min under identical conditions. In the case of $(dG\text{-}^{hexynyl5}dC)_6$, no degradation was detected up to 24 h.

DISCUSSION. Insertion of alkyl groups into position 5 of the pyrimidine rings of $(dA\text{-}dT)_{10}$ and $(dG\text{-}dC)_6$ decreased the initial rate of their degradation by PDE and human serum. In both series the rate was a function of the length of the alkyl chain, and oligomers having n-hexyl or longer alkyl substituent were resistant to hydrolysis during 30 min at 37 °C. A similar, but less expressed tendency was found for poly$(dA\text{-}^{alkyl5}dU)$ derivatives (8). Steric inhibition may provide the major contribution to the increased

TABLE 1. Initial rate of hydrolysis of self-complementary oligonucleotides $(dA\text{-}r^5dU)_{10}$ and $(dG\text{-}r^5dC)_6$ by PDE

5-substituent of the pyrimidine base	Hydrolysis rate[1] $(dA\text{-}r^5dU)_{10}$	rel. rate (%) $(dA\text{-}r^5dU)_{10}$	rate[1] $(dG\text{-}r^5dC)_6$	rel. rate (%) $(dG\text{-}r^5dC)_6$
hydrogen	-	-	137	100
methyl	573	100	117	85.4
ethyl	-	-	104	75.9
propyl	451	78.7	-	-
butyl	157	27.4	67	48.9
pentyl	16	2.8	40	29.2
hexyl	0	0	0	0
vinyl	230	40.1	-	-
(E)-butenyl	144	25.1	-	-
(E)-pentenyl	18	3.2	16	11.7
(E)-hexenyl	0	0	0	0
ethynyl	-	-	19	13.9
propynyl	217	37.9	-	-
pentynyl	0	0	0	0
hexynyl	0	0	0	0

[1] Initial rates were calculated from data obtained after 30 min incubation as described in Experimental

TABLE 2. Initial rate of hydrolysis of self-complementary oligonucleotides $(dA\text{-}r^5dU)_{10}$ and $(dG\text{-}r^5dC)_6$ in human serum

5-substituent of the pyrimidine base	Hydrolysis rate[1] $(dA\text{-}r^5dU)_{10}$	rel. rate (%) $(dA\text{-}r^5dU)_{10}$	rate[1] $(dG\text{-}r^5dC)_6$	rel. rate (%) $(dG\text{-}r^5dC)_6$
hydrogen	-	-	7.8	100
methyl	714.5	100	4.0	51.0
ethyl	-	-	0	0
propyl	256.9	36.0	-	-
butyl	174.5	24.4	-	-
pentyl	0	0	-	-
hexyl	-	-	0	0
vinyl	108.8	15.2	-	-
(E)-butenyl	0	0	-	-
ethynyl	-	-	0	0
propynyl	81.3	11.4	-	-
pentynyl	0	0	0	0
hexynyl	-	-	0	0

[1]Initial rates were calculated from data obtained after 30 min (for 10 mer) or 3 h (for 6 mer) incubation as described in Experimental

stability of base-modified oligonucleotides against nucleases. The 5-alkyl group protruding into the major groove of the double helical oligos may sterically inhibit the nuclease action. The longer is the alkyl group, the greater is the steric inhibition. On the other hand, free rotation of alkenyl or alkynyl substituents around the base may be hindered by conjugation of a double or triple bond of the side chain with the heteroaromatic ring. This rigid side chain may further increase the steric hindrance against nuclease action. The increased hydrophobicity and duplex stability of oligos derivatized by an alkynyl group (7) may also contribute to the observed nuclease resistance.

Results indicate that substitution of the pyrimidine ring at 5 position by n-(1-alkynyl) group of appropriate length may lead to oligonucleotide derivatives which can successfully be used in antisense technology.

EXPERIMENTAL. Self-complementary oligonucleotides (dA-r^{5}dU)$_{10}$ and (dG-r^{5}dC)$_6$ were synthesized, purified and characterized by UV spectrophotometry as described (9). PDE (5 units/mg protein) was from Merck. Human serum taken from a healthy, 26 year old female volunteer, 0RH$^+$, was centrifuged three times at 4 °C by 2000 g for 20 min. Hydrolyses were run at 37 °C using oligomers in concentrations of 1.0 A_{max} unit/mL (~ 5 nmol/mL). Hydrolyses by PDE were carried out in 20 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$ buffer, pH 7.2 with 0.0135 mg of PDE in a final volume of 0.12 mL. For hydrolyses in human serum 0.12 mL of the serum was used. Samples of 0.01 mL were taken from the incubation mixtures at different intervals and injected onto an ion-exchange HPLC column (Protein-Pak DEAE 5 PW, 7.5 x 75 mm, Waters) equipped with a guard column (Nucleosil C18, 5 x 4 mm, BST). In the case of serum analyses a Nucleosil 300-7 Protein RP (100 x 4.6 mm, Macherey-Nagel) column was inserted between the guard and the HPLC columns. The HPLC apparatus consisted of two Waters type 510 pumps, a Waters type 991 diode-array UV detector and a Millipore gradient controller. Separation was done by using a linear gradient of 0.5 M NaCl in 0.02 M sodium phosphate, pH 3.0 (0 → 80 % in 20 min, for (dA-r^{5}dU)$_{10}$) or that of 0.5 M NaCl in 0.02 M sodium phosphate, pH 7.0 (0 → 80 % in 60 min, for (dG-r^{5}dC)$_6$) with a flow rate of 1.0 mL/min. Elution was followed at 260 nm [for (dA-r^{5}dU)$_{10}$] or 280 nm, but absorption data were collected in the range of 250-285 nm (for (dG-r^{5}dC)$_6$).

Initial rate of hydrolysis ($\underline{v}$) was calculated by the formula $\underline{v} = \underline{A}/\underline{a} \times \underline{e} \times \underline{i}$, where $\underline{A}$ is the area of dAMP or dGMP peak, $\underline{a}$ is the amount of oligomer in A_{max} units, $\underline{e}$ is the amount of enzyme in microgram and $\underline{i}$ is the time in minutes [30 min for $(dA\text{-}r^5dU)_{10}$ and 180 min for $(dG\text{-}r^5dC)_6$].

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Received 1/20/99
Accepted 4/7/99