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HIV-1 Integrase Inhibitory Substances from *Coleus parvifolius*

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For the purpose of discovering anti-HIV-1 agents from natural sources, water and EtOH extracts of 50 Thai plants were screened for their inhibitory activity against HIV-1 integrase (IN), an enzyme essential for viral replication. Of these plants, an EtOH extract of *Coleus parvifolius* Benth. (aerial parts) showed potent activity against HIV-1 IN with an IC_{50} value of 9.2 μg/mL. From this extract, 11 compounds were isolated and identified as luteolin 5-*O*-β-D-glucopyranoside (1), luteolin (2), luteolin 7-methyl ether (3), luteolin 5-*O*-β-D-glucuronide (4), 5-*O*-β-D-glucopyranosyl-luteolin 7-methyl ether (5), rosmarinic acid (6), rosmarinic acid methyl ester (7), daucosterol (8), a mixture of α- and β-amyrin (9, 10) and phytol (11). Of these compounds, rosmarinic acid methyl ester (7), rosmarinic acid (6), luteolin (2) and luteolin 7-methyl ether (3) exhibited inhibitory activities against HIV-1 IN with IC_{50} values of 3.1, 5.0, 11.0 and 11.0 μM, respectively. Among rosmarinic acid derivatives, the HIV-1 IN inhibitory activity increased in turn for a dimer (IC_{50} = 5.0 μM), a trimer (IC_{50} = 1.4 μM), and a tetramer (IC_{50} = 1.0 μM).

Keywords: HIV-1; integrase; inhibition; anti-HIV activity; Thai plants; *Coleus parvifolius*; rosmarinic acid derivatives.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) has evolved rapidly into an epidemic and world-wide public health crisis. Following the identification of this human retrovirus, human immunodeficiency virus type 1 (HIV-1), many investigations have been carried out to discover anti-HIV-1 agents. HIV-1 encodes the enzymes that are essential for viral replication, protease (PR), reverse transcriptase (RT) and integrase (IN) (Katz and Scalka, 1994). For the first two enzymes, many synthetic inhibitors have been used for AIDS treatment in combination regimens or for use alone. However, they have limited or transient clinical benefits due to rapid development of HIV-1 resistance, side effects and toxicity (St. Clair *et al.*, 1991; Elfarrash *et al.*, 1994; Borman *et al.*, 1996; Wainburg, 1998). Therefore, HIV-1 IN is becoming an interesting target for the development of new anti-AIDS agents. Viral integrase (IN) is an enzyme that integrates the viral reverse transcribed DNA into host-cell DNA. During viral infection, IN catalyzes the excision of the last two nucleotides from each 3′-end of the linear viral DNA, leaving the terminal dinucleotide CA-3′-OH at the recessed 3′-ends (3′-processing). After transport to the nucleus as a nucleoprotein complex, IN catalyzes a DNA strand transfer reaction involving the nucleophilic attack at the ends on the host DNA, which is called strand transfer or joining (Fujiwara and Mizuuchi, 1988; Vink and Plasterk, 1993; Katz and Skalka, 1994). Searching for new agents as HIV-1 IN inhibitors from natural sources is necessary since clinically useful antiviral drugs targeting this enzyme still have not yet been developed. Moreover, it would be desirable if agents show potent inhibitory activities against both HIV-1 and HIV-1 IN. For the purpose of finding anti-HIV-1 agents, 50 Thai plants belonging to various families were tested for HIV-1 IN inhibitory activity and anti HIV-1 activity. It was shown that an ethanol extract of *Coleus parvifolius* exhibited potent antiviral and anti HIV-1 IN activities. In the present study, we report the isolation of constituents from *C. parvifolius*, and HIV-1 IN inhibitory activity of several of these and some related compounds using a multiplate integration assay (MIA).

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MATERIALS AND METHODS

General. The ^1H and ^{13}C-NMR spectra were measured with a JEOL-LA 400 Lambda (^1H, 400 MHz, ^{13}C, 100 MHz) spectrometer, the chemical shifts being represented as ppm, with tetramethylsilane as an internal standard. Optical rotations were measured on a JASCO DIP-360 automatic polarimeter at 21 °C. GC-MS were obtained on a GC-17 A gas chromatograph (Shimadzu Co., Kyoto, Japan) and Automass system II (JEOL Co., Tokyo, Japan). An automatic washing machine was used for the MIA assay (Bio-Rad Model 1250 Immunowash).

