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Biologically Active Oligodeoxyribonucleotides. Part 12:[1] N^2-Methylation of 2′-Deoxyguanosines Enhances Stability of Parallel G-Quadruplex and Anti-HIV-1 Activity

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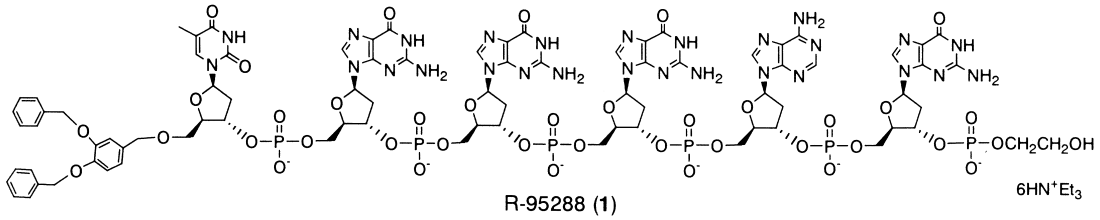
Abstract—2′-Deoxyguanosine residues of a 3′,5′-end-modified hexadeoxyribonucleotide (R-95288) with anti-HIV-1 activity were substituted with N^2-methyl-2′-deoxyguanosine (m^2dG). These modified oligodeoxyribonucleotides (ODNs) showed a 2-fold higher activity than R-95288. Also, the CD spectra of these ODNs indicated that the m^2dG modification stabilized the tertiary structure of the G-quadruplex.

Oligodeoxyribonucleotides (ODNs) for drug candidates, which can bind to nucleic acids, proteins and small organic compounds that are disease-related, are called antisense nucleic acids or aptamers.[2] We have found that a hexadeoxyribonucleotide (5′ TGGGAG) with a 3,4-dibenzyloxybenzyl (3,4-DBB) group at the 5′ end had high anti-HIV-1 activity and low cytotoxicity.[3] Modification of the 3′ end with a 2-hydroxyethyl phosphate group enhanced the anti-HIV-1 activity as well as stability of the ODN in human plasma.[4] This modified ODN (R-95288; **1**) also showed in vivo anti-viral activity using hu-PBL-SCID/beige mice as an animal model for HIV-1 infection.[5] This modified ODN most likely binds to the gp120 protein of the HIV-1 virus, inhibits absorption and entry of the virus, and acts as an aptamer.[6] Moreover, this kind of short, guanine-rich ODN forms a parallel-quadruplex based on *anti*-conformational G-quartet (Fig. 1(A,B)).[1,3,4,7]

In this paper, we describe the N^2-methylation of 2′-deoxyguanosine residues in R-95288 (**1**), stability of the G-quartet by these ODNs and their anti-HIV-1 activity. Although one of the protons of an exocyclic amine group of 2′-deoxyguanosine contributes to the hydrogen bonding in the G-quartet, the other is available for modification. Once a 2′-deoxyguanosine residue in the G-quadruplex is modified, this is the same as all other 2′-deoxyguanosine residues in the G-quartet plate being modified (Fig. 1(B)). Substitution at the 8 position of 2′-deoxyguanosine residues in the anti-parallel G-quadruplex stabilizes



R-95288 (**1**)

