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Biochemical and Biophysical Research Communications 313 (2004) 55–61
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Anti-HIV-1 activity and mode of action of mirror image oligodeoxynucleotide analogue of zintevir

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Received 17 November 2003

Abstract

Zintevir is an oligonucleotide analogue, which has the phosphorothioate modification at both termini, that forms a K^+-induced quadruplex structure and shows potent anti-human immunodeficiency virus (HIV)-1 activity. We synthesized the non-modified analogue (D-17mer) of Zintevir and its enantiomer (L-17mer), and compared their anti-HIV-1 activity and molecular mechanism of action. Although L-17mer forms the exact mirror image quadruplex structure of D-17mer, which has a very similar structure with Zintevir, L-17mer showed comparable anti-HIV-1 activity with Zintevir. The results obtained by the time-of-addition experiments and the immunofluorescence binding assay strongly suggest that the primary molecular target of L-17mer is the viral gp120 envelope protein as well as Zintevir, regardless of their reciprocal chirality.

Keywords: Anti-HIV-1 activity; Chirality; DNA; Enantiomer; Viral entry; Zintevir

The chirality of molecules plays important roles in the higher structural organization and specific ligand recognition of biomolecules [1]. Usually, the enantiomer of a molecule shows different behavior and action from the parental molecule in chiral environment such as living body. Recent discovery of 2′-deoxy-3′-thiacytidine (3TC), which has an unnatural L-configuration, brought about a breakthrough in nucleoside-based chemotherapy of viral diseases. This antiviral nucleoside analogue (3TC) is discovered as a racemic compound [2]. Coats et al. separated each optical isomer of 2′-deoxy-3′-thiacytidine and they evaluated their anti-HIV-1 activity. Unexpectedly, the unnatural stereoisomer (3TC) showed comparable anti-HIV-1 activity with natural one, in spite of its unnatural configuration [3,4]. Moreover, 3TC showed an extremely lower cytotoxicity for host cells than the natural stereoisomer [3–5]. Therefore, 3TC should be recognized by HIV-1 reverse transcriptase after phosphorylation and taken into the growing DNA strand to inhibit the further elongation of the viral DNA strand, although phosphorylated 3TC should not be easily recognized by host cell polymerases [6]. This means that HIV-1 reverse transcriptase is unable to recognize 3TC with a stereospecific manner. In addition to HIV-1 reverse transcriptase, D-peptide containing the basic-arginine rich region of the Tat protein was reported to specifically bind to the major groove of TAR RNA in a similar fashion to that observed for the natural L-Tat peptide [7,8]. Above facts suggest that these proteins derived from HIV-1 may recognize their specific ligands with low or even no stereospecificity.

Zintevir	d(G*TGGTGGGTGGGTGGG*T)
D-17mer	d(GTGGTGGGTGGGTGGGT)
L-17mer	L-d(GTGGTGGGTGGGTGGGT)

*: phosphorothioate linkage

Zintevir (T 30177) is a guanosine-quartet structure (G-tetrad)-forming single stranded oligodeoxynucleotide (Fig. 1), which is partially phosphorothioated at both the termini to raise its in vivo stability. Zintevir was discovered as a potent inhibitor for HIV-1 integrase [9,10], and this discovery seemed to promise the

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doi:10.1016/j.bbrc.2003.11.094

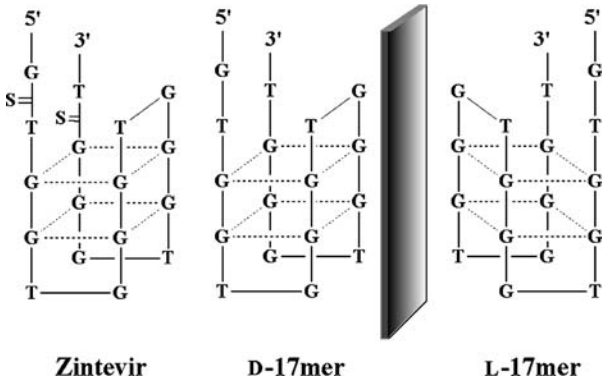

Fig. 1. Structures of Zintevir, D-17mer, and L-17mer.

