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Inhibition of HIV-1 replication by a two-strand system (FTFOs) targeted to the polypurine tract

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Abstract Reverse transcription of HIV-1 vRNA into the double-stranded DNA provirus involves initiation of plus-strand DNA synthesis at the polypurine tract (PPT) by reverse transcriptase (RT). The PPT is highly conserved among the known human immunodeficiency virus (HIV-1) strains and is a possible target for triplex formation. We show the effects of triple-helix formation by assays of primer extension inhibition in vitro, using a two-strand system (foldback triplex-forming oligonucleotides (FTFOs)) targeted to the 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 RT activity in a sequence-specific manner, i.e. the triplex actually formed at the PPT and blocked the RT. The FTFOs containing the phosphorothioate groups at the antisense sequences showed greater 3′-exonuclease resistance. In HIV-1-infected MOLT-4 cells, the FTFOs containing the phosphorothioate groups at the antisense sequence sites and guanosine rich parts within the third Hoogsteen base-pairing sequence inhibit the replication of HIV-1 more effectively than the antisense oligonucleotides, indicating sequence-specific inhibition of HIV-1 replication.

Key words: Triplex; Two-strand system; Inhibition of human immunodeficiency virus replication; Polypurine tract; MOLT-4 cell; MT-4 cell

1. Introduction

The human immunodeficiency virus (HIV-1), the etiological agent of the acquired immunodeficiency syndrome, behaves as a classical retrovirus with regard to many aspects of 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 dsDNA and allows for integration into the cellular genome [1–4]. This process involves multiple steps. The RNAse H activity of the RT catalyzes hydrolysis of the viral RNA from an RNA/DNA hybrid molecule [3]. A 16 nucleotide (nt) polypurine tract (PPT), which is 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 sequence, antisense oligonucleotides as well as triple-helix-forming oligonucleotides (TFOs) might be useful tools to interfere with retroviral replication [6–13]. 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.

In this paper, we describe the inhibition of the RT activities by a two-strand system (FTFOs) targeted to the PPT region (Fig. 1). 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) [14–20]. 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. The FTFOs inhibit the action of the RT 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 sequence sites and guanosine rich parts within the third Hoogsteen base-pairing strand.

2. Materials and methods

2.1. Oligonucleotides

The oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems Model 392 DNA/RNA synthesizer on the 1 μM scale and with controlled pore glass supports. RNA and DNA phosphoramidite units were purchased from PerSeptive Biosystems. 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/7 M urea gels.

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

2.2. Inhibition of reverse transcription by TFOs and FTFOs

Plasmid pSV2neo JRCSF-B, containing the PPT 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).

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2.2.1. RNA/DNA hybrid formation. In vitro transcribed RNA containing the PPT target sequence (430 nt) (0.1 μM), a ^{32}P-labelled primer (5′-TCAGGGAAGTAGCCTTGTGT-3′, complementary to HIV-1 mRNA, nt 252–271, 0.1 μM) and an antisense oligonucleotide (5′-TCCCCCCTTTTCTTTT-3′, H-20, 1 μM) were diluted in a buffer consisting of 25 mM Tris-acetate (pH 6.8), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM α-mercaptoethanol and 0.4 mM spermine, incubated for 5 min at 90°C and then cooled slowly to 37°C. For triple-helix formation, the RNA/DNA hybrid was incubated with D1 (0.1 μM). HIV-1 RT and dNTPs were then added to the samples. After an incubation for 1 h at 37°C, the samples were analyzed on a 10% polyacrylamide-TBE-urea gel. The reactions were quantified by radioanalytic imaging with a Bioimage analyzer, BAS 2000 (Fujifilm).

2.2.2. Triple-helix formation on a single-stranded RNA target. For analyzing FTFOs, the efficiency during RNA-directed cDNA synthesis was performed generally as described above. FTFOs were added in an equivalent molar to the RNA template. HIV-1 RT and dNTPs were added and samples were incubated for 1 h at 37°C. The samples were analyzed on a 10% polyacrylamide-TBE-urea gel. The reactions were quantified by radioanalytic imaging with a Bioimage analyzer, BAS 2000 (Fujifilm).

2.3. Thermal denaturation profiles

Thermal transitions were recorded at 260 nm using a Shimadzu UV-2200 spectrometer. The insulated cell compartment was warmed from 15 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 $MgCl_2$ and 50 mM 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 15°C. At temperatures below 15°C, N_2 gas was continuously passed through the sample compartment to prevent the formation of condensation.