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Plant materials. The plants were collected at the botanical garden of Prince of Songkla University and some areas at Songkhla, Thailand, and were identified by T.S. The voucher specimens are deposited at the herbarium of Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Preparation of extracts. Five grams of each dried plant were extracted two times with 100 mL of water and ethanol separately under reflux for 3 h. The solvents were removed under reduced pressure to give the respective dry extracts and dissolved in 50% DMSO for bioassay.

Extraction and isolation. The dried aerial parts of *C. parvifolius* (1.2 kg) collected at Songkhla, Thailand, were ground and extracted 4 times with EtOH by maceration at room temperature (each, 12 L) for 2 days. The EtOH extract was then concentrated and partitioned between water and hexane, and successively partitioned with chloroform and water. After that, the water layer was partitioned with EtOAc. Each partition was evaporated to dryness *in vacuo* to give residues of 33.6 g, 23.5 g, 3.5 g and 45.6 g for hexane, chloroform, EtOAc and water fractions, respectively. The water fraction (45 g) was chromatographed on Diaion (Mitsubishi Chemical Corporation, Tokyo, Japan) and eluted consecutively with water, 50% MeOH and MeOH. Column chromatography of the 50% MeOH fraction (8.0 g) over MCI gel (Mitsubishi Chemical Corporation, Tokyo, Japan) using MeOH and water (1:1) gave compound **1** (280 mg). The MeOH fraction (2.7 g) was chromatographed by Sephadex LH-20 using MeOH and $CHCl_3$ (9:1) followed by repeated chromatography on SiO_2 to afford compounds **2** (28 mg), **3** (15 mg), **4** (8 mg), **5** (16 mg) and **6** (38 mg). The EtOAc fraction (2.4 g) was chromatographed by Sephadex LH-20 using MeOH and $CHCl_3$ (9:1). The eluates were repeatedly chromatographed on Sephadex LH-20 and RP-18 to afford compounds **2** (34 mg), **6** (210 mg) and **7** (83 mg). The $CHCl_3$ fraction (15.0 g) was chromatographed over SiO_2 using $CHCl_3$ and MeOH to give compounds **8** (5 mg), **1** (10 mg) and **6** (12 mg). The hexane fraction (15 g) was chromatographed by SiO_2 using hexane and EtOAc and subjected to repeated column chromatography on RP-18 to afford a mixture of compounds **9** and **10** (44 mg) and compound **11** (10 mg). The structures of **1–11** were determined by comparing the ^1H and ^{13}C-NMR spectral data with those reported (Agrawal, 1989; Harborne, 1988; Kuhnt *et al.*, 1995; Kraut *et al.*, 1996).

HIV-1 IN inhibitory activity

a) HIV-1 IN. HIV-1 IN protein was expressed in *Escherichia coli*, purified according to the method of Goldgur *et al.* (1999), and stored at –80 °C before use.

b) Oligonucleotide substrates. Oligonucleotides of long terminal repeat (LTR) donor DNA and substrate DNA were stored at –25 °C before use. The sequence of biotinylated LTR donor DNA was 5′-biotin-ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3′ and its unlabelled complement, 3′-GAAAATCAGTCAACACTTTTAGAGATCGTCA-5′, while 5′-TGACCAAGGGCTAATTCACT-digoxigenin and its 3′-labelled complement, digoxigenin-ACTGGTTCCCGATTAAGTGA-5′, was the substrate DNA (digoxigenin labelled target DNA).

