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Alteration of cross-linking selectivity with the 2′-OMe analogue of 2-amino-6-vinylpurine and evaluation of antisense effects

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ABSTRACT

We previously reported that oligodeoxynucleotides containing 2-amino-6-vinylpurine (2-AVP: **1**) exhibit efficient selective cross-linking to cytosine. In this study, the 2′-OMe nucleoside analogue (**2**) of 2-AVP was designed in order to increase its affinity to RNA and enhance metabolic stability. It has been demonstrated that 2′-OMe oligonucleotides bearing **2** achieve highly selective cross-linking to the thymine base in DNA and show higher antisense effect on luciferase production in cell lysate.

An oligonucleotide (ON) with a sequence complementary to a specific mRNA can inhibit its expression, thereby inhibiting the transfer of genetic information from DNA to protein as an antisense strategy.[1] This method is therefore very effective not only for therapeutic purposes but also to investigate gene functions.[2] The use of unmodified ON for applications in cells, however, is very limited because of the following reasons: ON's poor cellular uptake efficiency and targeted delivery, its low specificity and affinity for the target sequence, and susceptibility to degradation by nucleases. Many modified ONs containing unnatural bases or modified sugars have been prepared for improving the efficacy of the antisense method.[3] One such example is ONs incorporating a 2′-modified nucleotide. These analogues are known to exhibit high binding affinity to target RNA because of their enhanced metabolic stability.[3b] Recently, 2′-modified antisense ONs have been shown to effectively inhibit microRNAs activity in cells.[4] Furthermore, cross-linking reactions are expected to enhance the antisense activity by irreversibly binding to the target RNA, based on the steric blocking mechanism. Several cross-linking ONs have been reported to react with the target DNA when triggered by photoirradiation[5] or chemical reactions.[6] We previously demonstrated that the 2-amino-6-vinylpurine (2-AVP) deoxynucleoside analogue was activated by the duplex formation with the target DNA and reacted to cytosine selectively.[7] In this study, we designed the 2′-O-methyl (2′-OMe) analogue (**2**) of 2-AVP (Fig. 1) to check its reactivity and affinity to RNA. The use of the 2′-OMe backbone was also expected to enhance metabolic stability. Herein, we describe the synthesis and evaluation of the cross-linking properties of 2'-OMe ON incorporating the 2-AVP (**2**), and the higher antisense effect with the use of **2** in non-cell translation assay.

Scheme 1 summarizes the synthesis of 2′-OMe ONs incorporating a reactive nucleoside analogue. 2-AVP derivative (**3**) was synthesized from 2′-OMe guanosine, as described previously.[7a] After protection of the vinyl group and the 2-amino group with methyl sulfide and with phenoxyacetyl, respectively, the conventional procedure produced the phosphoramidite precursor (**5**) in good yield. The sulfide protected 2′-OMe ON (**6**) was then synthesized from the phosphoramidite precursor through the use of an automated DNA synthesizer. The sequences of synthesized ONs were found to be complementary to firefly-luciferase mRNA (Scheme 1). After deprotection of the DMTr group, the mixture was purified by gel electrophoresis using denaturing polyacryl-

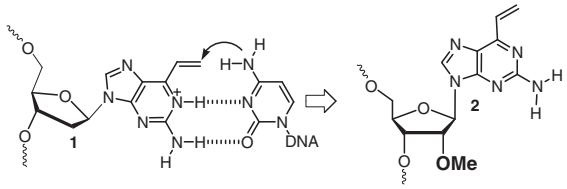

Figure 1. Structure of 2′-OMe analog of 2-AVP.

