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Biologically Active Oligodeoxyribonucleotides. Part 11:† The Least Phosphate-modification of Quadruplex-forming Hexadeoxyribonucleotide TGGGAG, Bearing 3′- and 5′-End-modification, with Anti-HIV-1 Activity

Makoto Koizumi,[a,*] Rika Koga,[a] Hitoshi Hotoda,[a] Toshinori Ohmine,[b] Hidehiko Furukawa,[b] Toshinori Agatsuma,[b] Takashi Nishigaki,[b] Koji Abe,[c] Toshiyuki Kosaka,[c] Shinya Tsutsumi,[c] Junko Sone,[c] Masakatsu Kaneko,[a] Satoshi Kimura[d,e] and Kaoru Shimada[d,f]

[a]*Exploratory Chemistry Research Lab., Sankyo Co., Ltd, Tokyo 140, Japan*
[b]*Biological Research Lab., Sankyo Co., Ltd, Tokyo 140, Japan*
[c]*Analytical and Metabolic Research Lab., Sankyo Co., Ltd, Tokyo 140, Japan*
[d]*Department of Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan*
[e]*Department of Infection Control and Prevention, The University of Tokyo Hospital, Tokyo 113, Japan*
[f]*Tokyo Senbai Hospital, Tokyo 108, Japan*

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Abstract—We have found that a hexadeoxyribonucleotide (5′TGGGAG3′, R-95288), Koizumi, M. et al. *Bioorganic & Medicinal Chemistry*, **1997**, *5*, 2235, bearing a 3,4-dibenzyloxybenzyl (3,4-DBB) group at the 5′-end and a 2-hydroxyethylphosphate at the 3′-end, has high anti-HIV-1 activity and the least cytotoxicity in vitro and in vivo. In order to synthesize more potent hexadeoxyribonucleotides, we substituted phosphodiester (P–O) bonds in the 6-mer with the least phosphorothioate (P–S), phosphoramidate (P–N), or methylphosphonate (P–Me) bonds. When more than two P–N or P–Me bonds were introduced into a 6-mer, the phosphate-modified 6-mers had weak or no anti-HIV-1 activity, in spite of quadruplex structure formation. However, when P–S bonds were substituted for P–O bonds, anti-HIV-1 activity of their 6-mers did not dramatically decrease, compared with compounds substituted with P–N or P–Me bonds. The results suggest that the formation of a quadruplex structure is not always sufficient for anti-HIV-1 activity of the 6-mer, and that net negative charges derived from P–O or P–S bonds in the quadruplex are important for anti-HIV-1 activity. Moreover, among various phosphate-modified ODNs, we found that the anti-HIV-1 activity of ODN PS7 with only one P–S bond was the same as that of R-95288, both having a high stability in human plasma. © 1998 Elsevier Science Ltd. All rights reserved.

Key words: Aptamer; 3,4-dibenzyloxybenzyl; 2-hydroxyethylphosphate; phosphorothioate; stability.
*Corresponding author. Tel: +8103-3492-3131; Fax: +8103-5436-8570; E-mail: koizum@shina.sankyo.co.jp
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Abbreviations: CD: circular dichroism, CPG: controlled pore glass, DBB: dibenzyloxybenzyl, DMTr: 4,4′-dimethoxytrityl, HIV: human immunodeficiency virus, ODN: oligodeoxyribonucleotide, PBS: phosphate-buffered saline, TEAA: triethylammonium acetate.

Introduction

We previously found that a pentadecadeoxyribonucleotide bearing a 4,4′-dimethoxytrityl (DMTr) group at the 5′-end had anti-HIV activity.[2,3] When the chain length of the oligodeoxyribonucleotide (ODN) was shortened,[4] and the acid-labile DMTr group was substituted with a variety of aromatic groups, a hexadeoxyribonucleotide (5′TGGGAG3′), bearing a 3,4-dibenzyloxybenzyl (3,4-

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DBB) group at the 5′-end had high anti-HIV-1 activity and low cytotoxicity.[5,6] Moreover, when various modified phosphate groups were introduced into the 5′-end-modified 6-mer at the 3′-end to promote resistance to 3′-exonuclease, these compounds had higher anti-HIV-1 activity and greater stability in human plasma than 6-mers without 3′-end-modification.[1] The 6-mer bearing a 3,4-DBB group at the 5′-end and a 2-hydroxyethylphosphate group at the 3′-end (R-95288) was the best compound from among the 6-mers tested for anti-HIV-1 activity.[1] We also showed that R-95288 had anti-HIV-1 activity using hu-PBL-SCID/beige mice as an animal model for HIV-1 infection.[7] The mechanism of action of such ODNs appears to be the inhibition of adsorption and the entry of HIV-1 to the $CD4^+$ cell.[3,8] It is thought that these 6-mers act as aptamers.