development of antiviral agents directed to a novel target of HIV-1 replication. Recently, the primary molecular target of Zintevir, however, was shown to be the HIV-1 gp120 envelope protein by the genetic analysis of the Zintevir-resistant strain, in which the resistant phenotype was associated with the emergence of mutations in the gp120 protein [11]. In this paper, to verify whether the rationale that HIV-1-related proteins recognize nonstereospecifically their specific ligands is also compatible to other viral proteins or not, we synthesized the all-phosphodiester analogue (D-17mer) of Zintevir and its mirror image 17mer (L-17mer) as well as Zintevir. Since oligonucleotides having an unnatural L-configuration show nearly complete resistance towards nucleases [12,13], L-oligonucleotide should have a more preferable properties as an antiviral agent than natural D-oligonucleotides. Here, we report the synthesis, physico- and biochemical characterization of the enantiomer (L-17mer) of Zintevir, and also its action mechanism associated with the anti-HIV-1 activity.

Materials and methods

General methods. L-Thymidine, L-deoxyguanosine, and their phosphoramidites were synthesized by the previously reported procedures [12,13]. Reagents for the DNA synthesizer other than L-thymidine and L-deoxyguanosine phosphoramidites were purchased from Applied Biosystems Japan (Tokyo, Japan). MALDI-TOF mass analyses of the 17mers were carried out on a PE Biosystems Voyager Linear DE or Elite spectrometer. Nuclease P1 and SVPD were purchased from Yamasa (Chiba, Japan) and Roche Diagnostics (Mannheim, Germany), respectively.

Synthesis of oligodeoxynucleotides. Oligodeoxynucleotides were synthesized by an Applied Biosystems model 392 DNA/RNA synthesizer. After usual deblocking, the purification was performed on a column of MonoQ HR 5/5 (Amersham Bioscience) with a linear gradient of NaCl in 10 mM NaOH by a Shimadzu LC-10A HPLC system. After neutralization, samples were desalted with Sep-Pak Plus C18 cartridge (Waters). The purity of the 17mers tested by the above system was more than 95%. The structures of the 17mers were confirmed by MALDI-TOF MS spectra: Zintevir, $C_{170}H_{210}O_{103}N_{70}P_{16}S_2$ calcd. *m/z* 5438.86 [M^-]; found 5439.84 (negative), D-17mer, $C_{170}H_{210}O_{105}N_{70}P_{16}$ calcd. *m/z* 5405.90 [M^+]; found 5406.66 (positive), and L-17mer, $C_{170}H_{210}O_{105}N_{70}$ P_{16} calcd. *m/z* 5405.90 [M^+]; found 5407.19 (positive).

Resistance against nuclease digestion. A solution (3 ml) of each 17mer (3OD units) containing 50 mM ammonium acetate (pH 5.0) for nuclease P1 or 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.0) for SVPD was placed in a 1 cm path-length quartz cell. With stirring at 37 °C, enzyme (nuclease P1, 1 mg/ml, 2 μl; SVPD, 2 mg/ml, 2 μl) was added into the solution, and absorbance at 260 nm was measured for 3 h by a JASCO Ubest-55 spectrophotometer equipped with a temperature controller.

Measurements of CD spectra. A solution (4 μM) of each 17mer containing 20 mM lithium phosphate (pH 7.0) and designated concentration of KCl was placed in a 1 cm path-length quartz cell. Spectra were measured by a JASCO J-820 spectropolarimeter equipped with a temperature controller.

Cells and virus. MT-4 and Molt-4 cell lines, which are human leukemic T-cell lines, were maintained in RPMI-1640 (Nikken Biomedical Laboratory, Kyoto Japan) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. HIV-1_{LAI} strain was obtained from culture fluid of Molt-4 cells persistently infected with HIV-1_{LAI} strain.

Anti-HIV-1 activity assay. MT-4 cells were infected with HIV-1_{LAI} strain at a multiplicity of infection (MOI) of 0.001 for 1 h and washed once with the culture medium mentioned above. The infected MT-4 cells were suspended in a culture medium containing inhibitors, which had been diluted stepwise, at a concentration of 1.5×10^5 cells/ml. The suspension was cultured at 37 °C for five days. Viable cell count was determined by the trypan blue dye exclusion methods and the 50% inhibitory concentration (IC_{50}) for each virus stock was calculated.

