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BIOPHYSICAL AND BIOCHEMICAL PROPERTIES OF OLIGODEOXYNUCLEOTIDES CONTAINING 4'-*C*- AND 5'-*C*-SUBSTITUTED THYMIDINES

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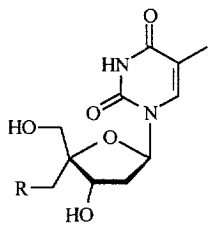
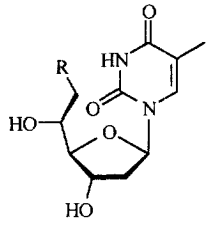
Abstract: We have previously reported oligodeoxynucleotides (ODNs) containing 4'-*C*- and 5'-*C*-substituted thymidines, which demonstrated certain favorable biophysical and biochemical properties. In this communication, the hybridization and nuclease stability data of the ODNs along with their capability to induce RNase H activity are presented.

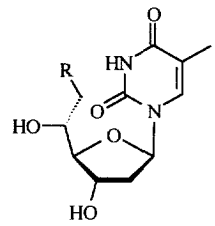
Antisense oligonucleotides (ONs) as potential human therapeutics have been explored for more than a decade.[1–3] In order to improve the binding affinity, nuclease stability, cell-uptake, and other pharmacokinetic properties, a variety of ON analogs has been synthesized and evaluated.[4–6] ONs containing sugar-modified nucleosides have also received considerable attention.[6] 2'-*O*-Alkyl ONs have shown superior hybridization to RNA and certain nuclease resistance.[7–9] ODNs containing 3'-*C*-branched thymidines,[10–12] 5'-*C*-branched nucleosides,[13–15] and 4'-*C*-substituted nucleosides[16–18] have also been reported. Most of these sugar-modified ODNs have satisfactory hybridization and enhanced nuclease stability compared to unmodified ODNs. However, among the sugar- and backbone-modified ODNs, only phosphorothioate ODNs were reported to induce RNase H activity,[3] which is important for antisense inhibition of gene expression. In search for ODN analogs possessing enhanced hybridization and nuclease stability as well as capability to induce RNase H activity, we have explored the ODNs containing 4'-*C*- and 5'-*C*-substituted thymidines (as shown below) as potential antisense inhibitors. In this communication some biophysical and biochemical properties of the ODNs will be presented.



R = OCH_3, NH_2



R = OH, NH_2, OCH_3, $CH{=}CH_2$
imidazole-4-CH_2CONH



R = CH_2CH_2OH, $CH{=}CH_2$

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Table 1. Hybridization and nuclease stability data of ODNs containing 4'-*C*- and 5'-*C*-substituted thymidines