On the other hand, it was previously reported that other guanine-rich ODNs also have anti-HIV activity.[9–12] Wyatt et al. found an anti-HIV-active 8-mer ($T_2G_4T_2$, ISIS-5320) with phosphorothioate (P–S) modification by means of combinatorial chemistry.[9,10] The homoguanylate, whose chain length was four or five, with or without P–S modification, also produce anti-HIV-1 activity.[11] Ojwang et al. reported that a 17-mer (AR-177), which was composed of thymidine and deoxyguanosine with two P–S bonds at the 3′- and 5′-ends, inhibited HIV integrase activity in HIV-infected cells.[12,13] The tertiary structures responsible for anti-HIV-1 activity of ISIS-5320, AR-177, and R-95288 were quadruplex structures formed by the guanine-quartet (G-quartet, Fig. 1).[19]

Although fully P–S-modified antisense ODNs have been evaluated in clinical trials, it has been reported that hemodynamic changes, such as hypotension and complement activation, were observed in monkeys upon iv-bolus administration of fully P–S-modified ODNs.[14,15] When some P–S bonds in ODNs were replaced by 2′-*O*-alkylated nucleoside phosphodiester bonds, complement activation decreased.[16] From these results, it seems that ODNs with reduced P–S bonds may be non-toxic and might have therapeutic prospects. In the research reported here, in order to synthesize more potent and non-toxic 6-mers, we replaced phosphodiester (P–O) bonds in the 6-mer with the least phosphorothioate (P–S), phosphoramidate (P–N), or methylphosphonate (P–Me) bonds.

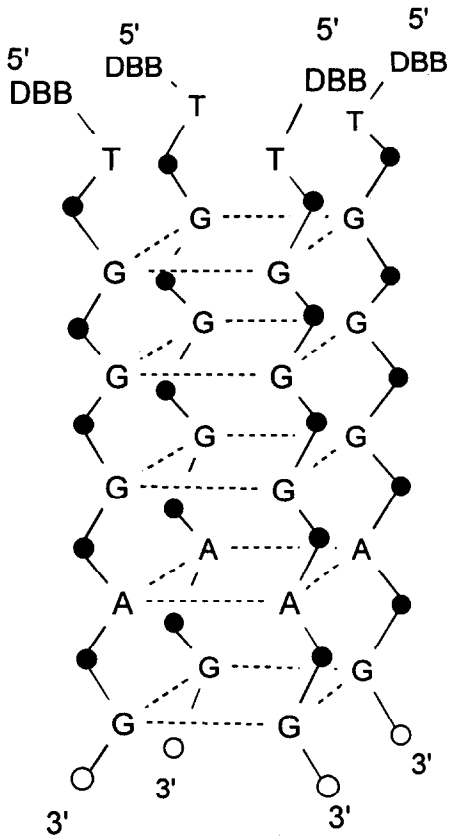


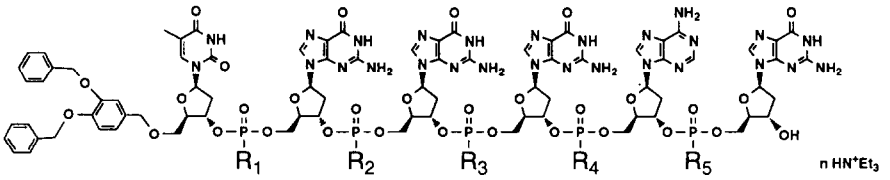
Figure 1. Proposed quadruplex structures of R-95288 and its derivatives. DBB: a 3,4-dibenzyloxybenzyl group, solid circles: P–O, P–S, P–N, or P–Me bonds, open circles: a 2-hydroxyethylphosphate, a 2-hydroxyethylthiophosphate, or a hydroxyl group.