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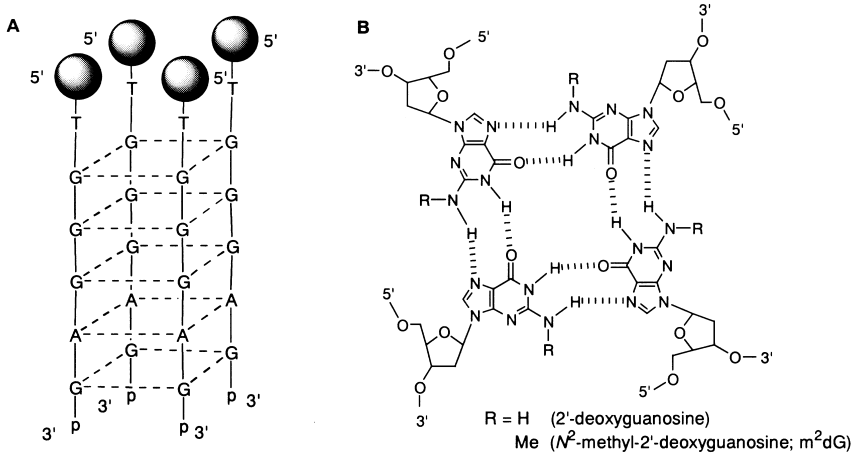
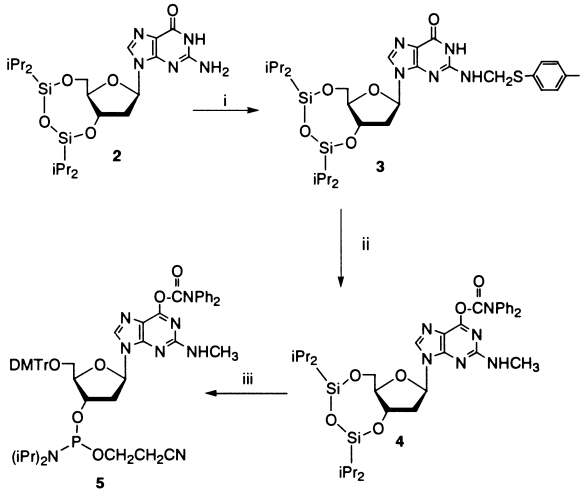


Figure 1. (A) Proposed parallel G-quadruplex structure of R-95288.[4] Circles and p show 3,4-DBB groups and 2-hydroxyethyl phosphate groups, respectively. Fifth 2′-deoxyadenosine from the 5′-end can also form the quartet structure.[16,17] (B) G-quartet structure by 2′-deoxyguanosine residues or N^2-methyl-2′-deoxyguanosine residues.

the tertiary structure, as the 2′-deoxyguanosine residues are now fixed in the *syn* conformation.[8,9] However, the actual effect of the N^2-methylation of 2′-deoxyguanosine residues in the parallel G-quadruplex is not yet known.

The synthesis of the N^2-methyl-2′-deoxyguanosine (m^2dG) unit to form modified R-95288 is shown in the Scheme 1. Selective methylation at the N-2 position was performed according to Reese's method for N^2-methylguanosine synthesis.[10,11] Mannich-like reaction of 3′,5′-TIPDS-2′-deoxyguanosine **2** by treatment with HCHO and thiocresol gave compound **3**. Reduction by $NaBH_4$, followed by protection of the O-6 position with *N,N*-diphenylcarbamonyl chloride, gave N^2-methylated 2′-deoxyguanosine **4**.[12] Subsequently, compound **4** was deprotected by TBAF in THF, treated with 4,4′-dimethoxytrityl chloride (DMTrCl) in pyridine and phosphitylated by 2-cyanoethyl *N,N,N,N*-tetraisopropylphosphordiamidite in the presence of *N,N*-diisopropylammonium tetrazolide to give phosphoramidite **5**.



Scheme 1. (i) HCHO, AcOH, thiocresol, 70%; (ii) (a) $NaBH_4$, DMSO; (b) $Ph_2NC(=O)Cl$, $(iPr)_2NEt$; two steps 49%; (iii) (a) TBAF, THF; (b) DMTrCl, pyridine; two steps 77%; (c) *N,N*-diisopropylammonium tetrazolide, 2-cyanoethyl *N,N,N,N*-tertaisopropylphosphordiamidite, THF, 91%.

A set of ODNs containing N^2-methyl-2′-deoxyguanosine was synthesized on a DNA synthesizer by the phosphoramidite method. 5′-*O*-(3,4-DBB)thymidine-3′-*O*-phosphoramidite[13] and ethylene glycol-modified controlled pore glass (CPG)[3] were used for the 5′ and 3′ end modification as reported previously. Phosphoramidite **5** was incorporated into the TGGGAG sequence instead of 2′-deoxyguanosine residues at various positions as shown in the Table 1. After chain-elongation, the modified ODNs were cleaved from the CPG support and deprotected with 28% aqueous ammonia solution. The purification of the ODNs was performed by C18 reversed phase column chromatography.[3] The structures of the modified ODNs were determined by negative MALDI TOF mass spectroscopy (Table 1).