c) Multiplate integration assay (MIA) procedure (see Fig. 1). Firstly, a 96 well plate was coated with 50 µL of a streptavidin solution containing 40 µg/mL streptavidin, 90 mM Na_2CO_3 and 10 mM KCl. Then, 50 µL of a biotinylated LTR donor DNA solution containing 10 mM Tris-HCl (pH 8.0), 1 mM NaCl and 40 fmol/µL of LTR donor DNA was added into each well, and the plate was shaken gently at room temperature for 30 min and washed with phosphate buffer saline (PBS) pH 7.3, four times. The mixture (45 µL) composed of 12 µL of IN buffer [containing 150 mM 3-(*N*-morpholino)propane sulfonic acid, pH 7.2 (MOPS), 75 mM $MnCl_2$, 5 mM dithiothritol (DTT), 25% glycerol and 500 µg/mL bovine serum albumin], 1 µL of 5 pmol/µL digoxigenin-labelled target DNA and 32 µL of sterilized water were added into each well. Subsequently, the plate was added with 6 µL of a sample solution and 9 µL of 1/10 dilution of IN enzyme and incubated at 37 °C for 80 min. The wells were washed with PBS four times and then 100 µL of 500 mU/mL alkaline phosphatase (AP) labelled anti-digoxigenin antibody were added and incubated at 37 °C for 1 h. The plate was washed again with washing buffer containing 0.05% Tween 20 in PBS four times and with PBS four times. Then, 150 µL of the AP buffer containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ and 10 mM *p*-nitrophenyl phosphate were added into each well and incubated at 37 °C for 1 h. Finally, the plate was measured by a microplate reader (Bio-Rad, model 3550 UV) at a wavelength of 405 nm. The positive control composed of the reaction mixture, 50% DMSO and IN enzyme, while the negative control was buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA.2Na), 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1 mM DTT and 4 M urea without the IN enzyme.

Anti HIV-1 activity

a) Cells. The HTLV-1-infected cell line MT-4 and human leukaemia T-cell line MOLT-4 were maintained at 37 °C under 5% CO_2 in RPMI-1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum (FCS, Flow Laboratories, North Ryde, Australia), 100 µg/mL of streptomycin (Meiji Seika, Tokyo, Japan) and 100 U/mL of penicillin G (Banyu Pharmaceutical, Tokyo, Japan).

b) Viruses. HIV-1 (strain HTLV-III_B) was obtained from the supernatant of MOLT-4/HTLV-III_B cells.

c) Primary screening for anti-HIV-1 activity. MT-4 cells were infected for 1 h with HIV-1 (HTLV III_B) at 50% tissue culture effective dose ($TCID_{50}$) of 0.001/cell. Then, the cells were resuspended at 1×10^5 cells/mL in RPMI-1640 medium and 200 µl/well of the cell suspension was cultured for 5 days in a 96-well culture plate, containing various concentrations (12 doses, maximum 1860–850 µg/mL and minimum 0.89–0.42 µg/mL) of the plant extracts. A control assay was performed in the absence of a plant extract with HIV-1-infected and -uninfected cultures. After 5 days, the inhibitory

