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5′-Linked Lipid-oligodeoxyribonucleotide Derivatives as Inhibitors of Human Immunodeficiency Virus Replication

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Abstract—The covalent attachment of a phospholipid moiety, bound to the 5′-ends of phosphodiester and phosphorothioate oligonucleotides (L-ODNs and LS-ODNs), was achieved using H-phosphonate chemistry, and the lipid-oligonucleotides were assayed for the inhibition of virus replication in HIV-1 infected MT-4 cells. In the anti-HIV activity test, lipid-phosphorothioate oligonucleotides showed higher anti-HIV activities than non-lipid-phosphorothioate oligonucleotides, at the low concentration of 0.04 μM. LS-ODNs can inhibit HIV-1 reverse transcriptase activity through interactions with the enzyme. We found that the covalent attachment of a phospholipid group to the 5′-end of the phosphorothioate oligonucleotide enhances its nonsequence specific anti-HIV activity. Copyright © 1996 Elsevier Science Ltd

Introduction

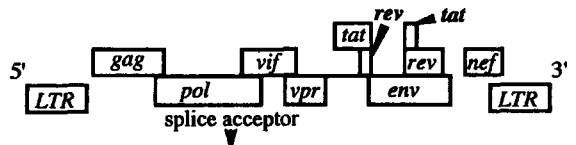
Antisense oligonucleotides and their analogues, which are complementary to a portion of a viral genome or some message fragment sequences, inhibit viral replication and regulate gene expression by hybridizing with viral nucleotide sequences.[1–7] It has also been reported that the modification of the phosphate backbones of oligonucleotides with a methyl group or a sulfur atom produced favorable antiviral agents in vitro as well as in vivo, because the phosphate-modified oligonucleotides are stable against cellular nucleases in the culture medium and in serum.[8–11] However, the use of antisense oligonucleotides introduces some problems, such as poor uptake by cells due to their short size and high charge density. A number of workers have proposed that DNA could be introduced into cells by microinjection,[12] liposome,[13] calcium phosphate,[14] and cationic lipid[15] methods. On the other hand, with the modification of internucleotidic bonds, nonionic oligomer analogues (methyl phosphonate,[16] amidate,[17] and triester[18]) diffuse passively through cell membranes. Recently, a cholesterol–nucleoside conjugate for incorporation into liposomes was reported.[19] Hydrophobic groups, such as a cholesteryl moiety covalently linked to either the 5′- or 3′-end of phosphodiester oligonucleotides (ODNs) and phosphorothioate oligonucleotides (S-ODNs), are more potent inhibitors of syncytia formation, reverse transcriptase activity, and p24 production.[20–22] The fact that the very short cholestryl-phosphorothioate derivatives bind poorly to complementary strands, yet exhibit appreciable activity, and the observation that the activity of the decamers is not sequence-dependent, point toward some mechanism other than antisense inhibition, at least in the case of the shorter oligonucleotides. In another study, a cholesteryl moiety covalently linked to the oligonucleotides was shown to enhance uptake into cells.[23] Furthermore, other workers have synthesized a series of oligonucleotides with a phospholipid moiety covalently linked at the 5′-end.[24] The oligomer structures were complementary to the VSV initiation codon region. The lipid-oligonucleotide should increase uptake and sequence-specific antiviral activity in L929 cells infected with VSV, with no cellular toxicity.

In this paper we describe the covalent attachment of a phospholipid group to the 5′-end of sense-, mismatched-, homo-, and antisense-phosphorothioate oligonucleotides (Fig. 1) and their activities as inhibitors of HIV replication in cultured cells. These results were compared with the inhibition observed by using an unmodified phosphorothioate oligonucleotide of the same target. We found that the covalent attachment of a phospholipid group to the 5′-end of the phosphorothioate oligonucleotide enhances its nonsequence specific anti-HIV activity.

Results

Synthesis and properties of lipid-oligonucleotides

The solid-phase synthesis of modified phosphodiester or phosphorothioate analogues bearing a terminal hydrophobic lipid group has been achieved (Fig. 2).