Anti-HIV-1 assays were performed using ODNs **6**–**10** containing m^2dG at various positions. The 50% inhibitory concentration (IC_{50}) of the cytopathic effect for MT-4 cells infected by an HIV-1_{IIIB} strain and the 50% cytotoxicity concentration (CC_{50}) were determined according to a procedure reported previously.[13] We reported that the IC_{50} of R-95288 (**1**) was 0.19 μg/mL (0.08 μM) and its CC_{50} was up to 100 μg/mL (>40 μM).[3] Relative IC_{50} values of m^2dG-containing ODN **6**–**10** to R-95288 were improved by approximately 2-fold (Table 1). These

Table 1. Anti-HIV-1 activity and characterization of m^2dG-containing ODNs

Compounds[a]	Relative IC_{50}[b]	MS [M–H]$^-$ Calcd	MS [M–H]$^-$ Found
1 (TGGGAG, R-95288)	1.0	2296.47	2296.30
6 (T(m^2dG)GGAG)	0.63	2310.49	2310.10
7 (TG(m^2dG)GAG)	0.63	2310.49	2310.21
8 (TGG(m^2dG)AG)	0.45	2310.49	2310.55
9 (TGGGA(m^2dG))	0.63	2310.49	2310.14
10 (T(m^2dG)$_3$AG)	0.46	2338.52	2338.57
11 (TGGG)	43	1654.36	1654.54
12 (T(m^2dG)$_3$)	2.0	1696.41	1696.38

[a]All ODNs have a 3,4-DBB group and a 2-hydroxyethyl group at the 5′ and 3′ end, respectively.
[b]Value of IC_{50} (μM)/IC_{50} value (μM) of R-95288 (**1**).

