Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 8, pp. 1029-1032, 1994

0960-894X/94 $6.00+0.00

0960-894X(94)E0095-V

EFFECTS OF 5-METHYL SUBSTITUTION IN 2'-*O*-METHYLOLIGO-(PYRIMIDINE)NUCLEOTIDES ON TRIPLE-HELIX FORMATION

Miho Shimizu,+ Tatsuhiko Koizumi, Hideo Inoue, and Eiko Ohtsuka*

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan

Abstract: A 2'-*O*-methyloligo(pyrimidine)nucleotide containing a 5-methyl-2'-*O*-methyluridine (**I**) forms a more stable triple-helix with double-stranded DNA than the 5-unmodified parent probe, whereas 5-methyl-2'-*O*-methylcytidine (**II**) destabilizes the triplex. It is also shown that at low salt concentrations, the triplexes with 2'-*O*-methyl RNAs are more stable than the corresponding DNA-triplex.

Oligonucleotides that bind sequence-specifically to double stranded DNA[1,2] have attracted much attention as potential chemotherapeutic agents, because they can be used to control gene expression at the level of transcription.[3,4] On the other hand, triple-helix forming oligonucleotides (TFOs) with DNA cleaving ability may be useful for mapping long chromosomal DNAs. Various modifications of TFOs at the base, sugar, or phosphate residues have been studied in order to obtain efficient biochemical tools for antisense strategies or genome analysis.[5]

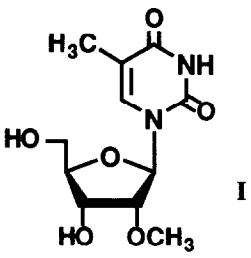
In the pyrimidine•purine•pyrimidine type DNA triplex, which has been more commonly described, a homopyrimidine-TFO can bind parallel to a homopurine sequence in the major groove of a homopurine•homopyrimidine duplex DNA through Hoogsteen bond formation.[1] The triplex contains T•AT and C^+•GC triplets; a protonated C is necessary to form two hydrogen bonds with a GC Watson-Crick base pair.

In the course of our studies of the synthesis and biochemical applications of oligo(2'-*O*-methylribonucleotides),[6-8] we recently demonstrated that the triplex of a 2'-*O*-methyl(pyrimidine)RNA oligomer with a duplex DNA oligomer is more thermally stable than the corresponding DNA triplex;[9] the same result was also obtained by Hélène and co-workers.[10] Although unmodified pyrimidine RNAs also stabilize the triplexes,[9-11] the 2'-*O*-methyl TFOs are more chemically stable, and are relatively nuclease resistant, as compared to the RNAs.[8] In this study, we introduced 5-methyl-2'-*O*-methyluridine (**I**, Um) and/or 5-methyl-2'-*O*-methylcytidine (**II**, Cm) (Figure 1) into a 2'-*O*-methyl(pyrimidine)TFO to find probes with higher binding affinity for duplex DNA, and examined the effects of the 5-methyl modification on the thermal stability of the triplex.

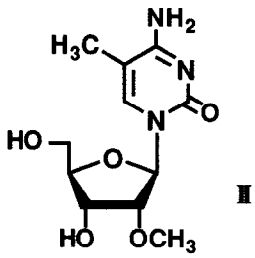
It has been shown that thymidine (dT) and 5-methyl-2'-deoxycytidine (dM) in a DNA-TFO enhance triplex formation relative to 2'-deoxyuridine and 2'-deoxycytidine (dC), and the introduction of the 5-methylcytosine residues extends the pH range for binding to duplex DNA.[12,13] The present results show that the introduction of **I** into 2'-*O*-methyl TFO stabilizes the triplex, but unexpectedly, the substitution with **II** leads to destabilization of the triplex.

Compound **I** was easily prepared from 5-methyluridine (ribothymidine) according to our method[6] described for 2'-*O*-methyluridine (Um). The synthetic intermediate, the 3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl) derivative of **I**, was also converted by the $POCl_3$/triazole procedure[14] to the cytidine counterpart, which upon N^4-benzoylation followed by sugar-deprotection, afforded N^4-benzoyl-5-methyl-2'-*O*-methylcytidine. This compound and **I** were protected and phosphitylated by standard procedures and incorporated into TFOs on a

DNA/RNA synthesizer using the phosphoramidite method. Oligonucleotide products were purified as described previously.[9]



I



II

TFOs		Target DNAs	
m9:	5' m(UCCCUUCUC) 3'	DD15:	
U-m9:	5' m(UCCCUUCUC) 3'	5' d(GGGAGGGAAGAGAAA) 3'	
C-m9:	5' m(UCCCUUCUC) 3'	3' d(CCCTCCCTTCTCTTT) 5'	
U,C-m9:	5' m(UCCCUUCUC) 3'		
r9:	5' r(UCCCUUCUC) 3'	HD34:	
d9:	5' d(TCCCTTCTC) 3'	5' d(GGGAGGGAAGAGAAA T T	
M-d9:	5' d(TMMMTTMTC) 3'	(CCCTCCCTTCTCTTT T T	

Figure 1: Structure of 5-methylpyrimidine 2'-*O*-methylribosides and sequences of TFOs and the target DNAs; m, r, d denote 2'-*O*-methyl ribooligomer, ribooligomer, 2'-deoxyribooligomer, respectively. The underlines indicate the presence of the 5-methyl substituents.