Results and Discussion

Anti-HIV-1 activity of phosphate-modified ODNs

ODNs, in which P–O bonds were modified with P–S, P–N, or P–Me bonds from the 3′-end, were synthesized using the solid-phase method (Table 1). A 50% inhibitory concentration (IC_{50}) of the cytopathic effect for MT-4 cells infected by HIV-1_{IIIB} and a 50% cytotoxic concentration (CC_{50}) of phosphate-modified ODNs were determined by an MTT assay according to a procedure reported previously.[5] We reported that a 3,4-DBB group exhibited no cytotoxicity up to 100 μg/mL.[6] The IC_{50} value of a 5′-*O*-(3,4-DBB)-modified ODN without 3′-end-modification (ODN PO) was 1.7 μg/mL. Anti-HIV-1 activity of phosphate-modified ODNs was measured, as shown in Table 1. When several P–O bonds of ODN PO from the 3′-end were substituted with P–S bonds, anti-HIV-1 activity of these ODNs decreased slightly compared with that of ODN PO. However, anti-HIV-1 activity of a fully P–S-modified ODN (ODN PS5) decreased by about six-fold (IC_{50} = 10.2 μg/mL). In the cases of P–N and P–Me modifications, more than two substitutions in ODNs caused a drastic decrease in anti-HIV-1 activity (IC_{50} > 40 μg/mL), as shown in Table 1. One of the reasons for low anti-HIV-1 activity was thought to be that phosphate-modification disrupted the formation of a G-quadruplex of these 6-mers,

Table 1. Structures of phosphate-modified ODNs and their anti-HIV-1 activity



ODN	n	R_1	R_2	R_3	R_4	R_5	IC_{50} (μg/mL)	CC_{50} (μg/mL)
PO	5	O^-	O^-	O^-	O^-	O^-	1.7	> 50
PS1	5	O^-	O^-	O^-	O^-	S^-	1.4	> 50
PS2	5	O^-	O^-	O^-	S^-	S^-	4.8	> 50
PS3	5	O^-	O^-	S^-	S^-	S^-	2.7	> 50
PS4	5	O^-	S^-	S^-	S^-	S^-	3.8	> 50
PS5	5	S^-	S^-	S^-	S^-	S^-	10.2	> 50
PN1	4	O^-	O^-	O^-	O^-	NHR_6	3.6	> 50
PN2	3	O^-	O^-	O^-	NHR_6	NHR_6	> 50	> 50
PMe1	4	O^-	O^-	O^-	O^-	CH_2	7.3	> 50
PMe2	3	O^-	O^-	O^-	CH_3	CH_3	40	> 50
PMe3	2	O^-	O^-	CH_3	CH_3	CH_3	39	> 50
PMe4	1	O^-	CH_3	CH_3	CH_3	CH_3	> 50	> 50

$R_6 = -CH_2CH_2OCH_3$.

as shown in Figure 1. Next, we investigated tertiary structures of these phosphate-modified 6-mers using CD spectroscopy.

CD spectra of phosphate-modified ODNs

We found that R-95288 has a quadruplex structure that consists of G-quartets using CD spectroscopy, which can show us tertiary structures of ODNs in physiological conditions (Fig. 1).[1] The CD spectra of R-95288 at 20, 30, and 40 °C had a maximum of 264 or 265 nm, which was consistent with the data for the parallel quadruplex, and those of R-95288 at more than 50 °C had a maximum of 254 nm, due to the structure of a single strand.[1,17] The CD spectra of ODN PO (Fig. 2(a)) were similar to those of R-95288 that formed the quadruplex structure.[1] When the CD spectra of P–S-modified ODNs with anti-HIV-1 activity were measured in PBS buffer at various temperatures to investigate the formation of a G-quadruplex structure, the CD spectra of P–S-modified ODNs at 20, 30, and 40 °C had a broad peak at 264 nm with a shoulder at about 254 nm, which was derived from a single strand, and those of P–S-modified ODNs at more than 50 °C had a maximum of 254 nm (the CD spectra of ODN PS2 are shown in Fig. 2(b), and the other data not shown). These data suggest that the G-quadruplex of P–S-modified ODNs is more unstable than that of ODN PO. In a recent study, P–S-modified $T_2G_4T_2$, ISIS 5320, showed strong anti-HIV-1 activity with the quadruplex structure. Unmodified ISIS 5320 without anti-HIV-1 activity also formed a quadruplex structure that was more stable than that of ISIS 5320.[9] The unstable structures of P–S-modified ODNs (ODN PS1–PS5) might be one reason these ODNs had less anti-HIV-1 activity than ODN PO.