CAAAAGCCUUAGGCAUCUCC (5529-5548)
3' d(GTTTTCGGAATCCGTAGAGG) ODNs-*rev*-sa 5'
d(GTTTTCGGAATCCGTAGAGG) S-ODNs-*rev*-sa
d(CAAAAGCCTTAGGCATCTCC) S-ODNs-*rev*-sa-sen
d(GTTTTCGGAATCAGTAGAGG) S-ODNs-*rev*-sa-8mis
d(TTTTTTTTTTTTTTTTTTTT) S-dT20
d(CCCCCCCCCCCCCCCCCCCC) S-dC20
*d(GTTTTCGGAATCCGTAGAGG) L-ODNs-*rev*-sa
*d(GTTTTCGGAATCCGTAGAGG) LS-ODNs-*rev*-sa
*d(CAAAAGCCTTAGGCATCTCC) LS-ODNs-*rev*-sa-sen
*d(GTTTTCGGAATCAGTAGAGG) LS-ODNs-*rev*-sa-8mis
*d(TTTTTTTTTTTTTTTTTTTT) LS-dT20
*d(CCCCCCCCCCCCCCCCCCCC) LS-dC20

Figure 1. (Upper part) The italicized bold characters are targeted regions in the HIV genome. (Lower part) The targeted RNA sequences and their complementary DNA sequences. Phosphorothioate portions are underlined. *Phospholipids are bound to the 5′-ends of the ODNs and S-ODNs. The numbering of the HIV RNA and the functional sites was taken from Sadaie *et al.*[25] for 5529–5548.

1,2-Di-O-hexadecyl-*rac*-glycerol was treated with tris-(1,1,1,3,3,3-hexafluoro-2-propyl)phosphite,[26] and was converted to an *H*-phosphonate monomer **1**. After conjugation of the phosphitylated phospholipid monomer **1** to the free 5′-hydroxyl terminus of the CPG support-bound oligonucleotide, I_2 oxidation or S_8 sulfuration was performed for the phosphodiester or the phosphorothioate linkage between the phospholipid and the oligonucleotide **2**. Fully deprotected lipid-oligonucleotides were eluted as a single peak by higher concentrations of acetonitrile than the non-lipid-oligonucleotides (60% and 12%, respectively) on reversed-phase HPLC. The lipid-oligonucleotides migrated as distinct bands of lower mobility than the non-lipid-oligonucleotides by denaturing PAGE. This post-synthetic strategy is practical and convenient for large scale 5′-end lipid-oligonucleotide synthesis with high purity and yield.

The lipid attachment at the 5′-ends of the oligonucleotides were confirmed by enzymatic digestion. The

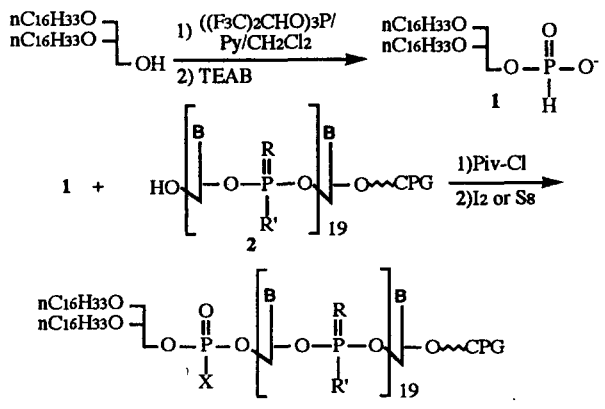


Figure 2. The chemical structure of unmodified (R=H, X=O: ODNs), phosphorothioate (R=H, X=S: S-ODNs), and lipid-conjugated (R=phospholipid, X=O: L-ODNs and R=phospholipid, X=S: LS-ODNs) oligodeoxynucleotides.

phosphorothioate oligonucleotide was oxidized with a 0.1 M I_2 solution to phosphodiesters. Nearly complete hydrolysis of the DNA internucleotidic bonds of the lipid-oligonucleotides was accomplished using snake venom phosphodiesterase, while bovine spleen phosphodiesterase had no effect.