We used seven kinds of 9-mer pyrimidine-TFOs, and as a target, a 15-mer duplex DNA (DD15) or a 34-mer hairpin DNA (HD34) for triplex formation (Figure 1). The thermal stabilities (T_m values) of the triplexes were evaluated by determination of the UV-absorbance (260 nm)-temperature profiles as described.[9] The pK_a values of the TFOs, which would indicate the apparent pK_a's of cytosine bases, were also measured: pK_a = 4.3 for m9; 4.2 for U-m9; 4.0 for C-m9; 4.2 for U,C-m9; 4.6 for r9; 4.5 for d9; and 4.8 for M-d9. The data revealed that the TFO with the lowest pK_a was C-m9, although a base-methyl substituent should be an electron-donating group; as expected, M-d9 showed higher pK_a value than d9.[15]

Tm measurements were carried out in a solution at pH 5.0. Under these conditions, the cytosines or 5-methylcytosines on the TFOs may be sufficiently protonated within the triplex.[16] The thermal denaturation profiles of the triplexes with DD15 showed monophasic or near-monophasic helix-coil transitions, which were similar to those observed for the triplexes of the 15-mer TFOs and the same DD15.[9] The target DD15 had a T_m of 46.2°C. The triplexes with HD34 showed a biphasic dissociation; the first transition corresponds to the melting of the third strand, and the second transition corresponds to the denaturation of the hairpin DNA (T_m = 72.5°C). The triplex formation (at pH 5.4) was also confirmed by a gel retardation assay[9] using a 20% native polyacrylamide gel (data not shown).

The triplexes with the 5-methyluracil-containing 2'-*O*-methyl TFO (U-m9) had the highest T_m values for the denaturation in both triplex-types, as shown in Table 1. The thermodynamic parameters for triplex formation were obtained[17] using the triplexes with DD15, and are listed in Table 2. The data clearly show that U-m9 forms the most stable triplex with DD15. On the other hand, the introduction of 5-methylcytosines to 2'-*O*-methyl TFOs

Table 1: Melting temperatures (T_m) of the triplexes of TFOs and the target DNAs

	m9	U-m9	C-m9	U,C-m9	r9	d9	M-d9
	T_m (°C)						
DD15 [a]	54.9	55.3	52.0	49.3	48.1	nd*	43.4
HD34 [b]	61.6	63.8	56.9	53.8	48.7	33.3	41.3

T_m values were determined in a buffer containing 10 mM sodium acetate (pH 5.0), 100 mM NaCl, and 0.1 mM EDTA and the concentration of TFO•target DNA was a) 1 μM or b) 2 μM. *not determined.

Table 2: Thermodynamic parameters for triplex formation of TFO•target DD15

	$-\Delta H°$	$-\Delta S°$	$-\Delta G°_{25}$
	kJ / mol	J / K mol	kJ / mol
m9	703	2024	100
U-m9	1539	4566	178
C-m9	630	1817	85
U,C-m9	317	864	60
r9	312	851	58
d9*	229	638	38
M-d9	289	792	53

Data were obtained in the buffer indicated in Table 1. The values were derived from the T_m^{-1} vs. log (C_t / 6) plots.[17] *HD34 was used.

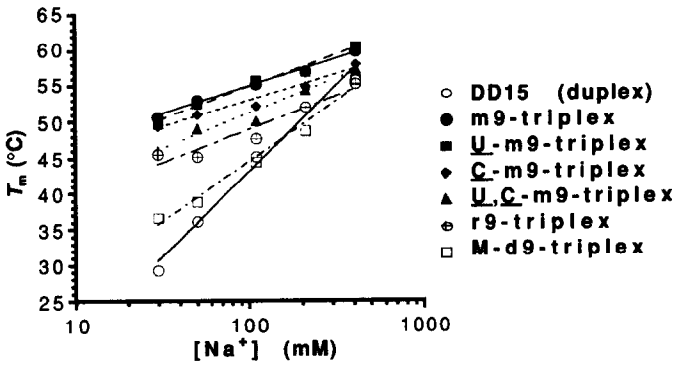


Figure 2: Salt dependence of T_m's for the duplex DNA (DD15) and triplexes with TFOs; Data were obtained in the buffer indicated in Table 1, with an additional 20-400 mM NaCl, and the concentration of either DD15 or TFO•DD15 was 2 μM.

destabilized the triplexes. The stabilities of various TFO-triplexes are in the order, U-m9 ($\Delta G°_{25°C}$ = −178 kJ/mol) > m9 ($\Delta G°$ = −100) > C-m9 ($\Delta G°$ = −85) > U,C-m9 ($\Delta G°$ = −60) > r9 ($\Delta G°$ = −58) > M-d9 ($\Delta G°$ = −53)

> d9 ($\Delta G°$ = –38*). At pH 6.0, triplex formation could hardly been observed under conditions similar to those indicated in Table 1.