The CD spectra of ODNs PN2 and PMe2 without anti-HIV-1 activity, in which two P–O bonds from the 3′-end were substituted with P–N and P–Me bonds, respectively, were measured at various temperatures, as shown in Figure 2(c) and (d). The CD spectra of these phosphate-modified ODNs at 20, 30, and 40 °C also had a maximum of 264 or 265 nm that was similar to that of ODN PO. These data show that these phosphate-modifications of the 6-mer, even P–N or P–Me bonds, do not influence the formation of the G-quadruplex structure, and that the formation of the G-quadruplex structure is not always critical for anti-HIV-1 activity. Similarly, it has also been shown that some 5′-DMTr-modified 15-mers containing $(G)_3$ sequences form a G-quadruplex without anti-HIV-1 activity.[18] In a recent study, ISIS 5320 with the quadruplex structure showed strong anti-HIV-1 activity. However, it was shown that the quadruplex structure was not always sufficient for antiviral activity, because the quadruplex-forming phosphodiester α-oligonucleotide had no activity.[9]

Inhibition of binding of gp120 with anti-V3 mAb by phosphate-modified ODNs

We have found that R-95288 binds to a V3 loop and CD4-binding sites on HIV gp120.[8] We investigated

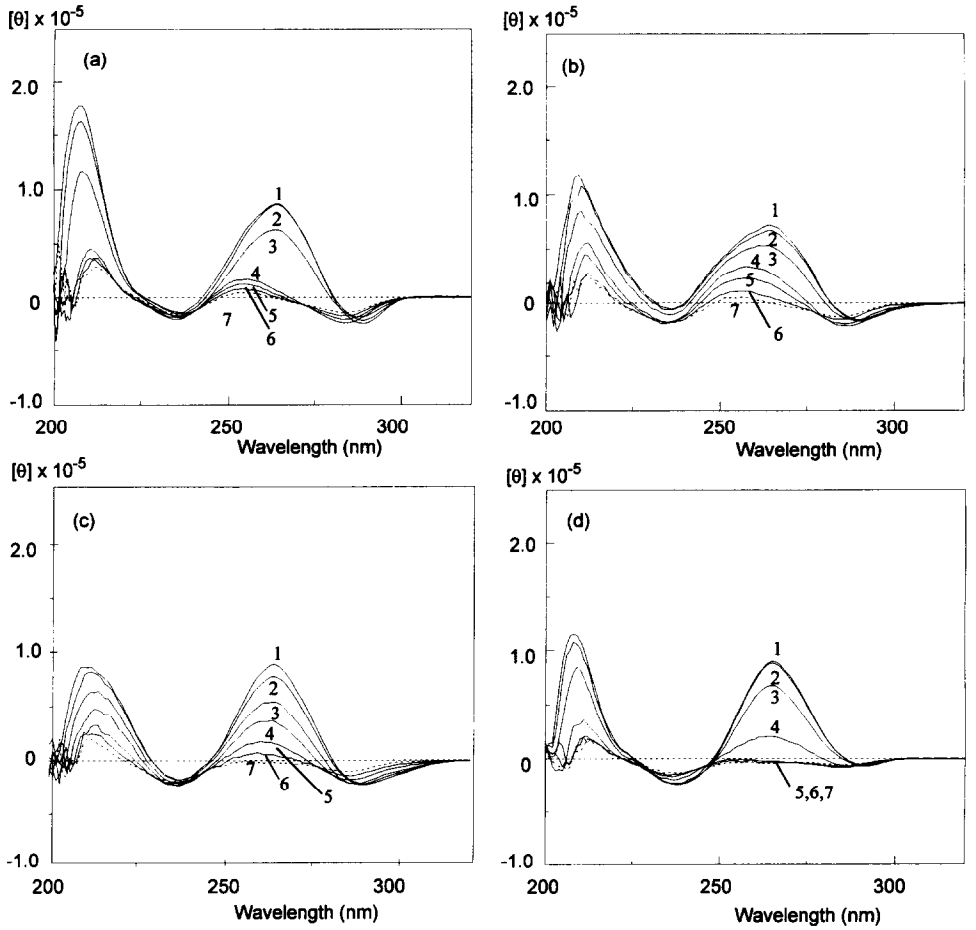


Figure 2. CD spectra of phosphate-modified ODNs (a) ODN PO, (b) ODN PS2, (c) ODN PN2, and (d) ODN PMe2. The measurement conditions are described in the Experimental. Line 1: 20 °C, line 2: 30 °C, line 3: 40 °C, line 4: 50 °C, line 5: 60 °C, line 6: 70 °C, line 7: 80 °C.

inhibition of binding of HIV-1 gp120 with anti-V3 mAb by three phosphate-modified ODNs, PS2, PN2, and PMe2, since anti-HIV-1 activity of the 6-mer seemed to be mainly due to binding of the gp120 V3 loop with the 6-mer.[8] The binding activity of ODNs to gp120 was shown as the value (EC_{50}) of inhibition of anti-V3 mAb binding by the 6-mer. The EC_{50} (mean ± SD) values for ODNs PO, PS2, PN2, PMe2, and R-95288 were 11 ± 2, 5.0 ± 0.9, 27 ± 2, 16 ± 1, and 2.5 ± 0.3 μg/mL, respectively. Although ODN PS2 with two P–S bonds had higher gp120-binding activity than ODN PO, probably due to its non-specific binding manner, anti-HIV-1 activity of ODN PS2 did not increase (Table 1). It has been reported that various P–S-modified ODNs, which bind to the basic V3 loop region of HIV-1 gp120, had anti-HIV-1 activity in their non-sequence-specific manner.