Time-of-addition experiments. At MOI of 0.5, HIV-1 was allowed to adsorb to 2×10^5 MT-4 cells in the absence or presence of the inhibitors (10 μM) on ice for 60 min. The cells were washed with cold culture medium three times to remove unadsorbed virus and then incubated at 37 °C. The inhibitors were added at 10 μM concentration at different times (0–6 h) after infection, as shown in Fig. 5. Some cells were harvested for PCR assay 12 h after virus inoculation.

Immunofluorescence binding assay. As many as 1×10^6 uninfected MT-4 cells were reacted with monoclonal antibody against CD4 (FITC-labeled anti-Leu3a antibody: Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA) at 37 °C for 30 min in the absence or presence of the inhibitors (100 μM). The cells were washed three times with 0.15 M PBS and then re-suspended in PBS containing 3% formaldehyde. A pair of anti-HIV-1 gp120 V3 (0.5β) [14] and Molt-4 cells persistently infected with HIV-1 was reacted in the same manner as described above. The cells were washed three times with 0.15 M PBS and then reacted with anti-mouse IgG1 antibody labeled with FITC (Research Diagnostics, Flanders, NJ, USA) at 37 °C for 30 min. The cells were washed three times with 0.15 M PBS and then re-suspended in PBS containing 3% formaldehyde. The fluorescence intensity of the suspension was measured using a flow cytofluorometer (Epics XL, Beckman Coulter, Fullerton, CA, USA) and the percentage of the fluorescence intensity of inhibitor-treated cells relative to that of untreated cells as a positive control was calculated.

Results and discussion

Synthesis and structure of 17mers

L-Thymidine, L-deoxyguanosine, and their phosphoramidites were synthesized by the previously reported procedure [13]. Synthesis of an L-oligodeoxynucleotide was achieved by the same methodology as that of D-oligodeoxunucleotides according to the conventional

solid-phase phosphoramidite chemistry. Since guanine-rich sequences, which show heterogeneity in their structure by intra- and inter-molecular hydrogen-bonding interactions, provide some difficulty in the purification step, we employed an anion exchange column with alkaline conditions for the purification of the 17mers. The chemical structures of the 17mers were confirmed by MALDI-TOF MS spectra.

In the presence of K^+ cations, Zintevir has been reported to effectively form an intramolecular G-tetrad structure [9,10]. To confirm the tertiary structure of L-17mer, circular dichroism (CD) spectra were measured. The positive Cotton band at around 265 nm of Zintevir is dramatically strengthened by increasing K^+ cation concentration, suggesting the formation of the stable G-tetrad structure, and the spectra of D-17mer under both low and high K^+ concentration conditions are very similar to those of Zintevir. On the other hand, L-17mer shows the same CD strength and the same behavior by increasing K^+ cation concentration as D-17mer except for its sign (Fig. 2). These results strongly suggest that L-17mer has the mirror image tertiary structure of D-17mer and Zintevir. Fig. 3 shows the temperature dependence of the CD strength at 260 nm of the 17mers at 0.2 M K^+ cation concentration. All the 17mers show the structural transition from the G-tetrad structure into a random coil by increasing temperature, whose midpoints are higher than 80 °C. Thus, all the 17mers similarly form the highly stable G-tetrad structure under these conditions.

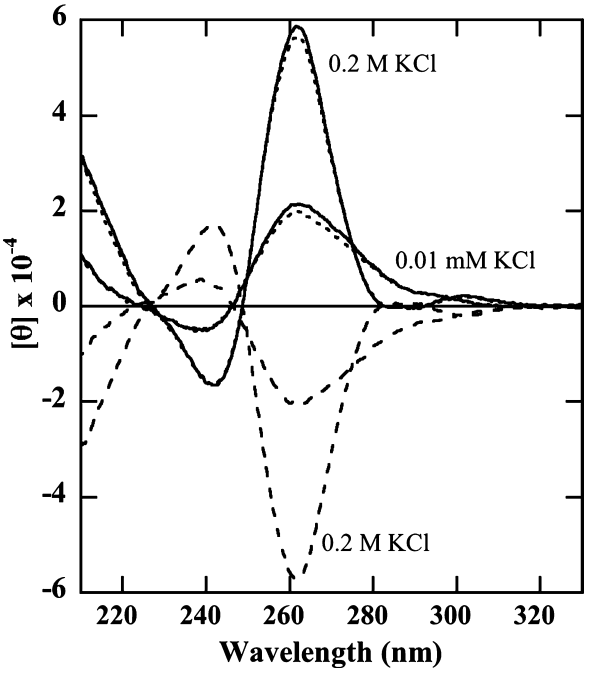

Fig. 2. CD spectra of Zintevir (solid lines), D-17mer (dotted lines), and L-17mer (broken lines) in 20 mM lithium phosphate (pH 7.0) containing 0.2 M or 0.01 mM KCl at 25 °C.

