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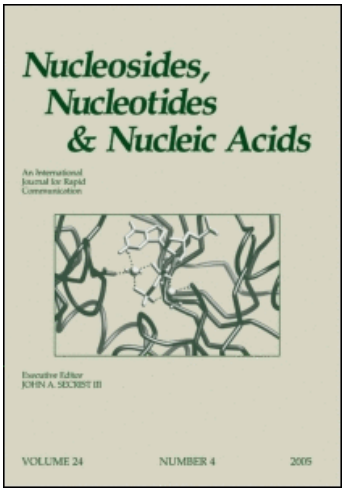

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Takashi Hiratou[a]; Satoru Tsukahara[a]; Naoko Miyano-kurosaki[a]; Kazuyuki Takai[a]; Takeshi Saito[b]; Naoki Yamamoto[c]; Hiroshi Takaku
[a] Department of Industrial Chemistry and Chiba, Institute of Technology, Narashino, Chiba, Japan [b] Institute for Consumer Kealthcare, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan [c] Department of Microbiology, Tokyo Medical and Dental University School of Medicine, Tokyo, Japan

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(10–12), 1721–1734 (2000)

ANTI-HIV-1 ACTIVITY BY A TRIPLE-HELIX FORMING OLIGONUCLEOTIDES TARGETED TO POLYPURINE TRACT ON VIRAL RNA

Takashi Hiratou[1], Satoru Tsukahara[1], Naoko Miyano-Kurosaki[1], Kazuyuki Takai[1], Takeshi Saito[2], Naoki Yamamoto[3], and Hiroshi Takaku[1]*

[1]Department of Industrial Chemistry and Chiba Institute of Technology, Tsudanuma, Narashino, Chiba 275-0016, Japan, [2] Institute for Consumer Kealthcare, Yamanouchi Pharmaceutical Co., Ltd., 3-17-1 Hasune, Itabashi-ku, Tokyo 174-8612, Japan, and [3]Department of Microbiology, Tokyo Medical and Dental University School of Medicine, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan,

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ABSTRACT: Reverse transcription of HIV-1 into double-stranded DNA involves initiation of plus-strand DNA synthesis at the polypurine tract, PPT, by reverse transcriptase (RT). The PPT is a possible target for triple-helix formation. We show the effects of triple-helix formation by assays of RNase H cleavage inhibition in vitro using two systems (two-strand-system (FTFOs) or three-strand-system (TFOs)) targeted to the polypurine tract (PPT) of HIV-1. The two-stranded composition of a triple-helix is thermodynamically and kinetically superior to the three-strand-system. The FTFOs inhibited the RNase H activity in a sequence-specific manner, i.e., the triplex actually formed at the PPT and blocked the RNase H. The FTFOs containing the phosphorothioate groups at the antisense strand showed greater 3'-exonuclease resistance. In HIV-1 infected MT-4 cells, the FTFOs containing the phosphorothioate groups at the antisense strand and guanosine rich parts within the third Hoogsteen base pairing sequence inhibit the replication of HIV-1 more effectively than the antisense phosphorothioate oligonucleotides, indicating sequence-specific inhibition of HIV-1 replication.

Replication of HIV-1 proceeds by means of the reverse transcriptase (RT), which catalyzes the conversion of the single-stranded viral RNA genome into double-stranded DNA and allows integration into the cellular genome.1-4 This process involves multiple steps. The RNase H activity of the RT catalyzes the hydrolysis of the viral RNA from an RNA/DNA hybrid molecule.3 A polypurine tract (PPT) consisting of 16 nt, which is

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#This article is dedicated to the memory of Professor Alexander Krayevsky.