Anti-HIV activity of lipid-oligonucleotides

We chose *rev* gene splice sites as targets for HIV-1 suppression in our previous work,[27,28] and we have now extended those ideas to lipid-oligonucleotides.

We could not detect any inhibitory effects of the lipid-oligonucleotides (L-ODNs-*rev*-sa) at a concentration of 5 μM (Table 1), but the L-ODNs showed 72% inhibition at 15 μM. On the other hand, the unmodified oligonucleotides (ODNs-*rev*-sa) with the same sequence showed no inhibitory activity at 15 μM (data not shown). The lipid-phosphodiester oligonucleotides (L-ODNs-*rev*-sa) would easily be degraded by cellular nucleases before displaying their function; otherwise these oligonucleotides were as effective as a phosphorothioates complementary to the *rev* gene of HIV-1 at higher concentrations. The phospholipid itself had very weak anti-HIV activity and it was not toxic up to 30 μM (data not shown).

On the other hand, the lipid-phosphorothioate oligonucleotides (LS-ODNs-*rev*-sa) exhibited a more potent inhibitory effect than the non-lipid-phosphorothioate oligonucleotides (S-ODNs-*rev*-sa) and the L-ODNs-*rev*-sa. The phosphorothioate oligonucleotide, S-ODNs-*rev*-sa, inhibited HIV replication by 97% at 1 μM (Table 1); however, the covalent attachment of a phospholipid to these oligonucleotides reduced their effective dosage for the HIV-1 infected MT-4 cells. Complete inhibition was observed with the LS-ODNs-*rev*-sa at the low concentration of 0.04 μM (Table 1).

Furthermore, the higher anti-HIV-1 activity of the LS-ODNs as compared to the S-ODNs was supported by uptake studies. Namely, the cellular uptake efficiencies of the LS-ODNs-*rev*-sa and the S-ODNs-*rev*-sa by MOLT-4 cells were compared using 3′-[^{32}P] labeled oligonucleotides and MOLT-4 cells. The uptake reached a plateau after 4 h of coincubation, and the cell-associated counts of the LS-ODNs-*rev*-sa (4 h: 0.185 pmol/10^5 cells; 6 h: 0.173 pmol/10^5 cells) were 2.5 times higher than those of the S-ODNs-*rev*-sa (4 h: 0.073 pmol/10^5 cells; 6 h: 0.062 pmol/10^5 cells).

To characterize the antisense inhibitory process of the LS-ODNs, we extended our studies to the inhibitory effects of sense- (LS-ODNs-*rev*-sa-sen), mismatched- (LS-ODNs-*rev*-sa-8mis), and homo- (LS-dT_{20} and LS-dC_{20}) phosphorothioate oligonucleotides noncomplementary to the HIV-1 mRNA. These compounds showed increased anti-HIV activity as compared to the non-lipid-antisense phosphorothioate oligonucleotides (Table 1). These results indicate that the lipid modification of the phosphorothioate oligonucleotide

Table 1. Comparison of anti-HIV activities of lipid- and non-lipid-oligodeoxyribonucleotides

Oligomer[a]	Inhibitory effect[b] (%)					Cytotoxicity[c] (%)				
	0.8	4	20	100	500	0.8	4	20	100	500
			$\times 10^{-2}$ μM					$\times 10^{-2}$ μM		
ODNs-*rev*-sa	0	0	0	0	3	0	0	0	0	8
S-ODNs-*rev*-sa	11	14	50	97	100	0	0	0	0	0
S-ODNs-*rev*-sa-sen	2	7	20	39	40	6	0	0	20	35
S-ODNs-*rev*-sa-8mis	5	0	31	40	42	0	0	0	18	29
S-dT_{20}	3	14	34	96	61	0	9	0	0	25
S-dC_{20}	0	0	28	89	65	0	0	0	0	29
Phospholipid	8	19	4	2	4	0	0	0	0	0
L-ODNs-*rev*-sa	9	3	0	8	5	0	0	0	0	8
LS-ODNs-*rev*-sa	25	91	100	100	100	6	0	0	0	0
LS-ODNs-*rev*-sa-sen	13	41	77	79	88	0	0	0	0	3
LS-ODNs-*rev*-sa-8mis	6	51	78	81	92	0	0	0	0	2
LS-dT_{20}	19	57	75	100	98	4	0	0	0	0
LS-dC_{20}	12	45	81	93	92	0	0	0	0	0