[19,20] Anti-HIV-1 activity of these P–S-modified ODNs was high in comparison with the unmodified P–O ODN, due to binding of sulfur atoms of P–S bonds to gp120. P–S bonds of ISIS 5320 with anti-HIV-1 activity played a role in augmenting binding to the V3 loop of HIV-1 gp120.[9,21] It was also shown that a derivative of a quadruplex-forming AR177 with all P–O bonds was less active than an all P–S-modified AR177 derivative.[12] Based on these results, we speculate that binding formations of ODNs PO and PS may be different from those for ISIS 5320, AR177, and other P–S-modified ODNs that were effective in their non-sequence-specific manner. Moreover, the greatest structural difference between ODNs PO and PS, and other P–S-modified ODNs with anti-HIV-1 activity, is that the former had a hydrophobic 3,4-DBB group, which promoted the formation of the G-quadruplex structure.[1,22] It has been shown that some cholesterol-linked ODNs have anti-HIV-1 activity.[19] HIV-1 gp120 seems to interact with a hydrophobic group such as a cholesteryl moiety or a 3,4-DBB group. A detailed study of the interaction with gp120 and the 6-mer is in progress.

On the other hand, ODNs PN2 and PMe2 with three negative charges showed a weaker inhibition of anti-V3 mAb binding than ODNs PO and PS2 with five negative charges and R-95288 with six negative charges. These data obviously suggest that anti-HIV-1 activity and gp120-binding inhibitory activity of the 6-mers depend on net negative charges in the quadruplex structure.

Stability of P–S-modified ODNs in human plasma

We investigated the stability of more effectively P–S-modified ODNs (ODN PS1–PS5, Table 1) in human plasma, because a high concentration of ODNs needs to be maintained in the body for a long time to produce effective anti-HIV therapy. ODNs PS1–PS5 were incubated with human plasma for 4 h, then these reaction mixtures were analyzed by reverse phase HPLC to quantify the amount of ODNs remaining (Fig. 3(a)). ODN PS1 with a P–S bond was very stable in human plasma when compared to ODN PO with all P–O bonds. The stability of the other P–S-modified ODNs was similar to that of ODN PS1. Therefore, it was found that the substitution of only one oxygen atom of ODN PO with a sulfur atom at the R_5 group was sufficient for stabilizing ODN in human plasma.