2.4. 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#8, were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μU/ml penicillin and 100 μg/ml 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.

2.5. 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 (3×10^5 cells/ml) 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 culturing 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 Multican 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 anti-viral protection [21,22].

The CD4+ T-cell line, MOLT-4 (3×10^5 cells/ml), was infected with HTLV-IIIB at a MOI of 0.01. After a 2 h infection, the cells were washed and treated with the synthetic oligonucleotides at a 1 μM concentration in the culture medium. After 2 days, the medium was removed and fresh medium containing the oligonucleotides at a 1 μM concentration was added. Virus replication was monitored at the cellular level by syncytia formation and in the culture supernatants by a p24 ELISA (Cellular Products). At the time points indicated, an aliquot of the culture supernatant was removed for p24 antigen analysis and was replaced by fresh medium. Every 4 days, viable cells were counted and passed at 3×10^5 cells/ml [23].

3. Results and discussion

3.1. Inhibition of reverse transcription by TFOs and FTFOs

The structure of the three-strand system with the 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 for the slow process of triple-helix formation with the target strand before their degradation [24,25]. 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 antigen 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 a greater sequence-specificity than conventional antisense and antigen oligonucleotides.

In order to test direct inhibition of reverse transcription by the FTFOs, primer extension assays were performed with a primer binding downstream of the PPT. A RNA of 430 nucleotides in length, transcribed in vitro from plasmid pSV2neo

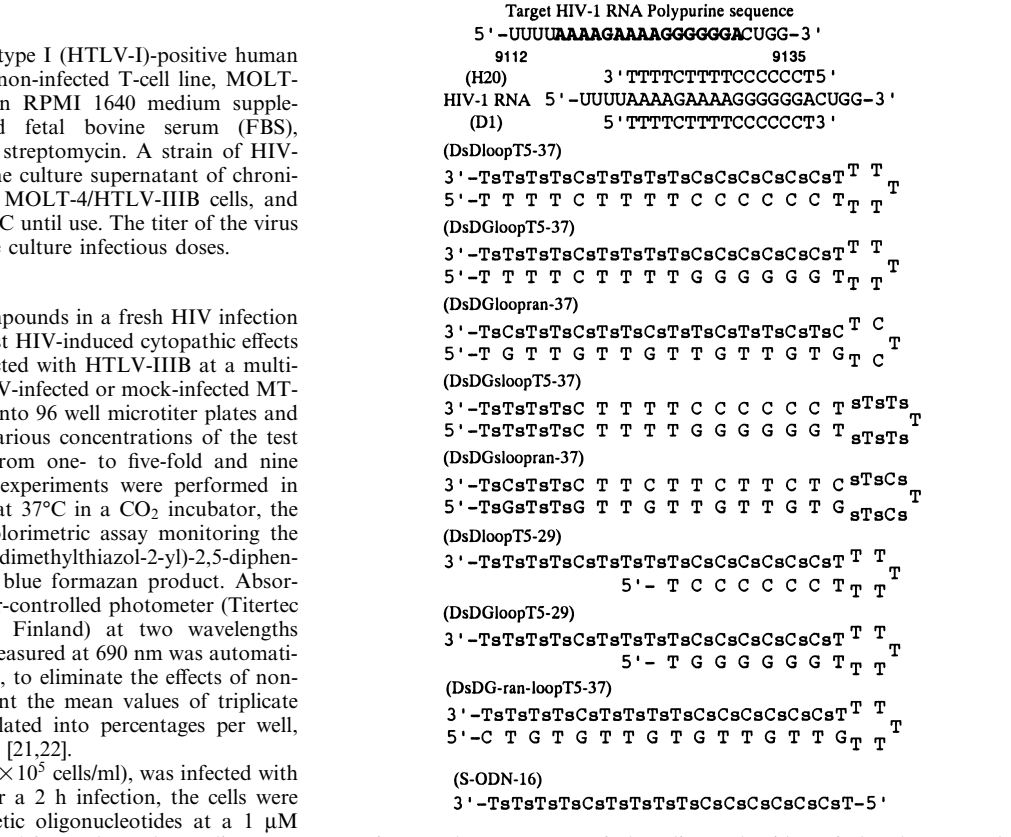

Fig. 1. The sequences of the oligonucleotides of the three-strand (TFOs) and two-strand (FTFOs) systems used in this work. The target PPT-RNA is indicated as bold face. The nucleotide sequences of the RNA/DNA hybrid substrate, consisting of the HIV-1 mRNA (430 nt) and the 20-mer antisense phosphodiester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