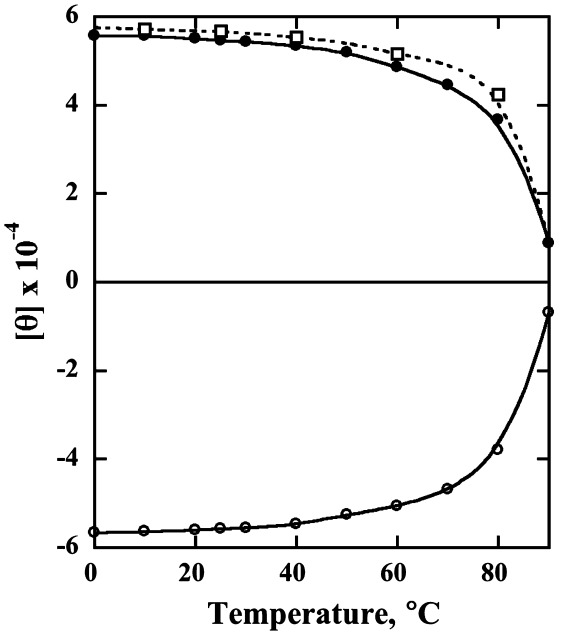

Fig. 3. Temperature dependence of molar ellipticity at 260 nm of Zintevir (open square), D-17mer (closed circle), and L-17mer (open circle) in 20 mM lithium phosphate (pH 7.0) containing 0.2 M KCl.

Anti-HIV-1 activity and nuclease resistance

Zintevir has been shown to possess potent anti-HIV-1 activity [9,10]. To evaluate the anti-HIV-1 activity of L-17mer, the in vitro inhibitory effects of the 17mers on the HIV-1-induced cytopathicity in MT-4 cells were tested. The inhibitory effects of Zintevir and L-17mer were almost the same, their IC_{50} values being 0.225 μM, despite having opposite chirality to each other, whereas that of D-17mer is 0.57 μM. The difference in the activity between the two former 17mers and the latter one was considered to be due to the difference of their resistance to nucleases in the culture medium and/or in the cells. Therefore, we compared the susceptibility of the 17mers towards snake venom phosphodiesterase (SVPD) and nuclease P1. Fig. 4 shows the time course of hyperchromicity at 260 nm induced by degradation of the 17mers with the enzymes. D-17mer was degraded rapidly with both of the enzymes. Under the same conditions, Zintevir showed significant resistance for SVPD, which is a 3′-exonuclease, yet not so much for nuclease P1 (Figs. 4A and B, respectively), because of its phosphorothioate modification at both termini. In contrast, L-17mer was completely resistant to both of the enzymes under the same conditions. Therefore, the lower anti-HIV-1 activity of D-17mer may be due to its susceptibility to degradation by nucleases.

Time-of-addition experiments

Although Zintevir has been reported to inhibit viral integrase, the primary molecular target of Zintevir was