	Sequence	Tm °C DNA	ΔTm °C/Mod.	Tm °C RNA	ΔTm °C/Mod.	$t_{1/2}$ min.
1.	5'-d(ATCTCTCCGCTTCCTTTC)-3'	58.3		64.4		5
2.	5'-d(ATCTCTCCGCTTCCTX_1X_1C)-3'	56.0	-1.1	63.3	-0.6	> 180
3.	5'-d(ATCTCX_1CCGCTX_1CCTTTC)-3'	55.8	-1.2	63.3	-0.5	
4.	5'-d(AX_1CTCTCCGCTTCCTTTC)-3'	58.0	-0.3	64.6	+0.2	
5.	5'-d(ATCTCTCCGCTTCCTX_2X_2C)-3'	56.4	-1.0			>180
6.	5'-d(ATCTCX_2CCGCTX_2CCTTTC)-3'	56.9	-0.7	61.0	-1.7	
7.	5'-d(AX_2CTCTCCGCTTCCTTTC)-3'	58.2	-0.1			
8.	5'-d(ATCTCTCCGCTTCCTX_3X_3C)-3'	57.1	-0.6	63.8	-0.3	144
9.	5'-d(ATCTCX_3CCGCTX_3CCTTTC)-3'	55.5	-1.4	63.3	-0.6	
10.	5'-d(ATCTCTCCGCTTCCX_4TX_4C)-3'	57.7	-0.3	63.6	-0.4	38
11.	5'-d(AX_4CTCX_4CCGCTX_4CCTTX_4C)-3'	56.7	-0.4	61.6	-0.7	
12.	5'-d(ATCTCTCCGCTTCCTX_5X_5C)-3'	55.6	-1.4	63.8	-0.3	114
13.	5'-d(ATCTCX_5CCGCTX_5CCTTTC)-3'	55.4	-1.5	63.3	-0.6	
14.	5'-d(AX_5CTCTCCGCTTCCTTTC)-3'	57.7	-0.6	65.1	+0.7	
15.	5'-d(ATCTCTCCGCTTCCX_6TX_6C)-3'	56.0	-1.2	63.6	-0.4	64
16.	5'-d(ATCTCX_6CCGCX_6TCCTTTC)-3'	56.8	-0.8	61.6	-1.4	
17.	5'-d(AX_6CTCTCCGCTTCCTTTC)-3'	57.9	-0.4	65.0	+0.6	
18.	5'-d(ATCTCTCCGCTTCCX_7TX_7C)-3'	58.0	-0.2			
19.	5'-d(ATCTCX_7CCGCX_7TCCTTTC)-3'	55.9	-1.2	62.8	-0.8	
20.	5'-d(AX_7CTCTCCGCTTCCTTTC)-3'	58.4	+0.1	63.3	-1.1	
21.	5'-d(CTTCCTGTCTGATGGCTTC)-3'	60.4		63.0		9
22.	5'-d(CX_1X_1CCX_1GX_1CX_1GAX_1GGCX_1X_1C)-3'	53.0	-0.9	59.7	-0.4	>>180
23.	5'-d(CX_1X_1CCX_1GTCTGAX_1GGCX_1X_1C)-3'	54.8	-0.9	60.6	-0.4	>>180
24.	5'-d(CX_3X_3CCX_3GX_3CX_3GAX_3GGCX_3X_3C)-3'	51.9	-1.1	58.8	-0.5	>>180
25.	5'-d(CX_3X_3CCX_3GTCTGAX_3GGCX_3X_3C)-3'	54.5	-1.0			>>180
26.	5'-d(CX_4X_4CCX_4GX_4CX_4GAX_4GGCX_4X_4C)-3'	57.1	-0.4	55.5	-0.9	>>180
27.	5'-dCX_4X_4CCX_4GTCTGAX_4GGCX_4X_4C)-3'	57.0	-0.6			>>180
28.	5'-d(CY_1Y_1CCY_1GY_1CY_1GAY_1GGCY_1Y_1C)-3'	58.2	-0.3	62.4	-0.1	>>180
29.	5'-d(CY_1Y_1CCY_1GTCTGAY_1GGCY_1Y_1C)-3'	58.8	-0.3			>>180
30.	5'-d(CY_2Y_2CCY_2GY_2CY_2GAY_2GGCY_2Y_2C)-3'	68.6	+1.0	62.4	-0.1	>>180
31.	5'-d(CY_2Y_2CCY_2GTCTGAY_2GGCY_2Y_2C)-3'	65.8	+0.9			>>180

X_1 = 5'(*S*)-*C*-hydroxymethylthymidine, X_2 = 5'(*R*)-*C*-hydroxypropylthymidine, X_3 = 5'(*S*)-*C*-methoxymethylthymidine, X_4 = 5'(*S*)-*C*-aminomethylthymidine, X_5 = 5'(*S*)-*C*-allylthymidine, X_6 = 5'(*R*)-*C*-allylthymidine, X_7 = 5'(*S*)-*C*-(imidazol-4-ylmethyl-carbamidomethyl)thymidine, Y_1 = 4'-*C*-methoxymethylthymidine, Y_2 = 4'-*C*-aminomethylthymidine. The samples for Tm measurements contain 2.0 μM of modified ODNs and 2.0 μM of either complementary DNA or RNA in a buffer (10 mM sodium phosphate, 0.1 mM EDTA, and 0.1 M sodium chloride, pH = 7.0). The half-lives of the ODNs were calculated from UV absorbance curves of ODN samples during degradation by SV phosphodiesterase at 25 °C.

The ODNs containing some 5'-*C*-substituted thymidines (Sequences 8, 10, 12, 15) were reported in our previous communication,[14] and the new ODN sequences (9, 11, 13, 14, 16, 17, 24-27) containing the same modifications were synthesized according to the same procedures. Sequences 2-7 and 22-23 were synthesized according to our previously published procedures.[15,19] The ODNs containing 5'(*S*)-*C*-(imidazol-4-ylmethyl-carbamidomethyl)thymidine (Sequences 18-20) were synthesized by using the corresponding phosphoramidite,[19] with 2,4-dinitrophenyl as the protecting group of imidazole. The coupling time in the ODN synthesis was 10 min for the modified phosphoramidite and 5 min for the unmodified phosphoramidites next to the modified one. The coupling yields (97–98%) were comparable to those for the unmodified phosphoramidites. The ODNs were deprotected and purified by a standard procedure as previously described.[14] The ODNs containing 4'-*C*-substituted thymidines were published in a previous communication,[18] and Sequence 28-31 were prepared by the same procedures.