*To Whom correspondence should be addressed:TEL:+81-474-78-0407; FAX:+81-474-71-8764; Email:takaku@ic.it-chiba.ac.jp

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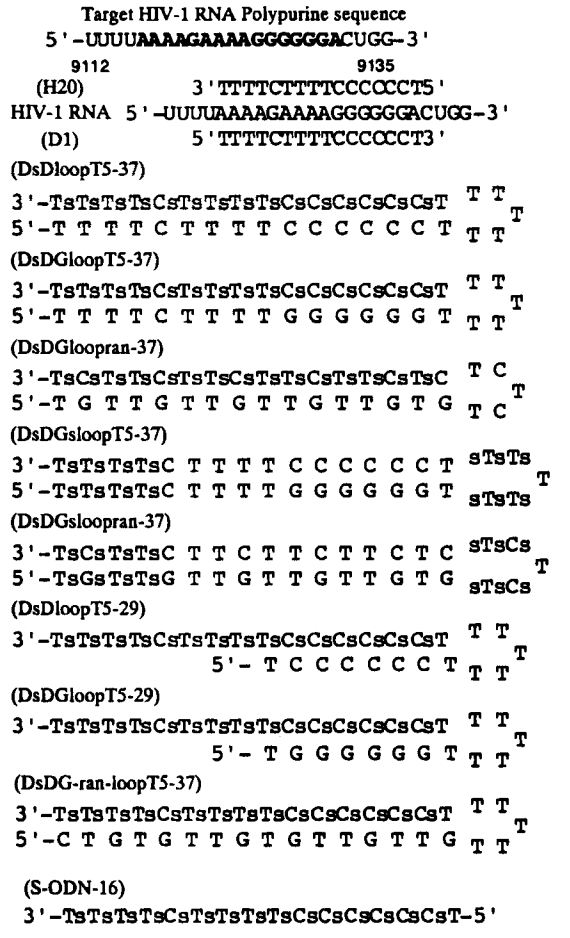

FIG. 1. The sequences of the oligonucleotides of the three-strand (TFOs) and two-strand (FTFOs) systems used in this work. The target polypurine track (PPT) RNA is indicated as boldface. The nucleotide sequences of the RNA/DNA hybrid substrate, consisting of the HIV-1 mRNA (430 nt) and the 20 mer antisense phosphodiester oligonucleotide (H 20) complementary to the PTT-region, are shown above.

Abbreviations: D, DNA; G, substitution of G for C^+ in the third Hoogsteen base-pairing strand; 37, 37 nucleotide; 29, 29 nucleotide; S, phosphorothioate group; S-ODN, antisense phosphorothioate oligonucleotide.

resistant to RNase H cleavage, serves as a primer for plus-strand DNA synthesis by the DNA-directed DNA polymerase activity of the RT. The PPT is a highly conserved region adjacent to the 3'-end (U3) of the viral RNA, and it has an essential function during reverse transcription.[5,6] When the homopolymeric PPT functions as a target

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sequence, antisense oligonucleotides as well as triple-helix-forming oligonucleotides (TFOs) might be useful tools to interfere with retroviral replication.[6-12] During reverse transcription, TFOs targeted against single-stranded PPT-RNA or PPT-RNA/DNA hybrids might interfere at various points. i) At the first stages of the RT reaction, DNA elongation during RNA-directed DNA synthesis might be arrested. ii) Hydrolysis of the RNA with RNase H might be blocked at the PPT region. iii) The initiation of plus-strand DNA synthesis can potentially be inhibited. iv) The RNA-polymerase of the host cell can be blocked during the transcription of double stranded DNA to mRNA. v) The triple-helix could also block the translation of viral mRNA. The sequences of the oligonucleotides of the three-strand (FTOs) and two-strand(FTFOs) systems used in this work. The terms anti and para refer to the antiparallel and parallel orientations of the third strand oligonucleotides. The nucleotide sequences of the RNA/DNA hybrid substrate, consisting of the HIV-1 mRNA (430 nt) and the 20 mer oligonucleotide (H 20) complementary to the PTT-region, are shown above.