Recently Froehler et al. reported that, while a 5-(1-propynyl)-2'-deoxyuridine in DNA-TFO significantly enhances triplex stability, the cytidine analog exhibits the opposite effect.[18] The low pK_a (3.30) of the cytosine derivative can explain this destabilization. In the present experiments, the relative instability of C-m9 as compared to m9 may be due partly to the lower apparent pK_a value of the Cm residue, although the value may be 2-3 units higher in triplex formation.[16] Studies of the structures of the Cm-containing triplexes to explain the relative stability of C-m9 > U, C-m9 will provide valuable information.

Thermal denaturations of the triplexes with DD15 were also investigated under various salt concentrations (Figure 2). It has been shown that the variation of the Tm's of triplex DNAs is similar to that for the corresponding duplex DNAs.[19] Such a tendency was observed for the M-d9-triplex and DD15, but the series of triplexes with RNA/2'-*O*-methyl RNAs was distinct and the salt dependance of their T_m's was found to be comparatively low. Thus, 2'-*O*-methyl TFOs and 5-methyluracil-containing 2'-*O*-methyl TFOs have high or higher binding affinity for duplex DNA, even at low salt concentrations.[20]

Acknowledgement: This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

References and Notes:

+Present address: Institute of Biosciences and Technology, Texas A & M University, 2121 W. Holcombe Blvd., Houston, TX 77030, USA.

1. Moser, H. E; Dervan, P. B. *Science* **1987**, *238*, 645-650.
2. Cooney, M.; Czernuszewicz, G.; Postel, E. H.; Flint, S. J.; Hogan, M. E. *Science* **1988**, *241*, 456-459.
3. For a review see: Hélène, C.; Toulmé, J.-J. *Biochimica et Biophysica Acta* **1990**, *1049*, 99-125.
4. Ing, N. H.; Beekman, J. M.; Kessler, D. J.; Murphy, M.; Jayaraman, K.; Zendegui, J. G.; Hogan, M. E.; O'Malley, B. W.; Tsai, M.-J. *Nucleic Acids Res.* **1993**, *21*, 2789-2796.
5. For a review see: Thuong, N. T.; Hélène, C. *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 666-690.
6. Inoue, H.; Hayase, Y.; Imura, A.; Iwai, S.; Miura, K.; Ohtsuka, E. *Nucleic Acids Res.* **1987**, *15*, 6131-6148.
7. Hayase, Y.; Inoue, H.; Ohtsuka, E. *Biochemistry* **1990**, *29*, 8793-8797.
8. For a review of antisense 2'-*O*-alkyl RNAs, see: Lamond, A. I.; Sproat, B. S. *FEBS Lett.* **1993**, *325*, 123-127.
9. Shimizu, M.; Konishi, A.; Shimada, Y.; Inoue, H.; Ohtsuka, E. *FEBS Lett.* **1992**, *302*, 155-158.
10. Escudé, C.; Sun, J.-S.; Rougée, M.; Garestier, T.; Hélène, C. *C. R. Acad. Sci. Paris, Ser. III* **1992**, *315*, 521-525.
11. Roberts, R. W.; Crothers, D. M. *Science* **1992**, *258*, 1463-1466.
12. Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 3059-3061.
13. Xodo, L. E.; Manzini, G.; Quadrifoglio, F.; van der Marel, G. A.; van Boom, J. H. *Nucleic Acids Res.* **1991**, *19*, 5625-5631.
14. Divaker, K. J.; Reese, C. B. *J. Chem. Soc., Perkin Trans. 1* **1982**, 1171-1176.
15. pKa's of the TFOs were obtained from UV-absorbance experiments as a function of pH. cf. pKa's = 4.4 for dM and 4.3 for dC: Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M. Data for Biochemical Research; Oxford University Press: New York NY, 1986; pp. 107.
16. Callahan, D. E.; Trapane, T. L.; Miller, P. S.; Ts'o, P. O. P.; Kan, L.-S. *Biochemistry* **1991**, *30*, 1650-1655.
17. Sugimoto, N.; Shintani, Y.; Tanaka, A.; Sasaki, M. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 535-540.
18. Froehler, B. C.; Wadwani, S.; Terhorst, T. J.; Gerrard, S. R. *Tetrahedron Lett.* **1992**, *33*, 5307-5310.
19. Shea, R. G.; Ng, P.; Bischofberger, N. *Nucleic Acids Res.* **1990**, *18*, 4859-4866.
20. Another advantage for 2'-*O*-methyl TFOs is that they may be useful to discriminate between matched and mismatched triplexes.[9]

(*Received* 13 *October* 1993)