Hybridization properties of these 4'-*C*- and 5'-*C*-substituted ODNs were studied by thermodynamic melting (Tm) experiments as previously described.[12,14,20] As can be seen from Table 1, most of the ODNs containing 5'-*C*-substituted thymidines have lower Tm with complementary DNA than the unmodified ODNs, approximately one degree drop per modification. Generally, the modifications in the middle and 3'-region of the ODNs destabilize DNA-DNA duplex more than those in the 5'-region, which have little effect on duplex stability. Sequences 22-25, which contain multiple modifications spreading over the sequences, also give an average drop in Tm by one degree per modification. It seems that the size and orientation (*R* or *S*) of the 5'-*C*-substituents do not affect the hybridization dramatically since Sequences 5-7 containing 5'(*R*)-*C*-hydroxypropyl and Sequences 18-20 containing 5'(*S*)-*C*-imidazol-4-ylmethylcarbamidomethyl have similar Tm values as Sequences 2-17 that contain smaller 5'(*S*)-*C*-substituents. Most of the ODNs containing 5'-*C*-substituted thymidine hybridize with the complementary RNAs slightly stronger than with DNAs, with Tm drop by around one-half degree per modification. The ODNs containing 4'-*C*-substituents generally have favorable hybridization to both the complementary DNA and RNA.[18] The ODNs containing multiple 4'-*C*-aminomethylthymidine (Sequences 30, 31) have stronger hybridization to the complementary DNA than the unmodified ODN, with an increase in Tm by one degree per modification. The ODNs containing multiple 4'-*C*-methoxymethylthymidine (Sequences 28, 29) have comparable hybridization to DNA as the unmodified ODN. These sequences (28-31) also hybridize well with the complementary RNA, with almost no drop in Tm.

Stability to snake venom phosphodiesterase (SVP) was measured according to a previously published procedure.[12,21,22] The ODNs having 5'(*S*)-*C*-hydroxymethyl and 5'(*R*)-*C*-hydroxypropyl modifications were resistant to SVP. With only two of these modified thymidines in the 3'-region, the ODNs had half-lives well above 3 h (Table 1). The ODNs containing 4'-*C*-methoxymethyl, 5'(*S*)-*C*-methoxymethyl, and 5'(*S*)-*C*-allyl modifications also showed significantly enhanced stability to SVP. It seems both the size and polarity affect the nuclease stability. The hydroxyl groups of 5'-*C*-hydroxyalkyl had an important contribution to the nuclease

stability while the amino groups of both 4'-*C*- and 5'-*C*-aminomethylthymidines led to only moderate increases in the half-lives of the ODNs. However, the stability to SVP was dramatically increased when the ODNs contained multiples of the modified thymidines. Sequence 22-31 in Table 1, which contain six or eight modified thymidines, were basically intact or only slightly degraded by SVP after 3 h.

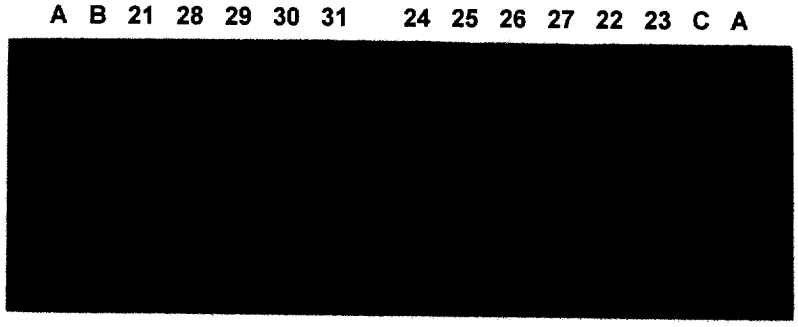

Figure 1. RNase H-dependent cleavage of human IL8R RNA fragment in the presence of ODNs.[23] The RNA (~350 bases) was treated with ODNs and *E. coli* RNase H at 37 °C for 20 min. The cleavage products were resolved on an 1.8 % agarose gel under denatured condition. Lane designations refer to the sequence number of the ODNs in Table 1 except A, B, and C, A: RNA ladder; B: no ODN; C: ODN having a random sequence.

Table 2. RNase H-dependent cleavage of human IL8R RNA in the presence of modified ODNs*

ODN sequence	21	28	29	30	31	22	23	24	25	26	27
% cleavage	87	67	83	neg	85	neg	80	neg	82	56	80

* The quantitation of the RNA cleavage products was performed on a BioRad Gel Doc 1000 System.
neg = negligible.