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In this paper, we have tested the effect of inhibition of the RNase H activities by a three-strand-system and a two-strand-system targeted to the PPT region. The oligonucleotides contained phosphorothioates at both the 3'-end and the 5'-end, or antisense strand parts (Fig. 1). The three-strand-system comprises an RNA/DNA hybrid and triplex-forming-oligonucleotides (TFOs), and the two-strand-system comprises a Watson-Crick and Hoogsteen base pairing sequence on a single strand connected by one hairpin loop (T)5. These are referred to as foldback triplex-forming-oligonucleotides (FTFOs).[13-15] The formation of the pyr/pur/pyr triple-helix, which is pH-dependent and unstable under physiological conditions, was avoided by the substitution of C^+ for G in the third Hoogsteen base-pairing strand. The FTFOs inhibit the action of the RNase H most effectively, and form a more stable triplex with the PPT-RNA *in vitro* than the TFOs. Furthermore, the modified FTFOs show enhanced exonuclease resistance. We also describe the anti-HIV-1 activities of the FTFOs containing phosphorothioate groups at the antisense strand and guanosine rich parts within the third Hoogsteen base-pairing strand.

RESULTS

Inhibition of reverse transcription by TFOs and FTFOs. In order to analyze whether triple-helix formation at the PPT, the RNase H cleavage protection

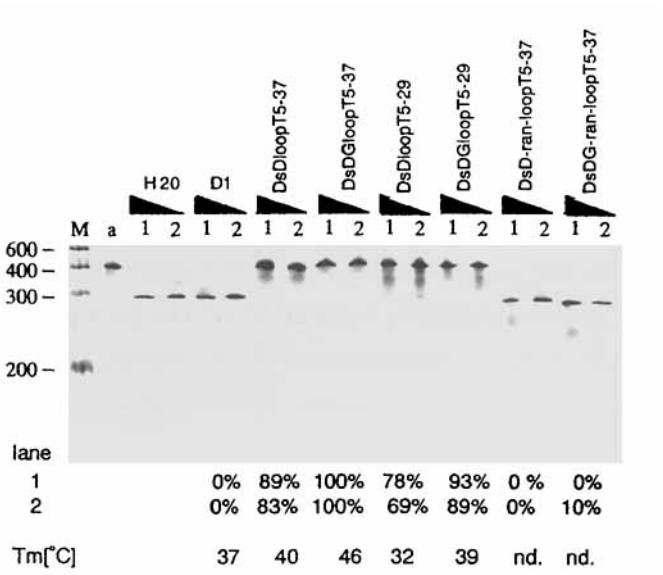

FIG. 2. RNase H cleavage protection by three-strand (TFOs) and two-strand (FTFOs) systems. 5'-P^{32}-end labeled HIV-1 mRNA (430 nt), transcribed in vitro, was treated with the antisense oligonucleotide (H 20), D1, and FTFOs. Triple-helix formation was performed at 4°C for overnight, then RNase H was added for 1 h at 37°C. Lane a shows HIV-1 mRNA (430 nt). Lanes 1 and 2, 100 nM HIV-1 mRNA (430 nt) with 100 nM and 1 μM of the antisense oligonucleotide (H 20), D1, and FTFOs. M, molecular size markers. Tm values and quantification of the amounts of uncut RNA are listed at the bottom.

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assays were performed with the TFOs and FTFOs. An RNA 430 nucleotides in length, transcribed could interfere with the enzyme activities of the RT especially the RNase H. Various phosphorothioated FTFOs were designed (Fig. 1). The motif involves guanosine binding, by reverse-Hoogsteen hydrogen bonding, to the guanosine of a G-C base pair (G-G-C base triplex).[16,17] The control RNase H cleavage reaction was performed with the antisense oligonucleotide (5'-TCCCCCCTTTTCTTTT-3', H-20) corresponding to the Watson-Crick parts of the PPT region. In vitro transcribed RNA containing the extended PPT target sequence was pre-incubated with increasing amounts of TFOs and FTFOs. All of those FTFOs (DsDloopT5-37, DsDGloopT5-37, DsDloopT5-29, and DsDGloopT5-29) can form an RNA-DNA hybride substrate for the RNase H activity with their Watson-Crick base-pairing sequence. RNase H cleavage resistance was analyzed by gel electrophoresis and autoradiography. Fig. 2 shows the results of the RNase H cleavage reaction by FTFOs and FTOs (D1). Quantitative