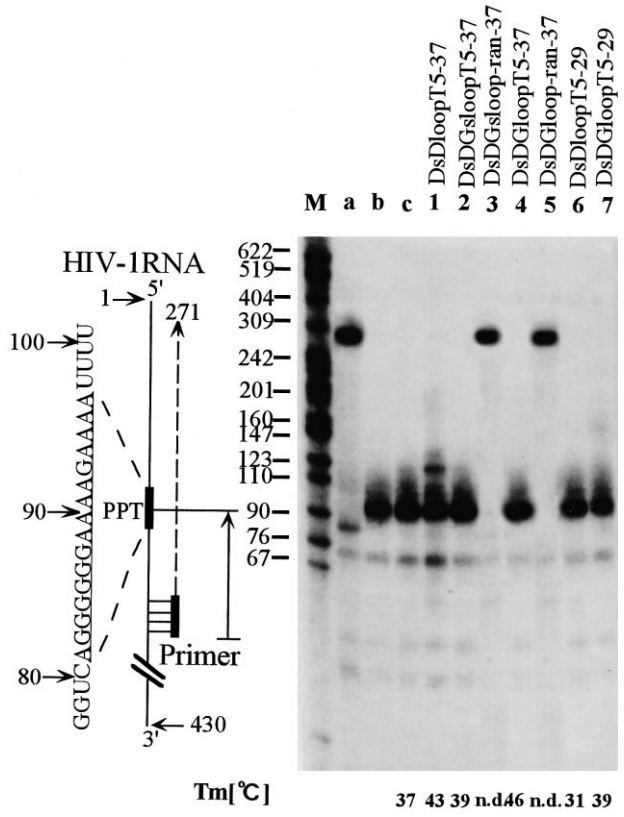

Fig. 2. Inhibition of primer extension by three-strand (TFOs) and two-strand (FTFOs) systems. Primer extension assay in the presence of FTFOs. HIV-1 mRNA (430 nt), transcribed in vitro, was primed with a radioactively labelled oligonucleotide primer (20-mer). The FTFOs were added and incubated. Lanes 1–7 contain the FTFOs as indicated, at 0.1 μM concentrations. Lane a shows a primer extension with the hybrid HIV-1 mRNA (430 nt) and a radioactively labelled oligonucleotide primer (20-mer), which binds downstream of the PPT. Lane b, control primer extension reactions with the antisense oligonucleotide (H 20) at a 0.1 μM concentration. Lane c, control primer extension reactions with the antisense oligonucleotide (H 20) and TFO, D1, at 0.1μM concentrations. M, molecular size markers. T_m values are listed at the bottom.

JRCS-B and encompassing the PPT region of the HIV-1 genome, was used as the template. We determined whether the three-strand system or the two-strand system could interfere with the enzyme activities of the RT. 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) [26,27]. Several studies [28–30] 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. The control primer extension reaction was performed with the antisense oligonucleotide (5′-TCCCCCCTTTTCTTTT-3′, H-20, 0.1 μM) corresponding to the Watson-Crick parts of the PPT region. Primer extension inhibition was assessed with a 5′-end-labelled primer DNA (5′-TCAGGGAAGTAGCCTTGTGT-3′, complementary to HIV-1 mRNA, nt 252–271) and was analyzed by gel electrophoresis and autoradiography. Fig. 2 shows the results of the FTFOs. All of the FTFOs (DsDloopT5-37, DsDGsloopT5-37, DsDGloopT5-37, DsDloopT5-29 and DsDGloopT5-29) showed similar inhibitory effects on cDNA synthesis as compared with the antisense oligonucleotide (0.1 μM). The in vivo target for the FTFOs, in the case of HIV-1 reverse transcription, is a single-stranded RNA. Interestingly, the shorter FTFOs, DsDloopT5-29 and DsDGloopT5-29, with the shorter Hoogsteen sequences, can protect against cDNA synthesis as well as the longer FTFOs (0.1 μM). The control sequences, DsDGsloop-ran-37 and DsDGloop-ran-37, had no detectable inhibitory effects on cDNA synthesis. These results suggest that the two-strand system was able to induce a full conversion to triple-helix formation. It is worth noting that the FTFOs conferred sequence-specific inhibition.