Another important property of antisense ODNs is their capability to induce RNase H activity, which cleaves the target RNA in a sequence-specific manner and fulfils the antisense task. The ODNs containing certain 4'-*C*- and 5'-*C*-subtituted thymidines were studied for the RNase H activation. The RNA fragment used in the RNase H-dependent cleavage reactions was a T7 sense transcript (~350 bases) transcribed from the linearized pCR 2.1-S-IL8R plasmid using Promega Ribomax Large Scale RNA Production System. Plasmid pCR 2.1-S-IL8R was constructed by sub-cloning the IL8R cDNA into the TA cloning vector pCR 2.1 (Invitrogen). The ODNs used in the studies were those containing multiple modifications (Sequences 22-31). As can be seen from the Figure 1 and Table 2, the ODNs having a gap of six unmodified nucleosides (Sequences 23, 25, 27, 29, 31) demonstrated similar capability to induce RNase H activity as the unmodified

ODN (Sequence 21). In our experiments,[22] about 80% of the RNA was cleaved by *E. coli* RNase H in the presence of these ODNs. The ODNs having eight modified thymidines spreading over the whole sequence (22, 24, 26 ,28, 30), which have a gap of three unmodified nucleosides (GGC) in the sequence, gave quite different results. Sequences 24, 26, and 30 did not effectively induce RNase H activity, and the RNA was almost intact in the cleavage reactions (Figure 1). However, Sequence 22 (the ODN containing 5'(*S*)-*C*-hydroxymethylthymidine) and Sequence 28 (the ODN containing 4'-*C*-methoxymethylthymidine), which have the same gap of unmodified nucleosides as that in Sequences 24, 26, and 30, did induce RNase H activity, with approximately 50-70% cleavage of the RNA, as shown in Figure 1 and Table 2. The data indicate that Sequences 22 and 28 can induce RNase H activity effectively although they are slightly less capable than the unmodified ODN (Sequence 21). The results reveal that 5'(*S*)-*C*-hydroxymethyl and 4'-*C*-methoxymethyl groups on ODNs do not decrease RNase H activity significantly while 4'-*C*-aminomethyl, 5'(*S*)-*C*-aminomethyl, and 5'(*S*)-*C*-methoxymethyl groups inhibit the RNase H activity very effectively. It seems that both size and polarity of substituents at C4' and C5' are important for the RNase H-dependent cleavage. More detailed studies are necessary to shed more light on the structure-activity relationship.

In summary, the ODNs containing 4'-*C*-substituted thymidines demonstrated favorable hybridization to the complementary DNA and RNA while the ODNs containing 5'-*C*-substituted thymidnes have shown a slightly weaker hybridization than the unmodified. However, all these modified ODNs are much more stable to SV phosphodiesterase than the unmodified ODNs. More importantly, the sugar-modified ODNs having no gap (no more than three unmodified nucleosides in a row) demonstrated, for the first time, the capability to activate RNase H. The ODNs containing 4'-*C*-methoxymethylthymidine and 5'(*S*)-*C*-hydroxymethyl-thymidine that hybridize well to RNA and activate RNase H effectively are potentially useful as antisense inhibitors. ODNs containing 4'-*C*-substituted and 5'-*C*-substituted nucleosides deserve further investigation.

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23. RNase H-dependent cleavage reactions were performed in tubes: 1 μL (4 mg/mL) of human IL8R RNA fragment (~ 35 pico mol), 55X excess of antisense ODN (~2000 pico mol in 1–18 μL of sterile water), 2.5 μL of 10X hybridization buffer (200 mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$, 1.0 M KCl and 10 mM DTT), and 1 μL (3 U/μL) of *E. coli* RNase H (BRL) were added successively. The final volume was brought to 25 μL with sterile water. The tubes were then incubated at 37 °C for 20 min, and then 100 μL of the sterile water added, followed by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The upper phase was precipitated with 1/10 volume of 3.0 M sodium acetate (pH 5.2) and 2.2 volume of absolute ethanol at –20 °C. One microliter of glycogen (20 mg/mL, Boehringer Mannheim) was added as a carrier to ensure full recovery of the RNA. After precipitation the RNA cleavage products were denatured and loaded on an 1.8% agarose gel in 2.2 M formaldehyde and 1X MOPS (pH 7.0). The gel was run at 80 volts for 1.5 hours in 1X MOPS (pH 7.0). Gel was stained with 0.5 μg/mL ethidium bromide for 30 min and then destained in dd water. The Gel was photographed and analyzed on a BioRad Gel Doc 1000 System.