[a]The antisense sequences are described in Figure 1.
[b]The inhibitory effect percentage of HIV-1 infected cells represents the complete protective effect by the oligomer, without cytotoxicity to MT-4 cells.
[c]Cytotoxicity of oligomers represents the percentage reduction of viable cell numbers in mock-infected MT-4 cells. Both inhibition and cytotoxicity assays were performed with the MTT assay, as described in the Experimental. Values are means of duplicate determinations.

enhances its nonsequence specific anti-HIV activity. Therefore, we have examined whether LS-ODNs, LS-dC_{20} oligomers, and 1,2-di-O-hexadecyl-*rac*-glycero-3-phosphate (phospholipid) can inhibit reverse transcriptase activity. As shown in Figure 3, HIV-1 reverse transcriptase activity was inhibited by LS-ODNs-*rev*-sa and LS-dC_{20}, by 98% and 99%, respectively, at a 10 μM concentration, whereas the inhibition by S-dC_{20} was 31% at a 20 μM concentration. In contrast, neither the S-ODNs-*rev*-sa nor the phospholipid were inhibitory at any concentration up to 20 μM. These results indicate that the lipid-phosphorothioate oligonucleotides are better inhibitors of the reverse transcriptase than the phosophorothioate oligonucleotides.

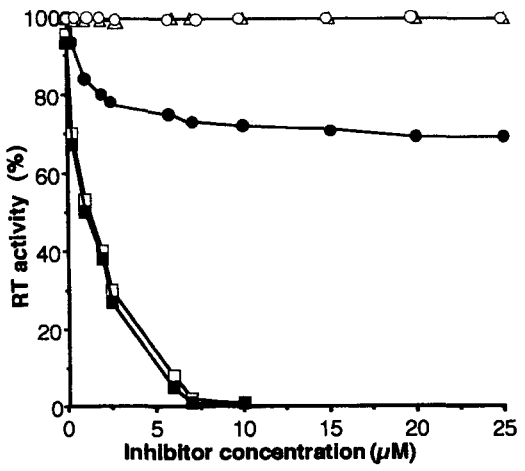


Figure 3. Inhibition of HIV-1 reverse transcriptase activity by oligomers. HIV-1 RT was incubated with the indicated concentration of either the LS-ODNs-*rev*-sa (■), LS-dC_{20} (□), S-ODNs-*rev*-sa (○), S-dC_{20} (●), or phospholipid (△) for 30 min at room temperature. The rest of the reaction components were then added to the mixture and the assay was performed at 37 °C for 1 h.

Discussion

We have developed a simple procedure for the synthesis of the 1,2-di-O-hexadecyl-*rac*-glycero-3*H*-phosphonate unit, which was coupled to the 5′-terminus of the oligonucleotide by the *H*-phosphonate method.

A recent study of L929 cells infected with VSV showed that lipid-oligonucleotides have increased uptake and antiviral activity, which occurred in a sequence-specific manner, with no cellular toxicity.[24]

In this paper, we have described the nonsequence specific mode of action of lipid-phosphorothioate oligonucleotides in inhibiting human immunodeficiency virus-1 replication. We have demonstrated that covalent modification of the phosphorothioate oligonucleotides by the addition of a 5′-phospholipid group makes these oligonucleotides much more potent inhibitors of HIV-1 infectivity than the corresponding unmodified phosphorothioate oligonucleotides.