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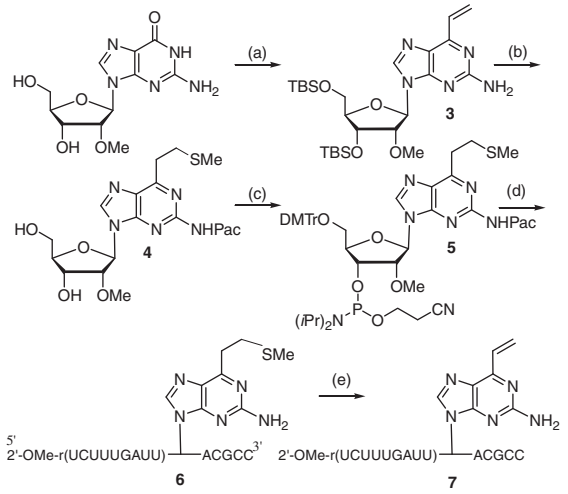

Scheme 1. Reagents and conditions: (a) (1) TBSCl, imidazole, DMF; (2) TsCl, TEA, DMAP, CH_2Cl_2; (3) $(C_2H_3BO)_3$, Pd(0), LiBr, dioxane–H_2O; (b) (1) CH_3SNa, CH_3CN, CH_2Cl_2; (2) $PhOCH_2COCl$, HBT, CH_3CN, pyridine; (3) *n*-Bu_4NF, THF; (c) (1) DMTrCl, Py; (2) (*i*-Pr)$_2$NP(Cl)OC_2H_4CN; (d) (1) synthesis with an automated DNA synthesizer, (2) 28% aqueous NH_3; (3) PAGE purification; (e) (1) 2 equiv MMPP, (2) aqueous NaOH.

amide gel to produce the sulfide protected 2′-OMe ONs in good yield. The ON (**6**) was then smoothly converted to **7** by oxidation with magnesium monoperphthalate, following elimination of the sulfoxide group under an alkaline condition. The structures of 2′-OMe ONs were confirmed by MALDI-TOF MS measurements.

The cross-linking reaction was then investigated under acidic conditions using the reactive 2′-OMe ON (**7**) and the target DNA (**8a**) (N = dG, dA, dC, dT) or RNA (**8b**) (N = rG, rA, rC, U) labeled with fluorescein at the 5′ end. The progress of the cross-linking was monitored by gel electrophoresis with 20% denaturing gel. The yields of the cross-linking reactions were calculated by comparing

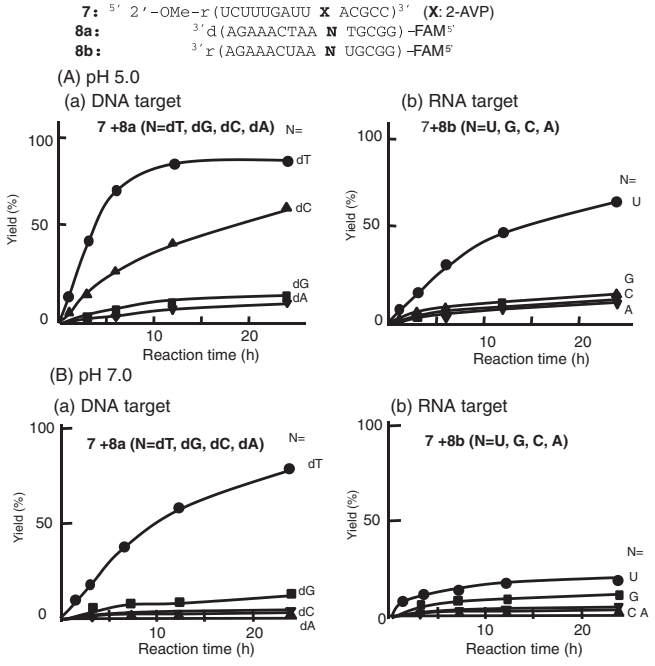

Figure 2. Comparison of the reaction yields calculated from the gel electrophoresis analysis of the cross-linking using **7** and target DNA (**8a**) (N = dT, dG, dC, dA) or RNA (**8b**) (N = U, G, C, A). The reaction was performed with 10 μM ODN (**7**) and 5 μM target ODN (**8**) in 0.1 M NaCl, 50 mM MES, pH 5.0 (A) or pH 7.0 (B), at 30 °C.