Anti-HIV-1 activity of partially P–S-modified R-95288

On the basis of the above information, we modified R-95288 ($IC_{50} = 0.19\ \mu g/mL$, $CC_{50} > 100\ \mu g/mL$),[1] which was a 6-mer, TGGGAG, bearing a 3,4-DBB group at the 5′-end and a 2-hydroxyethylphosphate group at the 3′-end, with some P–S bonds. We previously synthesized ODN PS6, in which the R_6 group at the 3′-end were substituted with a sulfur atom, and measured its anti-HIV-1 activity, and this value was similar to that of R-95288 (Table 2); however, ODN PS6 was unstable in plasma.[1] Because we found that modification of the R_5 group influenced the stability of ODNs in human plasma, where there were several 3′-exonucleases (Fig. 3(a)), we substituted an oxygen atom of R-95288 with a sulfur atom at the position of R_5 to obtain ODN PS7, as shown in Table 2. Even if a 2-hydroxyethylphosphate group at the 3′-end of ODN PS7 is digested with 3′-exonucleases, ODN PS7 becomes ODN PS1 that still maintains its anti-HIV-1 activity, as shown in Table 1, and that is stable in plasma, as shown in Figure 3(a). ODN PS8, in which P–O bonds were substituted with P–S bonds at two positions of R_5 and R_6, was also synthesized to provide more stable ODN in plasma. Although anti-HIV-1 activity of ODN PS7, in which the oxygen atom was substituted with the sulfur atom at the R_5 group, did not change in comparison to R-95288, another substitution failed to achieve high activity (Table 2). These results also suggest that the numerous substitutions of R-95288 with P–S bonds, as well as the P–S modifications of ODN PO (Table 1), cause a decrease in anti-HIV-1 activity, probably due to a disruption of the quadruplex structure. We found that ODN PS7 had a high stability in human plasma, as expected (Fig. 3(b)). This data shows that ODN PS7 as well as R-95288 may be candidates for possible use in therapy of anti-HIV-1. Yu et al. reported that ODNs with reduced P–S bonds were non-toxic.[16] Because R-95288 has no P–S modification and ODN PS7 has the least P–S modification, they may be therapeutic agents without side effects or toxicity. A pharmacokinetic study of R-95288 and ODN PS7 is in progress.

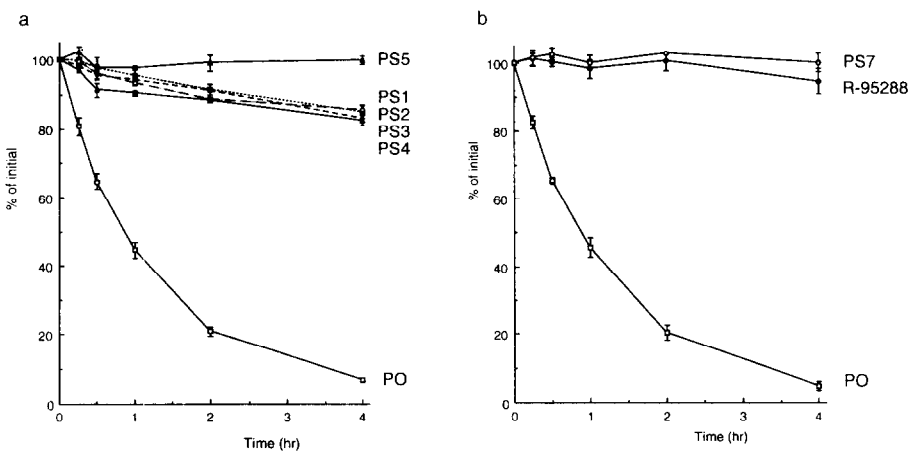
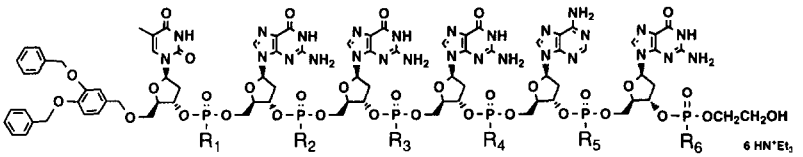


Figure 3. Measurement of stability of phosphate-modified ODNs PO, PS1, PS2, PS3, PS4, and PS5 in human plasma. (a) Percentages of the remaining ODNs PO, PS1, PS2, PS3, PS4, and PS5 are shown as open squares, solid squares, solid circles, open circles, open triangles, and solid triangles, respectively. (b) Measurement of stability of phosphate-modified ODNs PO, PS7, and R-95288 in human plasma. Percentages of the remaining ODNs PO, PS7, and R-95288 are shown as open squares, open circles, and solid circles, respectively.