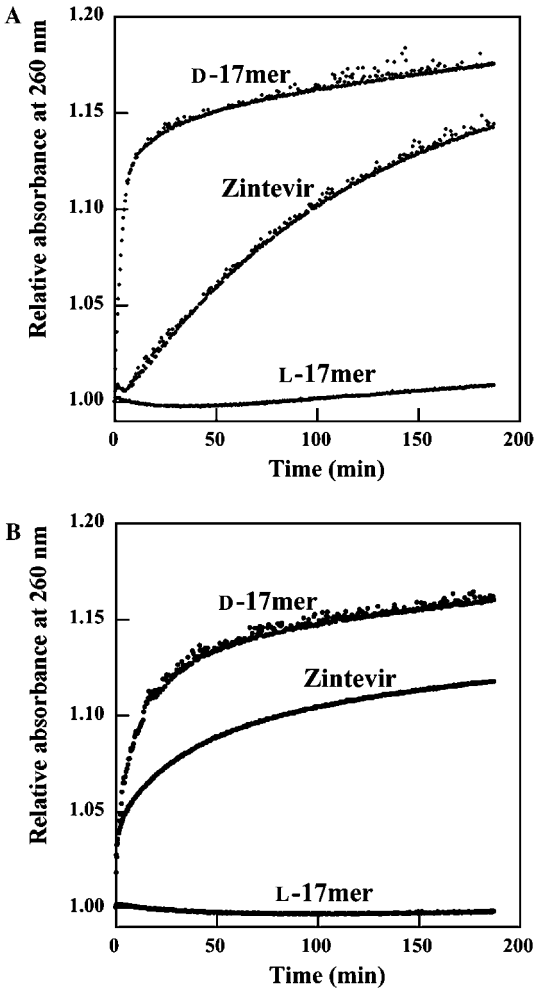

Fig. 4. Resistance of the 17mers against SVPD (A) and nuclease P1 (B). Reactions started by adding enzyme into the buffered solution of each 17mer at 37 °C. Hyperchromicity at 260 nm induced by degradation of an oligonucleotide is revealed periodically.

recently shown to be the viral gp120 envelope protein [11]. To verify the inhibitory step(s) of L-17mer within the HIV life cycle, time-of-addition experiments [15] were conducted, whereby the 17mers were added at different times after exposure of the MT-4 cells to HIV-1. In this assay system, the viral *gag* gene integrated into the host cell DNA can be observed by the electrophoresis when the 17mers cannot exhibit their inhibitory effects. The result is shown in Fig. 5. Zintevir could not show any inhibitory potency when it was added at 2 h or later after viral infection (lanes 14–16). However, when Zintevir was present only during the viral adsorption step, and also when virus-adsorbed cells were started to culture in the presence of Zintevir, it showed the inhibitory potency (lanes 12 and 13, respectively). Inhibition by AZT decreased when it was added at 4 h after viral infection (data not shown). This result strongly suggests that Zintevir inhibits the viral adsorption onto the host cells and the entry into the cells as reported by Esté et al. [11]. Similarly, D-17mer and L-17mer were

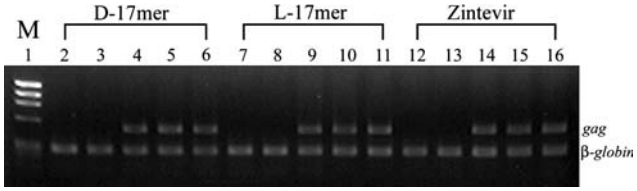

Fig. 5. Time-of-addition experiments for the 17mers. HIV-1 was adsorbed onto MT-4 cells on ice and the mixture was allowed for 1 h at this temperature in the presence or absence of each 17mer (adsorption). After free virus was washed out with ice-cold buffer, incubation was started at 37 °C and the 17mers were added after the designated incubation time as below. After 12 h, cells were harvested and amplified the viral *gag* gene together with the host *β-globin* gene by PCR: lane 1, size marker; lanes 2, 7, and 12, each 17mer existed only during the "adsorption" step; lanes 3, 8, and 13, each 17mer was added when the incubation started; lanes 4, 9, and 14, each 17mer was added at 2 h after the start of the incubation; lanes 5, 10, and 15, each 17mer was added at 4 h after the start of the incubation; lanes 6, 11, and 16, each 17mer was added at 6 h after the start of the incubation.

effective when they were present only during the viral adsorption step (lanes 2 and 7, respectively), and also when virus-adsorbed cells were started to culture in the presence of them (lanes 3 and 8, respectively). However, they lost their inhibitory activity if the treatment was delayed until 2 h after infection. (lanes 4 and 9, respectively). This strongly suggests that the inhibitory step of L-17mer is viral adsorption and/or viral entry within the HIV replicative cycle as well as Zintevir and D-17mer.