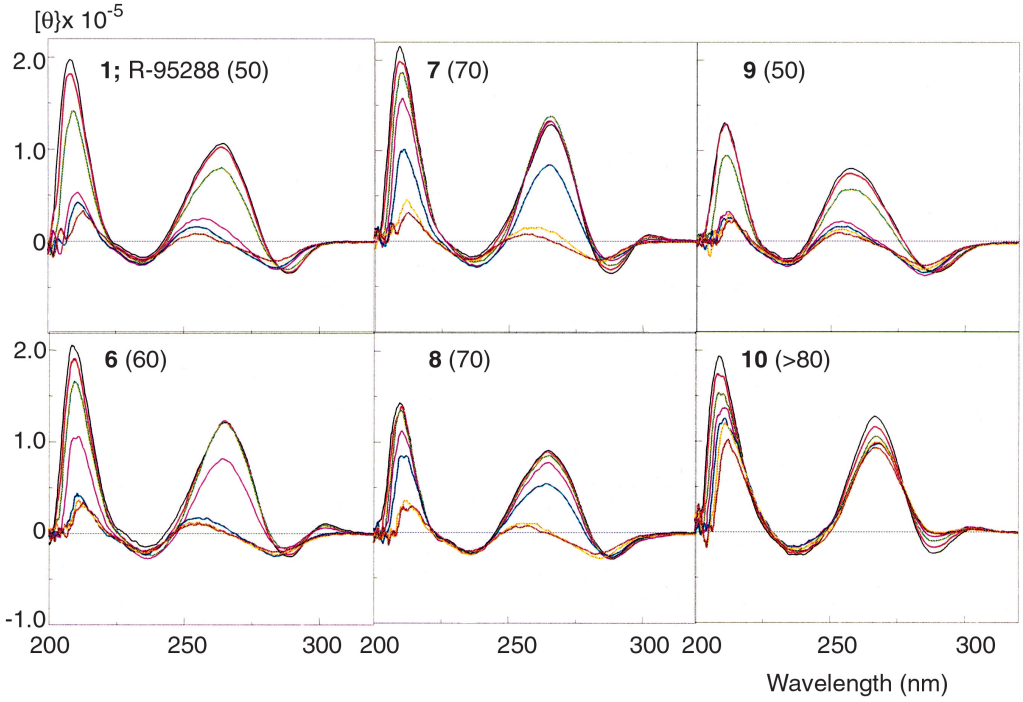


Figure 2. CD spectra of R-95288 and compounds **6**–**10** in phosphate-buffered saline (10 mM phosphate (pH 7), 138 mM NaCl, and 2.7 mM KCl; Sigma) at 20 °C (black), 30 °C (red), 40 °C (green), 50 °C (pink), 60 °C (blue), 70 °C (yellow), and 80 °C (orange). T_m values, which are temperatures at which the maximum wave length changed in the CD spectra, are shown in parentheses.

results indicate that the free proton of the exocyclic amino group of 2′-deoxyguanosine may not be involved in the interaction with the targeted protein, gp120. In particular, substitution at the fourth residue from the 5′ end (compounds **8** and **10**) seems to be effective for anti-HIV-1 activity. All modified compounds showed no cytotoxicity up to 100 μg/mL (>40 μM).

Guanine-rich ODNs can form parallel or anti-parallel quadruplex structures.[1,3,4,7–9] We have found that R-95288 also forms the parallel structure at less than 50 °C (this temperature is called T_m).[4] Oligonucleotides containing N^2-methylguanosine can form duplexes identical to that of normal oligonucleotides.[14] This means that methylation at the N-2 position of guanosine does not inhibit formation of hydrogen bonds. To investigate the effect of N^2-methylation on the stability of ODNs, we measured the CD spectra of these m^{2}dG-containing compounds in phosphate-buffered saline at various temperatures.

The CD spectra demonstrated that compounds **6**–**8**, in which one guanine residue of the internal contiguous guanines was substituted with m^{2}dG, formed the parallel G-quadruplex as well as did the parent compound R-95288 and had higher stability than R-95288 (Fig. 2). T_m values (70 °C) for compounds **7** and **8**, in which was substituted the second or the third 2-deoxyguanosine among the three contiguous 2′-deoxyguanosine residues, were much higher than that of compound **6** (60 °C), in which the first guanosine residue was substituted with m^{2}dG. On the other hand, terminal substitution with m^{2}dG (compound **9**) had no effect on the quadruplex due to the poor stacking of the terminal base. These results might be due to the improvement of base stacking potential by methylation in the nucleobase.[15] Interestingly, compound **10** with three contiguous m^{2}dGs dissociated the least, with little change, even at 80 °C. This stabilizing effect of a modification with three m^{2}dGs corresponds to that of five contiguous guanosine residues in an ODN such as (3,4-DBB)-T(G)$_5$.[3]

On the basis of these results, the structure R-95288 (**1**) was minimized down to three contiguous m^{2}dGs, that is, a TGGG sequence with three 2′-deoxyguanosines (compound **11**). This was the minimum required structure but had less anti-HIV-1 activity than R-95288.[3] Compound **11** was substituted with three modified nucleosides to obtain compound **12**. By comparing the IC$_{50}$s, the activity of compound **11** was 43 times lower than R-95288. However, as predicted, the activity of compound **12** with three contiguous m^{2}dGs was nearly identical to R-95288, due to the stabilization of the G-quadruplex.

In conclusion, we demonstrated that the N^2-methylation of 2′-deoxyguanosine on the parallel G-quadruplex enhanced the thermodynamic stability of the G-quartet, and that a set of ODNs modified with m^{2}dG had higher anti-HIV-1 activity than the parent R-95288, due to stabilizing effects. N^2-Alkylation of guanine residues is a promising way to improve the stability of the parallel G-quadruplex, as long as the modification does not sterically hinder the formation of the G-quadruplex.

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