Table 2. Structures of phosphate-modified R-95288 derivatives and their anti-HIV-1 activity



ODN	R_1	R_2	R_3	R_4	R_5	R_6	relative activity[a]
R-95288	O^-	O^-	O^-	O^-	O^-	O^-	1.0
PS6	O^-	O^-	O^-	O^-	O^-	S^-	0.8[b]
PS7	O^-	O^-	O^-	O^-	S^-	O^-	0.8[c]
PS8	O^-	O^-	O^-	O^-	S^-	S^-	1.8[c]

[a]Value of IC_{50}/IC_{50} of R-95288.
[b]Data from ref 1.
[c]This work.

Experimental

Synthesis of phosphate-modified ODNs

Phosphate-modified ODNs were prepared using a commercially available 2′-deoxyguanosine-bound CPG or an ethylene glycol-bound CPG[1] on a Cyclon DNA synthesizer (Milligen). 5′-*O*-(3,4-Dibenzyloxy)benzyl thymidine 3′-*O*-β-cyanoethylphosphoramidite[5] was finally coupled with ODN-linked CPGs for 5′-end-modification. In order to introduce P–S bonds into ODNs, 5′-*O*-(4,4′-dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine 3′-*O*-β-cyanoethylphosphoramidite or 5′-*O*-(4,4′-dimethoxytrityl)-*N*-benzoyl-2′-deoxyadenosine 3′-*O*-β-cyanoethylphosphoramidite (Milligen) was coupled in the presence of 1-*H* tetrazole on detritylated CPGs, and then sulfurized by tetraethylthiuram disulfide (Perkin Elmer).[23] 5′-*O*-(4,4′-Dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine-3′-methylphosphonamidite or 5′-*O*-(4,4′-dimethoxytrityl)-*N*-benzoyl-2′-deoxyadenosine-3′-methylphosphonamidite (Glen Research) was used to synthesize ODNs bearing P–Me bonds.[24] 5′-*O*-(4,4′-Dimethoxytrityl)-*N*-isobutyryl-2′-deoxyguanosine-3′-*H*-phosphonate or 5′-*O*-(4,4′-dimethoxytrityl)-*N*-benzoyl-2′-deoxyadenosine-3′-*H*-phosphonate (Milligen) was coupled on detritylated CPGs in the presence of 1-adamantanecarbonyl chloride, and then oxidative amination with 10% methoxyethylamine in CCl_4 was performed to introduce P–N bonds at the desired position of ODNs.[25] In the case of the synthesis of ODNs bearing P–S or P–N bonds, after coupling reaction, the CPG was treated with 29% aqueous ammonia at 60 °C for 5 h. Deprotection of ODNs bearing P–Me bonds was performed according to Hogrefes' method.[24] The CPGs were treated with 2 mL of ethanol:acetonitrile:29% aqueous ammonia (45:45:10 v/v/v) at room temperature. After 30 min, an equal volume of ethylenediamine was added to the reaction mixture. Then, after 6 h, the CPGs were filtered and washed with 2 mL of acetonitrile:H_2O (1:1 v/v) solution. The filtrate was neutralized with 6 N HCl containing 10% acetonitrile at 0 °C. The crude ODNs were purified by C18 column chromatography [Preparative C18, Waters, 1.5×15 cm, 50 mM triethylammonium bicarbonate (pH 7.5), 20–50% CH_3CN; linear gradient]. The purity of ODNs was analyzed by reverse phase HPLC [Inertsil ODS-2, GL Science Inc., Japan, 6×150 mm, solution A: 0.1 M triethylammonium acetate (TEAA, pH 7.5), solution B: CH_3CN (20 min), 1 mL/min, 260 nm]. The structures of the phosphate-modified ODNs were determined by negative ion LSI mass spectroscopy. ODN PS1 $[M-H]^-$, calcd: 2188.46, found: 2188.27. ODN PS2 $[M-H]^-$, calcd: 2204.44, found: 2204.48. ODN PS3 $[M-H]^-$, calcd: 2220.41, found: 2220.44. ODN PS4 $[M-H]^-$, calcd: 2236.39, found: 2236.38. ODN PS5 $[M-H]^-$, calcd: 2252.37, found: 2252.51. ODN PS7 $[M-H]^-$, calcd: 2312.45, found: 2312.59. ODN PN1 $[M-H]^-$, calcd: 2229.54, found: 2229.54. ODN PN2 $[M-H]^-$, calcd: 2286.60, found: 2286.37. ODN PMe1 $[M-H]^-$, calcd: 2170.50, found: 2170.43. ODN PMe2 $[M-H]^-$, calcd: 2168.52, found: 2168.50. ODN PMe3 $[M-H]^-$, calcd: 2166.54, found: 2166.52. ODN PMe4 $[M-H]^-$, calcd: 2164.56, found: 2164.38.