3.2. Foldback triplex formation with target sequence

The ability of the oligonucleotides to form triple-helices was examined by melting temperature studies. The T_m values at pH 6.8 of the FTFOs are listed at the bottom of Fig. 2. The T_m values of the pur/pur/pyr triplex (DsDGloopT5-37) and DsDloopT5-37 were 46 and 43°C, respectively. On the other hand, DsDGsloopT5-37, with phosphorothioates at the 3′- and 5′-ends (39°C), hybridized more weakly with the duplex DNA/RNA than DsDGloopT5-37. The oligomers with phosphorothioate internucleotidic bonds on the third strand, which exist as a mixture of diastereoisomers, have reduced abilities. The pur/pur/pyr triplex (DsDGloopT5-37) formed a more stable hybrid with the DNA/RNA duplex than the pyr/pur/pyr triplex (DsDloopT5-37) [31,32]. The DsDGloopT5-37 binds to the target sequence by the involvement of both Watson-Crick and Hoogsteen domains through foldback triplex formation. The T_m value of the D1 three-strand system (TFOs) was 37°C. Furthermore, the shorter FTFOs, DsDGloopT5-29 with guanosine rich parts at the Hoogsteen sequence, showed higher T_m values (39°C) than the D1 three-strand system (TFOs). In contrast, the DsDloopT5-29 had a lower T_m value (31°C). For the control sequences, DsDGsloopS-ran-37 and DsDGloop-ran-37, the T_m values were not detectable. These results support the notion that FTFOs are thermodynamically and kinetically superior to TFOs.

Another problem with such an affinity of oligonucleotides is their sensitivity to degradation by nucleases present in the

Table 1
Anti-HIV activity of FTFOs in MT-4 cells

Oligonucleotides	$EC_{50}^{a,c}$	$CC_{50}^{b,c}$
DsDloopT5-37	0.03 μM	>10 μM
DsDGloopT5-37	0.03 μM	>10 μM
DsDGloop-ran-37	5.00 μM	>10 μM
DsDGsloopT5-37	0.03 μM	>10 μM
DsDGsloop-ran-37	5.00 μM	>10 μM
DsDloopT5-29	0.08 μM	>10 μM
DsDGloopT5-29	0.04 μM	>10 μM
DsDG-ran-loopT5-37	0.20 μM	>10 μM
S-ODN-16	0.32 μM	>10 μM

[a]Anti-HIV-1 activity was monitored as percentage inhibition of HIV-1-induced cytopathogenicity in MT-4 cells. The EC_{50} 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 CC_{50} is the concentration of test compound that exhibits 50% cell toxicity.
[c]Data represent average values for at least three different experiments.

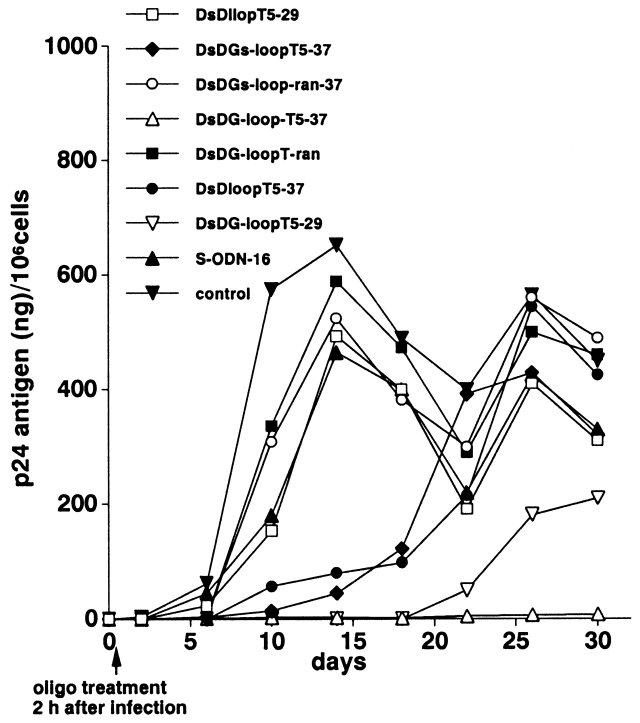

Fig. 3. Anti-viral activity of the FTFOs and antisense oligonucleotide at 1 μM. After 2 h, the virus was removed from MOLT-4 cells newly infected with HIV-1_{III-B} and the cells were treated with the synthetic oligonucleotides. The second treatment was performed 2 days later. Supernatants were collected and p24 expression was determined by the p24 antigen assay.

serum, especially 3′-exonucleases. After 24 h of incubation in medium containing 10% FBS, the two-strand system oligonucleotides (DsDloopT5-37, DsDGloopT5-37, DsDGsloopT5-37 and DsDGloopT5-29) show the same order of stabilities 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.