evaluation of amount of RNA cleavage is presented below and given percentage (Fig. 2). Furthermore, the ability of the oligonucleotides to form triple-helices was examined by melting temperature studies. The pur/pur/pyr triplex (DsDGloopT5-37) (46°C) formed a more stable hybrid with the RNA than the D1 three-strand-system (TFOs) (37°C) and other FTFOs (32-40°C). The DsDGloopT5-37 binds to the target sequence by the involvement of both Watson-Crick and Hoogsteen domains through foldback triplex formation. The DsDGloopT5-37 showed a highly RNase H protection effect (100%) at its lower concentration (0.1 μM) (Fig. 2). On the other hand, in the case of the DsDloopT5-37 containing pyrimidine residues, a protection from RNase H cleavage is caused 83% at the 0.1 μM concentration. Interestingly, the shorter FTFOs, DsDGloopT5-29 (89%) and DsDloopT5-29 (69%), with the shorter Hoogsteen sequences, can also protect RNase H activity at 0.1 μM concentration. In contrast, the D1 three-strand-system (TFOs) cannot protect the target RNA from RNase H cleavage (Fig. 2). The control sequences, DsDGsloop-ran-37 and DsDGloop-ran-37, had no detectable inhibitory effects on RNase H activity. These results suggest that the two-strand-system showed a highly RNase H protection effect at lower concentration.

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Another problem with such an affinity of oligonucleotides is their sensitivity to degradation by nucleases present in the serum, especially 3'-exonucleases. After 24 h of incubation medium containing 10% fetal bovine serum, the two-stand-system oligonucleotides (DsDloopT5-37, DsDGloopT5-37, DsDGsloopT5-37, and DsDGloopT5-29) shows the same order of stabilizes as that of the stability for antisense phosphorothioate oligonucleotide (S-ODN-16) (data not shown). This stabilization should help us to design much more efficient third strand oligonucleotides, which could be used as tools in cellular biology.

Cellular uptake of the FITC-labeled DsDGloopT5-37 in MOLT-4 and MOLT-4/HIV-1 cells. Efficient cellular penetration by the FTFOs is a critical step to enable targeting to the mRNA. We examined the cellular uptake of fluorescently (FITC)-labeled-DsDGloopT5-37 and S-ODN-gag-AUG with an AUG initiation codon, target to HIV-1 gag sequence[18] by treating MOLT-4/HTLV-IIIB cells with the oligonucleotide at 37°C. The cells were washed with PBS two times and then incubated sequentially for ten minutes each in FACSLyse and FACSPerm (Becton Dickinson, San Jose, California) solutions for permeabilization. The cells were washed, resuspended in PBS with 1% paraformaldehyde, and analyzed by FACSCalibur and CellQuest software. The

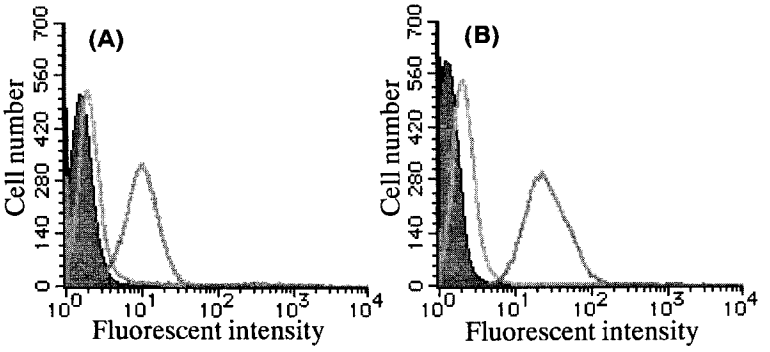

FIG. 3. Cellular uptake of FITC-labeled-DsDGloop-37 and FITC-S-ODN-gag-AUG by fluorescence activated cell sorter FACSCalibur. MOLT-4/ HTLV-IIIB cells were incubated with FITC-labeled-DsDGloop-37 and FITC-S-ODN-gag-AUG (1 and 2.5 μM) for 3 h. The thin-line histograms with shading is represented the control MOLT-4/ HTLV-IIIB cells in the absence of the FITC-labeled-oligonucleotides. The thin-line histograms are represented FITC-labeled-DsDGloop-37 and FITC-S-ODN-gag-AUG treatment of MOLT-4/ HTLV-IIIB cells .