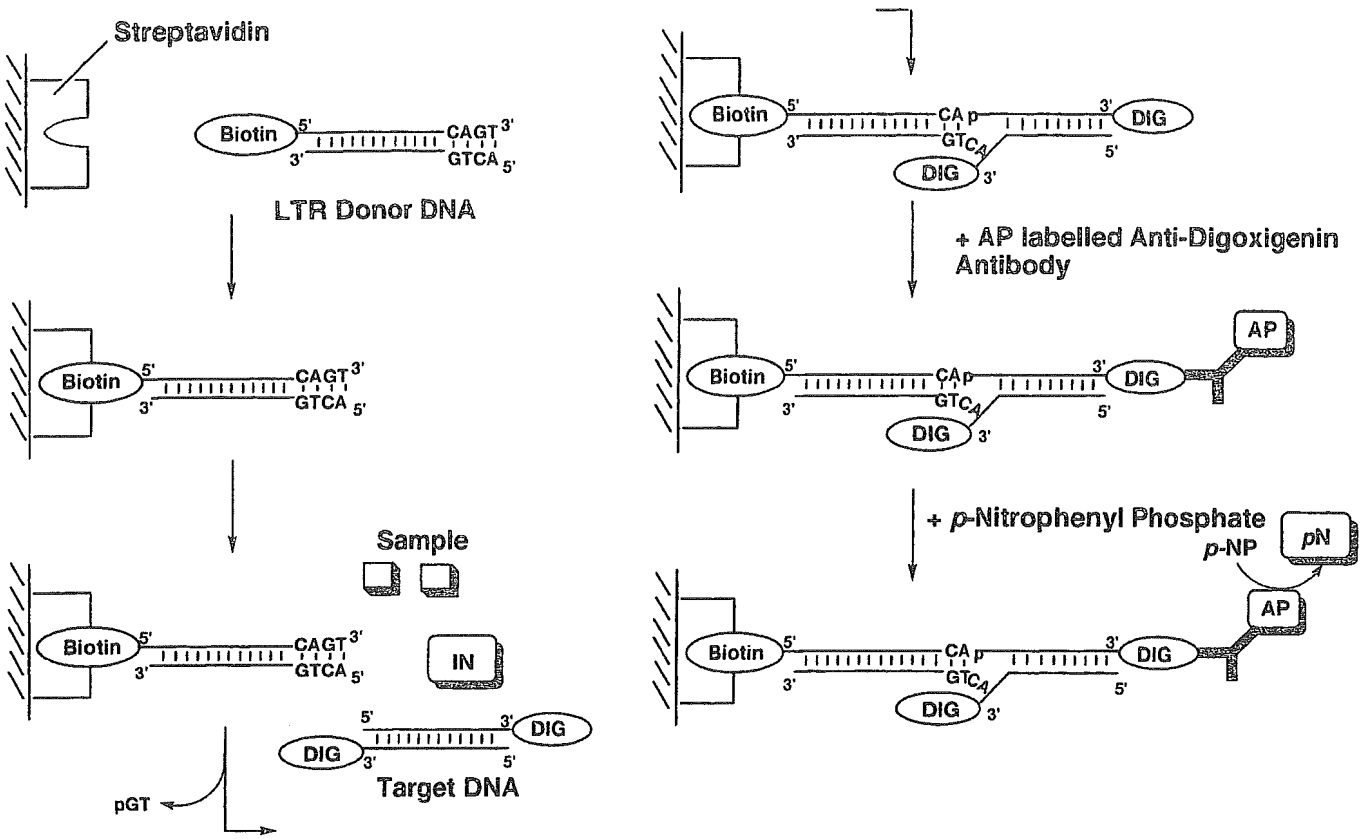

Figure 1. Schematic diagram of the multiplate integration assay (MIA) IN, integrase; DIG, digoxigenin; AP, alkaline phosphatase; *p*-NP, *p*-nitrophenyl phosphate; *p*N, *p*-nitrophenol

concentration (IC_{100}) of the test sample required to prevent HIV-1 induced cytopathic effect (CPE) completely (Harada *et al.*, 1985; Otake *et al.*, 1995) was examined through an optical microscope and the cell growth was visualized to give a cytotoxic concentration (CC_0) that reduces the viability of MT-4 cells. AZT and dextran sulfate 8,000 (DS 8,000) were used as an HIV-1 inhibitory control. IC and CC values of AZT were 0.0039 and >1 μg/mL, while those of DS 8,000 were 1.95 and >1,000 μg/mL, respectively.

RESULTS AND DISCUSSION

Of 100 ethanolic and water extracts of 50 Thai plants, it was showed that 11 extracts of 9 plants exhibited sig-nificant inhibitory activities on HIV-1 IN and HIV-1-induced cytopathic effects at non-cytotoxic concentrations on MT-4 cells, which were designated as IC_{50} and IC_{100} values, respectively (Table 1). The plants that showed high HIV-1 IN inhibitory activity were the water extracts of the fruits of *Cassia fistula* (IC_{50}: 2.8 μg/mL), the aerial parts of *Coleus parvifolius* (IC_{50}: 2.9 μg/mL), and the leaves of *Combretum quadrangulare* (IC_{50}: 2.9 μg/mL), *Ocimum basilicum* (IC_{50}: 6.0 μg/mL), *O. canum* (IC_{50}: 1.6 μg/mL), *Plumbago indica* (IC_{50}: 2.9 μg/mL) and *Psidium guajava* (IC_{50}: 1.7 μg/mL). The ethanol extracts that showed high inhibitory effects on HIV-1 IN were the aerial parts of *C. parvifolius* (IC_{50}: 9.2 μg/mL), the leaves of *C. quadrangulare* (IC_{50}: 2.5 μg/mL), *Croton sublyratus* (IC_{50}: 3.0 μg/mL) and *Thevetia peruviana* (IC_{50}: 12.0 μg/mL).