the bands of the target sequence to the cross-linked adducts. Comparison of the reactivity of the 2-AVP-containing 2′-OMe ON (**7**) towards the different bases at the target site of the DNA (**8a**) or RNA (**8b**) is shown in Figure 2. Surprisingly, **7** gave the highest yields in the reaction with the thymine base in DNA or with uracil base in RNA under acidic conditions, albeit it gave lower yields in the reaction with dC target (Fig. 2A). When the reactions of **7** with the DNA target were performed under neutral conditions, the cross-linked adduct was observed only with the thymine base, and no cross-link was formed with the cytosine base (Fig. 2B (a: vs DNA)). Cross-linking reactions of **7** with the RNA target did not occur under neutral conditions (Fig. 2B (b: vs RNA)). The reaction product between **7** and **8a** (N = dT) was purified by HPLC and proven to be the cross-linked one by MALDI-TOF MS measurement (calcd 10071.8 found 10072.3).

To check whether duplexes were formed under the reaction conditions, thermal stability was estimated by measuring the melting temperature (T_m) of the duplex formed between the 2′-OMe ON (**6**) containing stable precursor of 2-AVP and target DNA (**8a**) or RNA (**8b**) under acidic and neutral conditions (Supplementary data). The melting profile of 2′-OMe ON (**6**) /DNA or RNA duplex suggested that the duplexes of **7**/target DNA or **7**/RNA would be formed under the reaction conditions, and that the cross-linking reactions might occur in each duplex. In the case of the reactions shown in Figure 2B (b: vs RNA), where no cross-linking was observed, 2′-OMe ON/RNA duplexes were clearly formed, as evidenced by the melting profiles. However, differences in thermal stability could not explain the base selectivity of the cross-linking reactions when 2′-OMe ON (**7**) was used. The circular dichroism (CD) spectra were then measured to check the conformational change in the duplex between **6** and the DNA or RNA. These spectra were similar to that of the duplex between 2′-OMe ON containing adenosine instead of the 2-AVP derivative and target DNA (**8a**, N = dT) or RNA (**8b**, N = U). These CD spectra have indicated that the 2′-OMe ON/DNA duplexes lie between the A- and B-conformation, whereas the 2′-OMe ON/RNA duplexes lie in the A-conformation.[8] As the oligo DNA containing 2'-deoxy 2-AVP cross-linked to the cytosine base in the DNA/DNA duplex in the B-conformation,[7] the alteration of the base selectivity to the thymine base using 2'-OMe ON might be attributed to the difference of the duplex conformation.

To get more insight into the cross-linking reaction, the cross-linked nucleoside was isolated by enzymatic hydrolysis, and the structure of the cross-linked product was determined. After 6 h incubation of the purified cross-linked adduct with nuclease P1, snake venom phosphodiesterase and alkaline phosphatase were added to the mixture. HPLC analysis showed the formation of 8

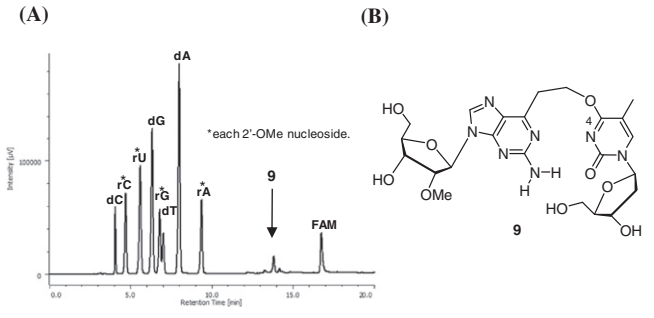

Figure 3. (A) HPLC analysis of the enzymatic hydrolysate. The purified adduct was digested with nuclease P1 in buffer (30 mM NaOAc, 5 mM $ZnCl_2$), snake venom phosphodiesterase and alkaline phosphatase in buffer (50 mM Tris–HCl, 10 mM $MgCl_2$). HPLC conditions: ODS column 1 mL/min; solvent; 0.1 M TEAA buffer, B: CH_3CN, B: 5–15%/10 min, 15–40%/20 min. (B) Speculative structure of the adduct (**9**).