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FITC-DsDGloopT5-37 content in the MOLT-4/HTLV-IIIB cells was relatively high as compared with that of the FITC-S-ODN-gag-AUG (Fig. 3). However, the extent of uptake of a 2.5 μM concentration of FITC-DsDGloopT5-37 and FITC-S-ODN-gag-AUG) was not significantly greater than that of the 1 μM concentration. DsDGloopT5-37 showed very highly taken up into MOLT-4 and MOLT-4/HTLV-IIIB cells, which might explain the enhanced antisense activity.

Anti-HIV-1 activity of foldback triplex forming oligonucleotides (FTFOs). In order to clarify the anti-HIV activities of the FTFOs, we tested the effects of FTFOs treatment in HIV-1 infected MT-4 cells. Control oligonucleotides were prepared for comparison, such as the FTFOs (5'-CTGTGTTGTGTTGTTGTTTTTTsCsCsCsCs-CsCsTsTsTsTsCsTsTsTsT-3', DsDG-ran-loopT5-37) containing a random sequence in the third Hoogsteen base pair strand and one with a base content identical to the FTFOs sequences and antisense phosphorothioate oligonucleotide (5'-TsCsCsCsCsCsCsTsTs-TsTsCsTsTsTsT-3', S-ODN-16), but in a random order (DsDGsloop-ran-37 and DsDGloop-ran-37). The FTFOs (DsDloopT5-37, DsDGsloop-T5-37, DsDGloopT5-37, and DsDGloopT5-29) inhibited virus replication in HIV-1 infected MT-4 cells (Table 1). These oligomers possessed EC_{50} values of 0.03 μM, whereas almost no cytotoxicity

TABLE 1. Anti-HIV activity of FTFOs in MT-4 cells.

oligonucleotides	EC50[a,c)]	CC50[b,c)]
DsDloopT5-37	0.03μM	>10μM
DsDGloopT5-37	0.03μM	>10μM
DsDGloop-ran-37	5.00μM	>10μM
DsDGsloopT5-37	0.03μM	>10μM
DsDGsloop-ran-37	5.00μM	>10μM
DsD loopT5-29	0.08μM	>10μM
DsDGloopT5-29	0.04μM	>10μM
S-ODN-16	0.32μM	>10μM

a) Anti-HIV-1 activity was monitored as percent inhibition of HIV-1-induced cytopathogenicity in MT-4 cells. The EC50 value is the concentration of test compound, that achieves 50% inhibition of viral antigen expression.
b) Cytotoxicity is expressed as the percentage of cell death of MT-4 cells cultured with test compounds. The number of viable cells was determined by the MTT assay. The CC50 is the concentration of test compound that exhibits 50% cell toxicity.
c) Data represent average values for at least three different experiments.

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was observed even at a 10 μM concentration of the compounds. However, the shorter FTFOs, DsDloopT5-29 with cytidine bases at the 5'-terminal sequences, protected against HIV-1 induced CPE at EC50 values of 0.08 μM. The deletion of 8 nucleotides (DsDloopT5-29) from the third Hoogsteen base pair strand sequences of the FTFOs (DsDloopT5-37) is responsible for the decreased anti-HIV-1 activity, whereas the deletion of 8 nucleotides (DsDGloopT5-29) from the third Hoogsteen base pair strand sequence had no influence on the anti-HIV-1 activity. The control sequences, DsDGsloop-ran-37 and DsDGloop-ran-37, had no effect (Table 1). On the other hand, the control oligomer with the random sequences in the third Hoogsteen base-pairing strand, DsDG-ran-loopT5-37, possessed EC50 values of 0.2 μM. The anti-HIV-1 activity of DsDG-ran-loopT5-37 was 7-fold lower than that of DsDGloopT5-37, but its potent inhibition is achieved in the antisense manner, without the triplex manner. Furthermore, antisense oligonucleotide, S-ODN-16 inhibited HIV-1 induced CPE at EC50 values of 0.32 μM, a substantially lower inhibitory effect than that of observed for its FTFOs, DsDGloopT5-37. FTFOs recognize the target sequence twice, unlike conventional antisense and antigene oligonucleotides. These results suggest that the FTFOs inhibited HIV-1 induced CPE, but the random-FTFOs failed to inhibit HIV-1 replication in acutely infected MT-4 cells.