In terms of inhibitory effect on HIV-1 replication, the following plants also exhibited high activity; the water extracts of the fruits of *Cassia fistula* (IC_{100}: 125 μg/mL), the aerial parts of *C. parvifolius* (IC_{100}: 250 μg/mL), and the leaves of *C. quadrangulare* (IC_{100}: 62.5 μg/mL), *O. basilicum* (IC_{100}: 500 μg/mL), *O. canum* (IC_{100}: 250 μg/mL), *P. indica* (IC_{100}: 125 μg/mL) and *P. guajava* (IC_{100}: 125 μg/mL). The ethanol extracts that showed high inhibitory effects on HIV-1 replication were the aerial parts of *C. parvifolius* (IC_{100}: 25 μg/mL), and the leaves of *C. quadrangulare* (IC_{100}: 12.5 μg/mL), *C. sublyratus* (IC_{100}: 3.13 μg/mL) and *T. peruviana* (IC_{100}: 1.56 μg/mL). The CC values of the above extracts ranged from 3.13 to >1,000 μg/mL. Of these extracts, the ethanol extract of *C. parvifolius* was selected for the isolation of active substances as potential anti-AIDS agents, since this plant extract possessed appreciable HIV-1 IN and HIV-1 inhibitory activities with low cytotoxicity (CC: 100 μg/mL). *Coleus parvifolius* is one of the plants in the family Labiatae. Preparations of *Coleus* species have long been used in Hindu and Ayurvedic traditional medicine, particularly for the treatment of heart disease, abdominal pain and convulsions (Evans, 1989). However, until now, there have been no reports on the chemical constituents or bioactivity of *C. parvifolius*.

Of six compounds isolated from the water-soluble fraction of *C. parvifolius*, rosmarinic acid (6), luteolin (2) and luteolin 7-methyl ether (3) showed potent HIV-1 IN inhibitory activities with IC_{50} values of 5.0, 11.0 and 11.0 μM, respectively, while luteolin 7-*O*-β-D-glucuronide (4), luteolin 5-*O*-β-D-glucoside (1) and 5-*O*-β-D-glucopyranosyl-luteolin 7-methyl ether (5) exhibited moderate activities with IC_{50} values of 20, 58 and 70 μM,

Table 1. HIV-1 Integrase inhibitory activity, antiviral activity and cytotoxicity of Thai plant extracts