When tested at a concentration of 5 μM, the lipid-oligonucleotides (L-ODNs-*rev*-sa) were not found to be active against HIV-1, but the L-ODNs at 15 μM were active. However, at the high concentration of 15 μM, the unmodified oligonucleotides (ODNs-*rev*-sa) with the same sequence had no detectable effect. This result suggests that the phospholipid modification of the phosphodiester oligonucleotides might improve the efficacy of their replication inhibition. However, the lipid-phosphodiester oligonucleotides (L-ODNs-*rev*-sa) would easily be degraded by cellular nucleases before displaying their function; otherwise this oligonucleotide should be as effective as a phosphorothioate complementary to the *rev* gene of HIV-1 at higher concentrations. Our results on the inhibition of HIV-1 replication by lipid phosphodiester oligonucleotides at

higher concentrations were in general agreement with their antiviral acitivity in L929 cells infected with VSV, as reported by Shea *et al.*[24] The phospholipid itself had very weak anti-HIV activity, and it was not toxic up to 30 μM (data not shown). Thus the inhibition of HIV production by the lipid-phosphodiester oligonucleotide is dependent on the lipid modification, but the L-ODNs are still nuclease sensitive because of the natural structure of the compound.

On the other hand, the phosphorothioate oligonucleotide, S-ODNs-*rev*-sa, at 1 μM, was as effective as the molecule with the covalent attachment of a phospholipid to this oligonucleotide, LS-ODNs-*rev*-sa, at the low concentration of 0.04 μM. This result suggests that modification of the 5′-ends of the phosphorothioate oligonucleotides by the addition of a phospholipid moiety makes them nuclease-resistant, so that they are much more potent inhibitors of HIV-1 replication than the corresponding unmodified phosphorothioate oligonucleotides. Furthermore, the higher anti-HIV-1 activity of the LS-ODNs, as compared to the S-ODNs, was supported by the uptake studies. The LS-ODNs-*rev*-sa yield 2.5 times more cell associated counts than the S-ODNs-*rev*-sa. In addition, the LS-ODNs showed anti-HIV activity, which was probably due mainly to the relative nuclease resistance of the L-ODNs and to the enhancement of their cellular uptake.

To demonstrate the antisense inhibitory process of the LS-ODNs, we used sense- (LS-ODNs-*rev*-sa-sen), mismatched-(LS-ODNs-*rev*-sa-8mis), and homo- (LS-dT_{20} and LS-dC_{20}) phosphorothioate oligonucleotides as nonsequence specific controls for the LS-ODNs-*rev*-sa. Surprisingly, all of these compounds showed increased anti-HIV activity as compared to the non-lipid-antisense phosphorothioate oligonucleotides (Table 1). These results indicate that the anti-HIV activity of the lipid-phosphorothioate oligonucleotides is not dependent on the base sequence of the oligonucleotides. Furthermore, the influence of the lipid modification on the activity of the phosphorothioate derivatives is especially striking with the phosphorothioate oligonucleotides noncomplementary to the HIV-1 mRNA sequences. The phospholipid moiety bound to the phosphorothioate oligonucleotide analogue enhances the antiviral property of the phosphorothioate derivatives. Thus, the lipid-phosphorothioate oligonucleotides appear to behave differently from the corresponding unmodified heterooligonucleotides, and may inhibit HIV-1 production by mechanisms other than by antisense competitive hybridization. A possible target is HIV-1 reverse transcriptase,[29,30] as shown in another laboratory.[31] Therefore, we examined whether LS-ODNs, LS-dC_{20} oligomers, and 1,2-di-O-hexadecyl-*rac*-glycero-3-phosphate (phospholipid) can inhibit reverse transcriptase activity. As clearly shown in Figure 3, the lipid-phosphorothioate oligonucleotides are better inhibitors than the phosphorothioate oligonucleotides for the reverse transcriptase, and the inhibition seems to be competitive with respect to the template primer.

Our results suggest that the lipid-phosphorothioate oligonucleotides may inhibit HIV-1 replication by inhibiting viral reverse transcriptase activity. Furthermore, the lipid-phosphorothioate oligonucleotides exhibited uptake and anti-HIV activity in cell culture systems. The anti-HIV activity of the phosphorothioate oligonucleotides is increased markedly by covalent modification with a phospholipid moiety at the 5′-end.