DISCUSSION

The structure of the three-strand system with the triplex-forming strand (TFO) was designed for applications in cell culture tests, which require high thermodynamic stability of the triple-helix and a sufficiently long half-life of the TFOs to allow the slow process of triple-helix formation with the target strand before their degradation.[19,20] These chemical modifications stringently require the physiological conditions in living cells for triple-helix formation. Here, another approach was taken. The FTFO recognizes the target sequence twice, unlike conventional antisense and antigene oligonucleotides. The FTFO recognizes the target sequence first when it binds through Watson-Crick hydrogen bonding, followed by the second recognition in which the other half of the oligonucleotide folds back onto the already formed duplex to form Hoogsteen hydrogen bonds (triplex). Therefore, in principle FTFOs are expected to exhibit greater sequence specificity than conventional antisense and antigene oligonucleotides.

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We determined whether the three-strand system or two-strand system targeted to the polypurine tract (PPT) of HIV-1 could interfere with the enzyme activities of the RT especially the RNase H. The formation of the pyr/pur/pyr triple-helix, which is pH-dependent and unstable under physiological conditions, was avoided by the substitution of G for C^+ in the third Hoogsteen base-pairing strand. Several studies[21-23] that investigated DNA triplexes with mixed purine-pyrimidine targets and containing G-G-C triplexes have suggested that in some instances, the oligonucleotides may bind in a parallel orientation. In vitro assays, DsDGloopT5-37 was shown to inhibit RNase H cleavage at the PPT-target site at lower concentration (0.1 μM) and the Tm values was 46°C. These results support the notion that FTFOs are thermodynamically and kinetically superior to TFOs. Furthermore we noted that the two-strand-system (FTFOs) inhibited the RNase H activity in a sequence-specific manner, i.e., the triplex actually formed at the PPT and blocked the target RNA from RNase H cleavage.

It is well known that the functional efficacy of oligonucleotides not only depends on the selection of efficient sequences and an appropriate target, but also on a sufficient intracellular concentration in cultured cells. The latter factor is affected by the cellular uptake, the intracellular distribution, and the degradation rate of the oligonucleotides by serum and cytoplasmic nucleases. The FTFOs containing the phosphorothioate groups at the antisense sequences showed greater 3'-exonuclease resistance. This stabilization

should help us to design much more efficient third strand oligonucleotides, which could be used as tools in cellular biology. On the other hand, the poor cellular uptake of oligonucleotides in cell culture is still a limiting factor, which may contribute to the lack of functional efficacy. Observations based on flow cytometry showed the higher uptake of FITC-labeled-DsDGloopT5-37 as compared with that of the FITC-S-ODN-gag-AUG as compared with that of the FITC-S-ODN-gag-AUG in the MOLT-4/HTLV-IIIB cells (Fig. 3). These results suggest that the DsDGloopT5-37 showed very highly uptake into MOLT-4 and MOLT-4/HTLV-IIIB cells, which might explain the enhanced antigene activity. Taking this observation into account, we thought that the FTFOs could penetrate both MOLT-4 and MOLT-4/HTLV-IIIB cells, and that the FTFOs might hybridize to the target sequence in the viral RNA. However, since MOLT-4 cells do not express the viral mRNA, the FTFOs could neither hybridize with nor remain in the cells.