Immunofluorescence binding assay

Both viral envelope proteins and cellular receptors are closely related to the viral adsorption and entry process, and are considered as a candidate for the molecular target of the 17mers. To verify this point, the immunofluorescence binding assay was conducted. Zintevir had no effect on the binding of a monoclonal antibody against the gp120-binding domain of CD4 (anti-Leu3a) to the CD4 cellular receptor of MT-4 cells (Fig. 6C), whereas it significantly inhibited the binding of monoclonal antibody (0.5β) [14] (directed to an epitope in the V3 loop region of gp120) to MOLT-4 cells persistently infected with HIV-1_{LAI} (Fig. 7C). These results strongly suggest that Zintevir binds not to the CD4 receptor but to the gp120 viral envelope protein, and thus the results support the conclusion of Esté et al. obtained by the genetic analysis of the Zintevir-resistant strain of HIV-1 [11]. Similarly, D- and L-17mers showed the inhibitory effects for the binding of anti-gp120 mAb (0.5β) to MOLT-4 cells (Figs. 7D and E, respectively), but not for the binding of anti-CD4 mAb (anti-Leu3a) to the CD4 cellular receptor of MT-4 cells (Figs. 6D and E, respectively). The above findings suggest that the primary molecular target of L-17mer would be the viral gp120 protein as well as Zintevir, although both 17mers have the opposite chirality to each other.

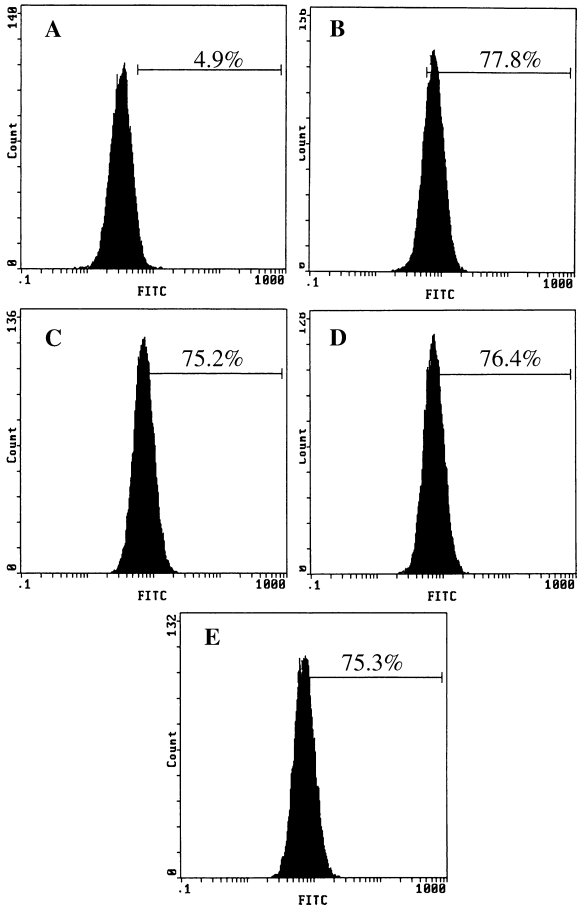

Fig. 6. Flow cytometric histograms of the binding of anti-CD4 mAb (Leu3a) on MT-4 cells: (A) fluorescence of MT-4 cells incubated only with FITC-labeled anti-mouse IgG1 antibody; (B) fluorescence of MT-4 cells incubated with anti-CD4 mAb (Leu3a), then with FITC-labeled anti-mouse IgG1 antibody; (C,D,E) fluorescence of the cells incubated as (B) in the presence of Zintevir, D-17mer, and L-17mer (100 μM each), respectively.