Experimental

Oligonucleotide synthesis

^{31}P NMR spectra were recorded using a Bruker AMX 400 spectrometer with 80% H_3PO_4 as the internal standard. UV Spectra were recorded on a Shimadzu UV-240 spectrophotometer. All DNA/RNA oligonucleotides were prepared by the phosphoroamidite method, using an Applied Biosystems Model 381A DNA synthesizer. Sulfuration for the phosphorothioate bonds was performed after each coupling step using 0.5 M tetraethylthiuram disulfide (TETD) dissolved in acetonitrile, according to Vu et al.[32] Oligonucleotides were purified according to published procedures.[33] Inseparable stereoisomeric mixures of phosphorothioate oligonucleotides characteristically peaked at about 55.60 ppm in the ^{31}P NMR spectra.

The structures and sequences of the synthesized unmodified, phosphorothioate, and lipid-bound oligonucleotides with the sense-, mismatched-, homo-, or antisense-sequences are shown in Figures 1 and 2, respectively.

Synthesis of 1,2-di-O-hexadecyl-*rac*-glycero-3-hydrogenphosphonate (1). 1,2-Di-O-hexadecyl-*rac*-glycerol (161 mg, 0.3 mmol) was dried with pyridine three times, and then redissolved in pyridine:methylene chloride (3.5 mL, 1:1, v/v). To this solution, 1.25 M tris-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite[26] in tetrahydrofuran (108 mL, 0.36 mmol) was added under nitrogen gas. After 2 h, the reaction mixture was poured into methylene chloride (5 mL) and washed several times with 1 M triethylammonium bicarbonate (TEAB, pH 7.5). The organic layer was dried with sodium sulfate, filtered, and evaporated in a vacuum. The crude phosphitylated lipid was purified by means of column chromatography on 300 mesh silica-gel. The product was eluted in a 3 to 15% MeOH–CH_2Cl_2 gradient containing 1% Et_3N, which was repeated using AcOH in place of Et_3N. The eluted portion was additionally extracted with 1 M TEAB, and afforded 169.2 mg (0.24 mmol, 80% yield) of triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3*H*-phosphonate (**1**) as a glacial, white solid. The product was visualized by means of an acidic molybdenum stain. ^1H NMR ($CDCl_3$): 0.84–0.89 (t, 6H), 1.16–1.17 (d, 6H), 1.19–1.20 (d, 6H), 1.24 (s, 26H), 2.60–2.64 (t, 2H),

2.80–2.85 (q, 6H), 3.40–3.66 (m, 7H), 3.81–3.85 (m, 2H), 6.83–6.85 (d, H-P). ^{31}P NMR ($CDCl_3$): 6.01(s).

Synthesis of phospholipid-conjugated oligodeoxynucleotides (L-ODNs or LS-ODNs) (2). 5′-Detritylated CPG-bound oligonucleotides, 1 μmol, were rinsed twice with acetonitrile and dried using an oil pump. The phosphitylated lipid **1** (15–20 mg, 21.3–28.4 μmol in 100 mL 1:1 pyridine:acetonitrile) was added under nitrogen gas, and treated with pivaloyl chloride (1 mL, 100 mM in 1:1 pyridine:acetonitrile) for 5 min, after which the solvent was removed. The CPG support was washed with pyridine:acetonitrile (1:1, v/v), treated with 0.1 M I_2 in THF:pyridine: H_2O (44:3:3, v/v) for 1 min or 5% elemental sulfur in carbon disulfide: pyridine:triethylamine (14.3:14.3:1.5, v/v) for 15 min, and then repeatedly washed with carbon disulfide: pyridine (1:1), acetonitrile, methylene chloride, and diethyl ether. After treatment with concentrated ammonium hydroxide, the oligonucleotides were directly purified by reversed-phase HPLC, using a sep-pack column. The samples were eluted in triethyl ammonium acetate buffer (pH 7.0) containing a 20–60% acetonitrile gradient, desalted, extracted, and precipitated in ethanol at −78 °C. The samples were then analyzed by electrophoresis through a 20% polyacrylamide gel containing 7 M urea and by HPLC (detected at 254 nm) or by ^{31}P NMR. The product was over 97% pure. ^{31}P NMR (D_2O), (−0.2–0.4) (s, P-O on L-ODNs), 55.99–60.01 (s, P-S on LS-ODNs).