Botanical name	Accession number	Family	Part used	Extract	IN	Anti-HIV	
					IC_{50}[a] (μg/ml)	IC_{100}[b] (μg/ml)	CC[c] (μg/ml)
Acacia consinna DC.	SKP.1150103	Mimosaceae	Leaf	Ethanol	22.0 ± 5.0	NE[d]	100
				Water	3.8 ± 0.4	NE	>1000
Adhatoda vasica Nees	SKP.0010122	Acanthaceae	Leaf	Ethanol	12.0 ± 2.1	NE	100
Alpinia galanga Sw.	SKP.2060107	Zingiberaceae	Rhizome	Water	18.0 ± 8.5	NE	1000
Alyxia nitens Kerr	SKP.0130114	Apocynaceae	Leaf	Ethanol	30.0 ± 6.2	NE	100
Andrographis paniculata Wall. ex. Nees	SKP.0010116	Acanthaceae	Leaf	Ethanol	12.0 ± 2.9	NE	12.5
				Water	1.5 ± 0.3	NE	1000
Baleria lupulina Lindl.	SKP.0010212	Acanthaceae	Leaf	Ethanol	10.0 ± 2.0	NE	100
				Water	10.0 ± 1.8	NE	>1000
Bixa orellana L.	SKP.0260215	Bixaceae	Leaf	Ethanol	2.2 ± 0.4	NE	25
				Water	0.7 ± 0.1	NE	>1000
Bixa orellana L.	SKP.0260215	Bixaceae	Seed	Ethanol	3.0 ± 0.6	NE	100
				Water	0.3 ± 0.1	NE	125
Calophyllum inophyllum L.	SKP.0830309	Guttiferae	Leaf	Ethanol	4.5 ± 0.8	NE	100
				Water	4.0 ± 0.5	NE	1000
Capsicum frutescens L.	SKP.1800306	Solanaceae	Fruit	Ethanol	33.0 ± 4.9	NE	>100
Cassia angustifolia Vahl	SKP.0340301	Caesalpiniaceae	Leaf	Ethanol	4.9 ± 1.4	NE	100
Cassia fistula L.	SKP.0340306	Caesalpiniaceae	Fruit	Ethanol	10.0 ± 2.0	NE	>100
				Water	2.8 ± 0.5	125	>1000
Clinacanthus nutans Lindau	SKP.0010314	Acanthaceae	Leaf	Ethanol	2.8 ± 0.2	NE	100
				Water	2.5 ± 0.3	NE	>1000
Coleus parvifolius Benth.	SKP.0950316	Labiatae	Aerial parts	Ethanol	9.2 ± 2.9	25	100
				Water	2.9 ± 0.6	250	500
Combretum quadrangulare Kurz	SKP.0490317	Combretaceae	Leaf	Ethanol	2.5 ± 0.2	12.5	25
				Water	2.9 ± 0.6	62.5	125
Croton sublyratus Kurz	SKP.0710319	Euphorbiaceae	Leaf	Ethanol	3.0 ± 0.4	3.13	6.25
				Water	12.0 ± 2.3	NE	250
Curcuma longa L.	SKP.2060312	Zingiberaceae	Rhizome	Ethanol	80.0 ± 20.5	NE	31.3
				Water	18.0 ± 3.4	NE	>1000
Curcuma zedoaria Roscoe	SKP.2060326	Zingiberaceae	Rhizome	Ethanol	62.0 ± 16.7	NE	5
				Water	50.0 ± 12.5	NE	>1000
Derris scandens Benth.	SKP.1410419	Papilionaceae	Leaf	Ethanol	3.9 ± 1.2	NE	100
				Water	13.0 ± 1.7	NE	500
Garcinia atroviridis Griff.	SKP.0830701	Guttiferae	Fruit	Water	26.0 ± 3.7	NE	>1000
Glycyrrhiza glabra L.	SKP.1410707	Papilionaceae	Root	Water	16.0 ± 2.5	NE	500
Hibiscus sabdariffa L.	SKP.1090819	Malvaceae	Flower	Water	1.4 ± 0.2	NE	> 1000
Kaempferia galanga L.	SKP.2061107	Zingiberaceae	Rhizome	Ethanol	42.0 ± 5.9	NE	125
				Water	30.0 ± 4.9	NE	>1000
Lawsonia inermis L.	SKP.1061209	Lythraceae	Leaf	Ethanol	2.1 ± 0.4	NE	25
				Water	3.3 ± 0.4	NE	250
Morinda citrifolia L.	SKP.1651303	Rubiaceae	Leaf	Ethanol	1.2 ± 0.3	NE	>100
				Water	6.0 ± 1.2	NE	>1000
Myristica fragrans L.	SKP.1211306	Myristicaceae	Leaf	Ethanol	3.0 ± 0.4	NE	50
				Water	2.3 ± 0.3	NE	250
Ocimum basilicum L.	SKP.0951502	Labiatae	Leaf	Ethanol	14.0 ± 2.1	NE	>100
				Water	6.0 ± 2.0	500	1000
Ocimum canum Sims	SKP.0951503	Labiatae	Leaf	Ethanol	12.0 ± 1.2	NE	50
				Water	1.6 ± 0.3	250	Water
Piper betle L.	SKP.1461602	Piperaceae	Leaf	Ethanol	4.0 ± 0.4	NE	3.13
				Water	34.0 ± 5.7	NE	250
Piper chaba Vahl.	SKP.1461603	Piperaceae	Fruit	Ethanol	16.0 ± 2.6	NE	100
Piper nigrum L.	SKP.1461614	Piperaceae	Fruit	Ethanol	70.0 ± 10.3	NE	50
				Water	8.0 ± 1.2	NE	>1000
Piper ribesoides Wall. (A*)	SKP.1461618	Piperaceae	Stem	Ethanol	42.0 ± 7.4	NE	15.6
				Water	0.9 ± 0.2	NE	125
Piper ribesoides Wall. (A*)	SKP.1461618	Piperaceae	Leaf	Ethanol	0.6 ± 0.3	NE	15.6
				Water	0.5 ± 0.1	NE	>1000
Piper ribesoides Wall. (B**)	SKP.1461618	Piperaceae	Stem	Ethanol	19.0 ± 3.4	NE	125
				Water	0.4 ± 0.2	NE	62.5
Piper ribesoides Wall. (B**)	SKP.1461618	Piperaceae	Leaf	Ethanol	0.1 ± 0.2	NE	62.5
				Water	4.1 ± 0.5	NE	1000
Piper sarmentosum Roxb.	SKP.1461619	Piperaceae	Leaf	Ethanol	1.2 ± 0.4	NE	>100
				Water	39.0 ± 8.2	NE	1000
Plumbago indica L.	SKP.1481609	Plumbaginaceae	Leaf	Ethanol	6.0 ± 1.2	NE	50
				Water	2.9 ± 0.4	125	500