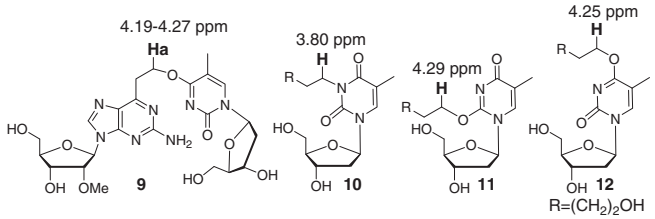

Figure 4. Structure of the thymine adducts.

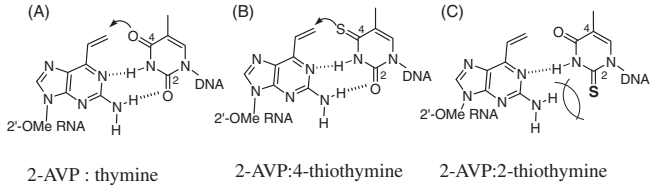

Figure 5. Speculative complex between 2-AVP and thymine or 2-or 4-thiothymine in duplex.

nucleosides together with an additional peak labeled with an arrow in the HPLC chart (Fig. 3).

The HR ESI MS spectrum of the isolated new product indicated the cross-linking between the thymine base and the 2-AVP derivative (calcd for $C_{23}H_{31}N_7O_9Na$ (M+Na): 572.2075 found 572.2073). In the ^1H NMR and ^1H–^1H cosy spectra of **9** in DMSO-d_6, multiplet peaks were observed around 4.19–4.27 ppm, which are assigned to Ha (Fig. 4. and Supplementary data).

The methylene proton adjacent to nitrogen in N3-alkylated thymine (**10**) appeared at 3.80 ppm, and the methylene proton adjacent to oxygen in O2 (**11**) and O4-alkylated thymine (**12**) appeared at around 4.3 ppm as has been reported in literature.[9] These results showed that the cross-link formed with either the 2- or 4-oxygen, and not with the 3-nitrogen of the thymine base. The alkylation site at 2-O or 4-O of the thymine base, however, could not be unambiguously determined. It was anticipated that 2-AVP in the 2′-OMe ON might react with the thymine base because of the effective proximity effect, by forming hydrogen bonds, as shown in Figure 5A. Based on such a hypothesized complex, the assumption was made that the cross-linking reactions in the 2-AVP:4-thiothymine complex would produce higher reactivity because of the high nucleophilicity of the sulfur atom (Fig. 5B). In contrast, the 2-thiothymine base would inhibit the cross-link formation because of the steric repulsion between the 2-thio group[10] and the 2-amino group of AVP (Fig. 5C).

The reactivities of (**7**) towards DNA bearing dT, 4-thio, or 2-thiothymine are compared in Figure 6. The rate enhancement was observed with the 4-thiothymine base, and reaction with the 2-thiothymine base was inhibited. These results accord with our assumption (shown in Fig. 5) that the cross-linking reaction of the 2-AVP derivative in 2′-OMe ON might occur with the O4 of the thymine base at the target site. More investigation is needed to clarify the origin of the selectivity change from the cytosine base in the DNA/DNA duplexes to the thymine base in the 2′-OMe ON/DNA or RNA duplexes observed in this study.