3.3. Anti-HIV-1 activity of FTFOs

In order to clarify the anti-HIV activities of the FTFOs, we tested both acutely and chronically infected cells. Control oligonucleotides were prepared for comparison, such as the FTFOs (5′-CTGTGTTGTGTTGTTGTTTTTTsCsCsCsCsCsCsTsTsTsTsCsTsTs-TsT-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 oligonucleotide (5′-TsCsCsCsCsCsCsTsTsTsT-3′), but in a random order (DsDGsloop-ran 37 and DsDGloop-ran-37). The FTFOs (DsDloopT5-37, DsDGsloopT5-37, 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 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 EC_{50} values of 0.08 μM. The deletion of 8 nt (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 nt (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 EC_{50} 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, not without the triplex manner. Furthermore, antisense oligonucleotide S-ODN-16 inhibited HIV-1-induced CPE at EC_{50} values of 0.32 μM, a substantially lower inhibitory effect than that observed for its FTFOs, DsDGloopT5-37. FTFOs recognize the target sequence twice, unlike conventional antisense and antigen 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. Phosphorothioate oligonucleotides have been shown to block the proliferation of HIV-1 in acutely infected cells in a non-sequence-specific manner [33], probably by the inhibition of RT [34,35] and/or the viral entry process [36,37]. However, the FTFOs inhibited HIV-1-induced CPE in acutely infected MT-4 cells in a sequence-specific manner. It is noteworthy that the FTFOs inhibit the replication of HIV-1 more effectively than the antisense phosphorothioate oligonucleotides.

Next, we examined the long-term effects of FTFOs treatment in HIV-1-infected MOLT-4 cells. The MOLT-4 cells were incubated with HIV-1_{III-B} for 2 h to allow absorption. The cells were then washed to remove the virus from the medium and the modified FTFOs were added with fresh medium. After 2 days, new medium supplemented with the oligonucleotides was added. The virus production in the culture supernatant was monitored by the HIV-1 p24 antigen assay (Fig. 3). In this assay system, the phosphorothioate-modified oligonucleotides cannot interfere with retroviral binding at the CD4 receptor, because the cells were incubated with an infectious HIV-1 supernatant for 2 h before the application of the oligonucleotides. The control-infected cells (no oligomer added) exhibited maximal HIV-1 replication at 14 days. However, in the cells treated with the DsDGloopT5-37 (1 μM), p24 expression was inhibited by 100%, as compared to the untreated control at 30 days. In contrast, the DsDloopT5-37-treated cells, containing cytidine rich parts within the third Hoogsteen base-pairing strand, expressed high levels of p24 products after 26 days. On the other hand, the DsDGsloopT5-37 with phosphorothioate groups at the 3′- and 5′-ends inhibited HIV-1 replication by 64%, as compared to the untreated control at 18 days. The oligonucleotide with phosphorothioate groups in the third Hoogsteen base-pairing strand has less ability to form a triple-helix. After 14 days, the random oligomer (DsDGsloop-ran-37 and DsDGloop-ran-37) and antisense oligonucleotide-treated cells (S-ODN-16) expressed high levels of p24. Interestingly, the short FTFOs, DsDGloopT5-29, inhibited virus replication by 100%, as compared to the untreated control at 18 days, but the treated cells expressed high levels of p24 products after 26 days. The DsDloopT5-29 had no detectable inhibitory effect. These results suggest that the thermodynamically extractable Hoogsteen base-pairing structure is apparently the best structure for the inhibitory efficiency. The greatest inhibitory effects on

HIV-1 replication were detected with the DsDGloopT5-37 at the 1 μM concentration.

In conclusion, the two-stranded composition of a triple-helix is thermodynamically and kinetically superior to the three-strand system. The FTFOs containing the phosphorothioate groups at the antisense sequences showed greater 3′-exonuclease resistance. In HIV-1-infected MOLT-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. In particular, the phosphorothioated FTFOs may be useful in nucleic acid-based anti-viral therapies with triple-helix approaches in vitro or in vivo.

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