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In order to clarify the anti-HIV activities of the FTFOs, we tested the effects of FTFOs treatment in HIV-1 infected MT-4. We found that the FTFOs (DsDloopT5-37, DsDGsloopT5-37, DsDGloopT5-37, and DsDGloopT5-29) inhibited HIV-1-induced CPE, but the shorter FTFOs, DsDloopT5-29 failed to inhibit HIV-1 replication in acutely HIV-1 infected MT-4 cells (Table 1). Interestingly, the deletion of 8 nucleotides (DsDGloopT5-29) from the third Hoogsteen base pair strand sequence had no influence on the anti-HIV-1 activity. The control sequences, DsDGsloop-ran-37 and DsDGloop-ran-37, had no effect (Table 1). On the other hand, the control oligomer with the random sequences in the third Hoogsteen base-pairing strand, DsDG-ran-loopT5-37 was 7-fold lower than that of DsDGloopT5-37, but its potent inhibition is achieved in the antisense manner, without the triplex manner. Furthermore, antisense phosphorothioate oligonucleotide, S-ODN-16 was 11-fold lower than that that of DsDGloopT5-37. FTFOs recognize the target sequence twice, unlike conventional antisense and antigene oligonucleotides. Phosphorothioate oligonucleotides have been shown to block the proliferation of HIV-1 in acutely infected cells in a non-sequence specific manner[24] probably by the inhibition of reverse transcriptase[25,26] and/or the viral entry process[27,28]. However, the FTFOs inhibited HIV-1 induced CPE in acutely infected MT-4 cells in a sequence specific manner without the inhibition of reverse transcriptase and/or the viral entry process such as an antisense phosphorothioate oligonucleotide (S-ODN-gag-AUG).[19] The FTFOs had very highly taken up into pMOLT-4/HTLV-IIIB cells, which

might explain the enhanced antigene activity. It is noteworthy that the FTFOs inhibit the replication of HIV-1 more effectively than the antisense phosphorothioate oligonucleotides.

Volkmann et al.[12] have also demonstrated that the TFOs was an efficient inhibitor of HIV-1 replication, leading to a block of p24 synthesis and inhibition of syncytica formation in newly infected cells. However, the phosphorothioated TFOs (54 mer) were designed for an extended 25 mer purine-rich target sequence, including the 16 mer PPT. In contrast, we designed the TFOs (DsDGloop-T5-37, 37mer) against the shorter PPT comprising only 16 nt containing phosphorothioate groups at antisense strand parts (Fig. 1). The DsDGloop-T5-37 showed the highest inhibitory effect on the HIV-1 replication. Furthermore, we found the short TFOs, DsDGloop-T5-29 to be at least as active as the DsDGloop-T5-37.

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In conclusion, the two-stranded composition of a triple-helix is thermodynamically and kinetically superior to the three-strand-system. The FTFOs inhibited the RNase H activity in a sequence-specific manner, i.e., the triplex actually formed at the PPT and blocked the RNase H activity. The FTFOs containing the phosphorothioate groups at the antisense sequences showed greater 3'-exonuclease resistance. In HIV-1 infected MT-4 cells, the FTFOs containing the phosphorothioate groups at the antisense sequence sites and guanosine rich parts within the third Hoogsteen base-pairing strand inhibit the replication of HIV-1 more effectively than the antisense oligonucleotides, indicating sequence-specific inhibition of HIV-1 replication without the inhibition of reverse transcriptase and/or the viral entry process such as antisense phosphorothioate oligonucleotides (S-ODN-gag-AUG). In particular, the phosphorothioated FTFOs may be useful in nucleic acid based anti-viral therapies with triple-helix approaches *in vitro* or *in vivo*.

EXPERIMENTAL

Oligonucleotides. The oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems Model 392 DNA synthesizer on the 1 μM scale, and with controlled pore glass supports. DNA phosphoramidite units were purchased from PerSeptive Biosystems, Inc. The support was treated with concentrated ammonia for 15 h at 55°C. The deprotected oligomers were purified by reverse phase HPLC or by electrophoresis on 20% polyacrylamide/7M urea gels.

The nucleoside compositions were determined after snake venom phosphodiesterase/bacterial alkaline phosphatase hydrolyses.