Antisense effects of the reactive ONs were evaluated by a translation assay in the cell lysates. The ONs were incubated for 5 h with target mRNA of firefly-luciferase under acidic conditions (pH 5.0), and then subjected to the translation reactions with a wheat-germ extract for 2 h at 30 °C. Two sequences of ONs (**13b**) and (**14b**) complementary to luciferase mRNA, were used in these experiments; the former was designed to react with rC, and the latter

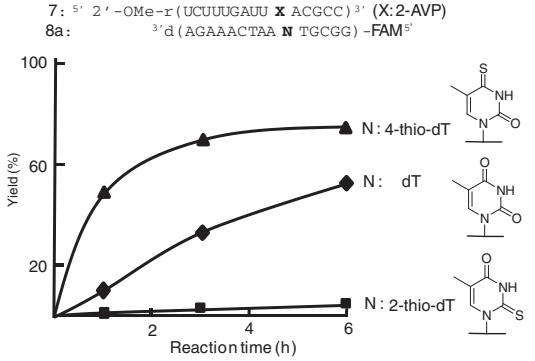

Figure 6. Cross-linking ability of **7** for the target DNA with thymine or thiothymine derivatives at the complementary site. The reaction was performed with 10 μM ODN (**7**) and 5 μM target ODN (**8a**) in 0.1 M NaCl, 50 mM MES, pH 7.0 at 30 °C.

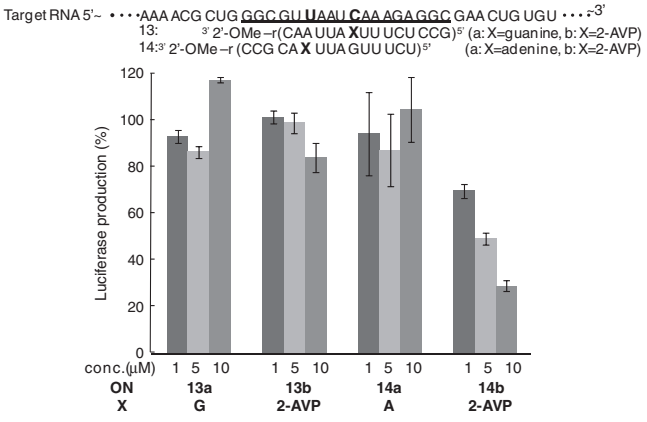

Figure 7. Antisense effects on luciferase production in an assay without cells. The extent of luciferase production relative to that in the control, which is performed translation in the absence of ON, is shown in the ordinate.

was designed to react with U. Production of luciferase was monitored by measuring luminescence. Antisense inhibitory effects are summarized in Figure 7. The 2′-OMe ONs, (**13a**) and (**14a**) as the non-reactive control sequences and 2-AVP-containing 2′-OMe ON (**13b**) did not show antisense inhibition. In contrast, 2-AVP-containing 2′-OMe ON (**14b**) with the uracil-targeting sequence showed higher antisense inhibition. The effect of antisense inhibition by **14b** accords with the chemical reactivity of **7** to rU in the RNA target under acidic conditions (Fig. 2A (b, vs RNA)). These results suggest that the cross-linking reactions between the 2′-OMe analogue of 2-AVP and U might be responsible for enhancement of antisense inhibition.

In this study, we have synthesized the 2′-OMe analogue of 2-AVP and the ONs containing this derivative. These ONs have exhibited highly selective cross-linking reactivity to dT in the DNA substrate under neutral conditions. It should be noted that change of the sugar moiety from deoxyribose to 2′-OMe caused a drastic change in base selectivity. We speculate that the conformational differences of the hetero duplex may contribute to the alteration of base selectivity.

It is not currently clear, however, why the base selectivity of the cross-linking reaction is changed. The reactive 2′-OMe ONs with U-targeting sequence showed higher antisense effects on luciferase production in cell lysates. These results suggest that cross-linking reactions of 2′-OMe ON might increase the antisense inhibition in cells; this is now being studied in our laboratory.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.027.

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