Table 1. (Continued)

Botanical name	Accession number	Family	Part used	Extract	IN	Anti-HIV	
					IC_{50}[a] (μg/ml)	IC_{100}[b] (μg/ml)	CC[c] (μg/ml)
Plumbago indica L.	SKP.1481609	Plumbaginaceae	Root	Ethanol	29.0 ± 7.4	NE	50
				Water	18.0 ± 3.4	NE	1000
Psidium guajava L.	SKP.1231607	Myrtaceae	Leaf	Ethanol	2.5 ± 0.5	NE	25
				Water	1.7 ± 0.3	125	250
Quisqualis indica L.	SKP.0491709	Combretaceae	Leaf	Ethanol	2.0 ± 0.2	NE	>100
				Water	1.2 ± 0.2	NE	1000
Rhinacanthus nasutus Kurz	SKP.0011814	Acanthaceae	Leaf	Ethanol	0.8 ± 0.1	NE	62.5
				Water	0.7 ± 0.1	NE	1000
Terminalia citrina Roxb. ex. Flemming	SKP.0492003	Combretaceae	Fruit	Ethanol	2.7 ± 0.5	NE	62.5
				Water	0.3 ± 0.1	NE	62.5
Theobroma cacao L.	SKP.1832003	Sterculiaceae	Leaf	Ethanol	8.0 ± 1.0	NE	100
				Water	2.5 ± 0.6	NE	250
Thevetia peruviana Schum.	SKP.0132016	Apocynaceae	Leaf	Ethanol	12.0 ± 2.3	1.56	3.13
				Water	8.8 ± 1.0	NE	62.5
Thunbergia laurifolia L.	SKP.1922012	Thunbergiaceae	Leaf	Ethanol	3.0 ± 0.4	NE	>100
				Water	2.8 ± 0.3	NE	500
Tribulus terristris L.	SKP.2072020	Zygophyllaceae	Aerial parts	Ethanol	8.0 ± 1.4	NE	50
				Water	16.0 ± 4.5	NE	1000
Trichosanthes anguina L.	SKP.0582001	Cucurbitaceae	Fruit	Ethanol	16.0 ± 3.3	NE	62.5
Zingiber cassumunar Roxb.	SKP.2062603	Zingiberaceae	Rhizome	Water	23.0 ± 3.8	NE	>1000
Zingiber officinale Roscoe	SKP.2062615	Zingiberaceae	Rhizome	Ethanol	4.0 ± 0.8	NE	50
				Water	1.8 ± 0.3	NE	1000
Zingiber zerumbet Smith	SKP.2062626	Zingiberaceae	Rhizome	Water	2.8 ± 0.4	NE	>1000

The results are the mean ± SD (n = 4). The following plant extracts were inactive against HIV-1 IN (IC_{50} > 100 μM); the water extracts of *A. vasica, A. nitens, C. frutescens, C. angustifolia, P. chaba, T. anguina*; and the ethanol extracts of *A. galanga, G. atroviridis, G. glabra, H. sabdariffa, Z. cassumunar* and *Z. zerumbet.*