Enzymatic digestion of phospholipid-conjugated oligodeoxynucleotides

The phospholipid-conjugated oligonucleotide (0.2 A_{260}) was dissolved in 0.5 mL buffer (10 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, and 100 mM NaCl) and incubated with snake venom phosphodiesterase (SVPD) at 37 °C for 2 h. The incubation of the phospholipid-conjugated oligonucleotides with bovine spleen phosphodiesterse was for 4 h. The extents of the enzymatic reactions were assessed using reversed-phase HPLC as described above for the analysis of the phospholipid-conjugated oligonucleotides.

Cell line

For the anti-HIV assay, the human T lymphotropic virus type-1 (HTLV-III)-positive human T cell line, MT-4, was subcultured twice a week at a concentration of 3×10^5/mL in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin, and 100 μg/mL streptomycin.

Virus

The HIV-1 strain, HTLV-IIIB, was used for the anti-HIV assay. The virus was prepared from culture supernatants of MOLT-4/HTLV-IIIB cells, which were persistently infected with HTLV-IIIB. The HIV stock solution was titrated in MT-4 cells, as determined by 50% tissue culture infectious doses (TCID50) and plaque forming units, and was stored at −80 °C until use.

Anti-HIV assay

The anti-HIV activities of test compounds in a fresh, cell-free HIV infection were determined by protection against HIV-induced cytopathic effects (CPE). Briefly, MT-4 cells were infected with HTLV-IIIB at a multiplicity of infection (MOI) of 0.01. HIV-infected or mock-infected MT-4 cells (1.5×10^5/mL, 200 μM) were placed into 96-well microtiter plates and were incubated in the presence of various concentrations of the test compounds (the dilutions ranged from one to five-fold, and nine concentrations were examined. All experiments were performed in triplicate). After 5 days of culture at 37 °C in a CO_2 incubator, the cell viability was quantified by a colorimetric assay monitoring the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a blue formazan product. Absorbances at 540 and 690 nm were read in a microcomputer controlled photometer (Titertec Multiscan®; Labsystem Oy, Helsinki, Finland). The absorbance measured at 690 nm was automatically subtracted from that at 540 nm, to eliminate the effects of nonspecific absorption. All data represent the mean values of triplicate wells. These values were then translated into percentages per well, cytotoxicity, and antiviral protection.[34,35]

3′-End labeling and cellular uptake

The 3′-ends of the LS-ODNs-*rev*-sa and the S-ODNs-*rev*-sa were labeled with [α-^{32}P]dATP and terminal deoxynucleotidyl transferase, and then purified using a Quick spin column (G-25). MOLT-4 cells were diluted to 1×10^5 cells/mL in RPMI-1640 medium containing 10% heat-inactivated FCS, and were dispensed in 24-well plates. After 48 h at 37 °C in a CO_2 incubator, the RPMI-1640 medium was changed. The cells were further incubated with the 3′-end labeled oligomers for the stated periods. After washing the cells four times with chilled phosphate-buffered saline (PBS), the cells were pelleted by centrifugation and lysed in 0.5 mL of 1% sodium dodecyl sulfate (SDS). The cellular uptake of ^{32}P-oligodeoxyribonucleotides was measured in a β-scintillation counter.

Reverse transcriptase assay

HIV-1 reverse transcriptase (5 μg) was mixed with various concentrations of LS-ODNs, LS-dC_{20}, S-ODNs, S-dC_{20} oligomers, and phospholipid in a reaction mixture (50 μL) consisting of 50 mM Tris–HCl (pH 8.4), 2 mM dithiothreitol, 100 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X100, 50 μg of poly(rA)-oligo(dT) per mL, and 1.25 μCi of [^{3}H]dTTP (57 Ci/mmol). The incubation was carried out at 37 °C for 1 h and was terminated by the addition of 200 μL of 5% trichloro-

acetic acid containing 0.02% sodium pyrophosphate. The acid-insoluble precipitates were collected on glass fiber filters, washed, dried, and their radioactivity was determined with a Beckman scintillation spectrometer.

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