Measurement of anti-HIV-1 activity in MT-4 cells

The inhibition of the cytopathic effect of HIV-1 by ODNs was assayed using MT-4 cells, as described previously.[1,5]

Measurement of stability of phosphate-modified ODNs in human plasma

To each solution of 30 μg of ODNs in 100 μL of PBS, 1.4 mL of human plasma (final concentration; 93.3% plasma) was added at 37 °C. The reaction mixture (100 μL) was added into 100 μL of lysis buffer (Perkin–Elmer) at 0, 0.25, 0.5, 1, 2, and 4 h after the initial mixing. To the sampling mixture was added 70 μL of PBS buffer

(pH 7.4), 10 μL of Tris–HCl (pH 8), 10 μL of 25 mg/mL proteinase K (Perkin–Elmer), and a 6-mer, TGGGAG, bearing a 3,4-dibenzyloxybenzyl group at the 5′-end and a 2-chlorophenylphosphate group at the 3′-end as an internal standard at 37 °C.[1] After 30 min, the reaction mixture was washed with 300 μL of phenol:$CHCl_3$:isoamylalcohol (25:24:1 v/v/v) and with 300 μL of $CHCl_3$. The reaction mixture was analyzed by reverse phase HPLC [Wakopak WS-DNA, Wako Co., Ltd, Japan, 4.6×150 mm, 0.1 M TEAA (pH 7.0):CH_3CN (75:25 v/v), 1 mL/min, 260 nm]. The amount of remaining 6-mer was calculated from the peak area of the internal standard (retention time of ODN PO: 15.7 min, ODN PS1: 16.2 min, ODN PS2: 16.4 min, ODN PS3: 16.2 min, ODN PS4: 16.6 min, ODN PS5: 35.2 min, ODN PS7: 15.3 min, R-95288: 15.0 min, internal standard: 20.3 min).

Measurement of CD spectra of phosphate-modified ODNs

CD spectra were recorded with a JASCO J-500C Spectropolarimeter. Phosphate-modified ODN (about 1 A_{260} unit/mL) was dissolved in PBS buffer.

Anti-V3 loop monoclonal antibody (mAb) binding assay

Capture antibody D7324 (Aalto) was adsorbed onto a 96-well plate (Nunc-Immuno Plate PolySorp; Nunc) by incubation at 4 °C overnight at a concentration of 10 μg/mL in 100 μL of 0.1 M Tris–HCl (pH 9.6). The wells were washed three times with PBS containing 0.05% Tween 20 (0.05% Tween 20-PBS), and then blocked for 2 h at room temperature with 200 μL of PBS containing 1% BSA (Sigma). Recombinant gp120 (Intracel, 200 ng/mL) was bound to D7324 by incubation in 100 μL of PBS containing 1% BSA and 1% normal sheep serum (Sigma) at room temperature for 2 h. After washing the wells five times with 0.05% Tween 20-PBS, anti-V3 loop mAb (ABi-13-105, Advanced Biotechnologies) was added in triplicate to PBS containing 1% BSA and 1% normal sheep serum at a concentration of 0.25 μg/mL in the presence of different concentrations of ODNs. The wells were incubated at room temperature for 30 min. Unbound antibodies were removed by washing five times with 0.05% Tween 20-PBS. Bound antibodies were detected by goat anti-mouse IgG peroxidase conjugates (Sigma) and 3,3′,5,5′-tetramethylbenzidine solution (Sumilon).

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