Thermal denaturation profiles. Thermal transitions were recorded at 260 nm using a Shimadzu UV-2200 spectrometer. The insulated cell compartment was warmed from 5°C to 90°C, with increments of 1°C and equilibration for 1 min after attaining each temperature, using the temperature controller SPR-8 (Shimadzu). Samples were heated in masked 1 cm path length quartz cuvettes fitted with Teflon stoppers. Each thermal denaturation was performed in 25 mM Tris/acetate buffer (pH 6.5), 0.4 mM spermine, 10 mM MgCl2, 50mM NaCl, containing 1 μM of each strand. The mixture of duplex and single strands was kept at 90°C for 5 min, and was then cooled to 22°C. At temperatures below 15°C, N_2 gas was continuously passed through the sample compartment to prevent the formation of condensate.

In vitro transcription. Plasmid pSV2neo JRCSF-B, containing the polypurine tract of the HIV-1 genome, was digested by Xho I and Nhe I to yield a dsDNA fragment (0.47 kb). The 23 base T7 promoter sequence was incorporated into the dsDNA by a PCR strategy. The PCR product was transcribed with T7 RNA polymerase according to the manufacturer's instructions (Ambion).

RNase H cleavage protection by TFOs and FTFOs. RNA/DNA hybrid formation.In vitro transcribed 5'-end-32P-labeled-RNA containing the PPT-target sequence (430 nt) was hybridyzed with an antisense oligonucleotide (5'-TCCCCCCTTTTCTTTT-3', H-20) in a buffer consisting of 25 mM Tris-acetate (pH6.8), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM a-mercaptoethanol, and 0.4 mM spermine, The mixture was incubated for 5 min at 90°C, and then cooled slowly to 37°C. For triple-helix formation the RNA/DNA hybrid was incubated overnight at 37°C with increasing amount (100 nM and 1 μM) of D1 . Finally, each samples was treated with 12 ng enzyme in RNase H-standard buffer for 1 h at 37°C. RNase H cleavage reactions were analyzed on a 10% polyacrylamide-TBE-urea gel. The reactions were quantified by radioanalytic imaging with a Bioimage analyzer, BAS 2000 (Fujifilm).

Exonuclease stabilities of FTOs and FTFOs. The oligonucleotides (0.2 A_{260}) were incubated with 200 μl of culture medium containing 10% fetal bovine serum for 24 h at 37°C. Aliquots were taken at 0, 3, 6, 9, 12, and 24 h and were analyzed by PAGE (20% polyacrylamide containing 7 M urea). Densitomeric analysis of gels stained with silver nitrate was performed on a Millipore BioImage 60 S.

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Cells and virus. The human T lymphotropic virus type I (HTLV-I)-positive human T-cell line, MT-4, and the HTLV-I-non-infected T cell line, MOLT-4, were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100μU/mL penicillin, and 100 μg/mL of streptomycin. A strain of HIV-1, HTLV-IIIB, was obtained from the culture supernatant of chronically HIV-1-infected MOLT-4 cells, MOLT-4/HTLV-IIIB cells, and was stored in a small volume at –80°C until use. The titer of the virus stocks was determined by 50% tissue culture infectious doses (TCID50).

Laser Flow Cytometry. Oligonucleotide uptake was monitored by fluorescence-activated cell sorting (FACS) analysis. MOLT-4/HTLV-1-IIB cells were incubated with FITC-labeled-DsDGloopT5-37 and S-ODN-gag-AUG, in culture medium at 37°C for 3 h. The cells were washed twice with PBS and then were incubated sequentially for ten minutes each in FACSLyse and FACSPerm (Becton Dickinson, San Jose, California) solutions for permeabilization. The cells were washed, resuspended in PBS with 1% paraformaldehyde, and analyzed by the FACSCalibur and CellQuest software (both from Becton Dickinson, San Jose, California).

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Anti-HIV assay. The anti-HIV activities of test compounds in a fresh, 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 (3 x 105 ml-1) 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 were read in a microcomputer-controlled photometer (Titertec MulticanR; Labsystem Oy, Helsinki, Finland) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically subtracted from that at 540 nm, to eliminate the effects of non-specific absorption. All data represent the mean values of triplicate wells. These values were then translated into percentages per well, cytotoxicity, and antiviral protection.[29,30]

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