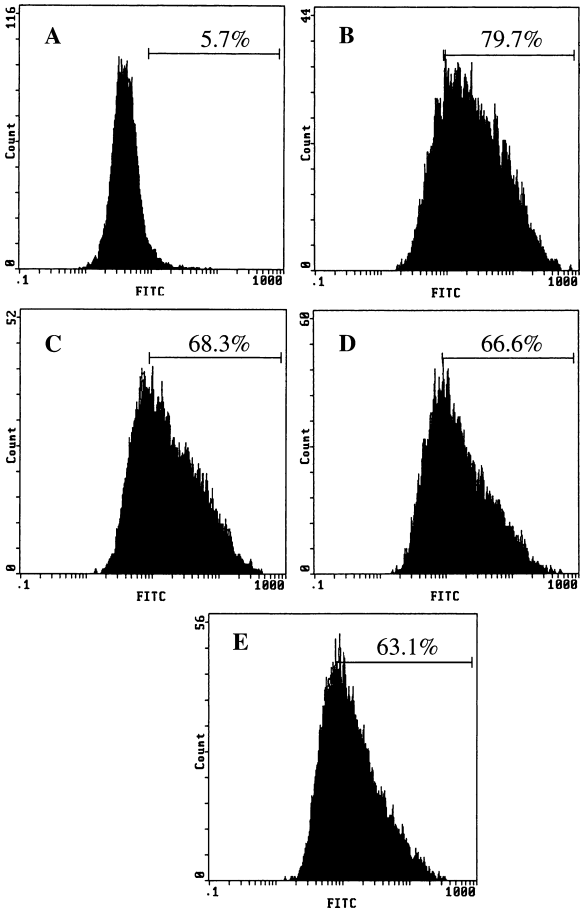

Fig. 7. Flow cytometric histograms of the binding of anti-gp120 mAb (0.5β) on Molt-4 cells persistently infected with HIV-1_{LAI}: (A) fluorescence of Molt-4 cells incubated only with FITC-labeled anti-mouse IgG1 antibody; (B) fluorescence of Molt-4 cells incubated with anti-gp120mAb (0.5β), then with FITC-labeled anti-mouse IgG1 antibody; (C,D,E) fluorescence of the cells incubated as (B) in the presence of Zintevir, D-17mer, and L-17mer (100 μM each), respectively.

Generally, chiral biomolecules show a strict enantiospecificity toward their substrates and ligands. Recently, some cases, in which L-nucleotides are recognized by natural enzymes, have been shown, such as T4 DNA ligase [16], human and viral deoxynucleoside kinases [17,18], viral reverse transcriptases [19,20], and HIV-1 integrase [21]. From a chemotherapeutic point of view, viral proteins that are recognized by L-nucleic acids are attractive molecular targets, since L-nucleic acid-based drugs can be expected to have the enhanced biological stability and the reduced cytotoxicity. Zintevir possesses the phosphorothioate modification at both the termini to raise its in vivo stability. This modification for oligonucleotides is also applied for antisense molecules, but it has been reported to cause nonspecific binding to some proteins, which should lead to undesirable side effects [22]. The toxicity of Zintevir should be partly owing to such nonspecific binding to some proteins. In contrast, L-17mer has no such modification and is expected to have superior chemotherapeutic nature to Zintevir.

Although Zintevir has been developed as a potent inhibitor for HIV-1 integrase, which was thought to be possibly associated with the anti-HIV-1 activity, the primary molecular target of Zintevir was shown to be the viral gp120 protein by the genetic analysis of Zintevir-resistant strain [11]: yet, there is a possibility that Zintevir also inhibits the later stage(s) of the HIV-1 replicative cycle. In the time-of-addition assay described here, we could not observe such activities for L-17mer as well as Zintevir and D-17mer. This result not only confirms the conclusion of Esté et al. [11] that the primary target of Zintevir is the viral adsorption and fusion, both of which are mediated in part by gp120, but also suggests that L-17mer inhibits these stages to exhibit the anti-HIV-1 activity. Zintevir was reported to be internalized by cells during prolonged incubation and it takes 4–6 h that the intracellular concentration of Zintevir reaches the extracellular levels [23]. Although integration of viral DNA starts at around 6–9 h after the viral infection [24], we could not observe intracellular

inhibitory effects even when the 17mers were added at 2 h after infection (Fig. 5, lanes 4, 9, and 14). It is thus plausible that the anti-HIV-1 activity of the 17mers is primarily associated with the gp120 inhibition but not the integrase inhibition.

In conclusion, the binding mode of Zintevir with the gp120 molecule has not yet been manifested. The polyanionic compounds such as dextran sulfate and heparin also bind to the gp120 molecule [25] to exhibit potent inhibitory effects on HIV replication [26]. The polyanionic nature of the 17mers may correlate with their anti-HIV-1 activity as dextran sulfate and heparin. However, the anti-HIV-1 activity of G-quartet-forming oligonucleotides is so significantly sequence-dependent [9,27,28] that the folded tertiary structure of the 17mers should be a critical factor for their binding to the gp120 molecule. Nevertheless, our results described here reveal that the interaction of Zintevir with the gp120 molecule does not depend on its chirality at all. Thus, L-17mer is an attractive molecule to facilitate to manifest the mode of the characteristic interaction of Zintevir with the gp120 molecule, together with its therapeutic potency. The comparative investigations for the binding mode of the 17mers with gp120 are currently under way.

Acknowledgments

This work was supported by a Grant-in-Aid for High Technology Research from the Ministry of Education, Science, Sports and Culture, Japan.

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