*A = lanceolate shaped leaf and ** B = cordate shaped leaf, [a] IC_{50} = 50% inhibitory concentration on HIV-1 Integrase, [b] IC_{100} = the minimum concentration for complete inhibition of HIV-1 induced CPE in MT-4 cells by microscopic observation, [c] CC = the minimum concentration for appearance of MT-4 cell toxicity by microscopic observation. [d] NE = not effective.

respectively. Rosmarinic acid methyl ester (**7**) which possessed an IC_{50} value of 3.1 μM, was obtained from the EtOAc fraction, together with **2** and **6**. Daucosterol (**8**) from the $CHCl_3$ fraction, and α- and β-amyrin (**9**, **10**) from the hexane fraction were inactive against HIV-1 IN (IC_{50} > 100 μM) (Fig. 2 and Table 2). Suramin was used as a positive control which exhibited a HIV-1 IN inhibitory activity with an IC_{50} value of 2.4 μM.

Rosmarinic acid methyl ester (**7**) was the most potent compound (IC_{50} = 3.1 μM), followed by rosmarinic acid (**6**, IC_{50} = 5.0 μM), luteolin (**2**, IC_{50} = 11.0 μM), luteolin 7-methyl ether (**3**, IC_{50} = 11.0 μM), luteolin 7-*O*-β-D-glucuronide (**4**, IC_{50} = 20 μM), luteolin 5-*O*-β-D-glucopyranoside (**1**, IC_{50} = 58 μM) and 5-*O*-β-D-glucopyranosyl-luteolin 7-methyl ether (**5**, IC_{50} = 70 μM). Substitution with a methyl group (**7**, rosmarinic acid methyl ester) slightly increased the activity (by 1.5 times), when compared with rosmarinic acid (**6**). Of the tested flavonoids, luteolin (**2**) showed the highest activity. Substitution with a glucosyl group at C-5 (**1**) resulted in a dramatic decrease of activity, more than that observed with a glucuronide residue (**4**) at C-7. When substituted at these two positions, the activity significantly reduced, as shown for 5-*O*-β-D-glucopyranosyl-luteolin 7 methyl ether (**5**). Thus, this may imply that hydroxy groups at C-5 and C-7 are required for this type of activity and a hydroxy group at C-5 seems to be more important than that at C-7 within this compound class.

Table 2. Inhibitory activity of compounds isolated from *C. parvifolius* against HIV-1 IN.

Compound	IC_{50} (μM)
Luteolin 5-*O*-β-D-glucopyranoside (**1**)	58.0 ± 8.2
Luteolin (**2**)	11.0 ± 0.8
Luteolin 7-methyl ether (**3**)	11.0 ± 1.5
Luteolin 7-*O*-β-D-glucuronide (**4**)	20.0 ± 0.7
5-*O*-β-D-Glucopyranosyl-luteolin 7-methyl ether (**5**)	70.0 ± 6.4
Rosmarinic acid (**6**)	5.0 ± 0.9
Rosmarinic acid methyl ester (**7**)	3.1 ± 0.8
Daucosterol (**8**)	>100
α-Amyrin (**9**)	>100
β-Amyrin (**10**)	>100
Phytol (**11**)	>100
Suramin (positive control)	2.4 ± 0.3

The results are the mean ± SD (*n* = 4).

Since rosmarinic acid (**6**) and rosmarinic acid methyl ester (**7**) showed high anti-HIV-1 IN activity among the isolated compounds from *C. parviflolius*, rosmarinic acid derivatives which are dimers, trimers and tetramers, together with their metal-binding derivatives were also investigated for inhibitory effects on HIV-1 IN. Of the tested compounds (Fig. 3 and Table 3), magnesium lithospermate (**15**), a magnesium salt of a trimer of caffeic acid, was the most potent HIV-1 IN inhibitor, followed by calcium rosmarinate (**12**), magnesium rosmarinate (**13**), lithospermic acid B (**16**), lithospermic acid (**14**), rosmarinic acid methyl ester (**7**) and rosmarinic acid (**6**) with IC_{50} values of 0.7, 0.8, 1.0, 1.0, 1.4,

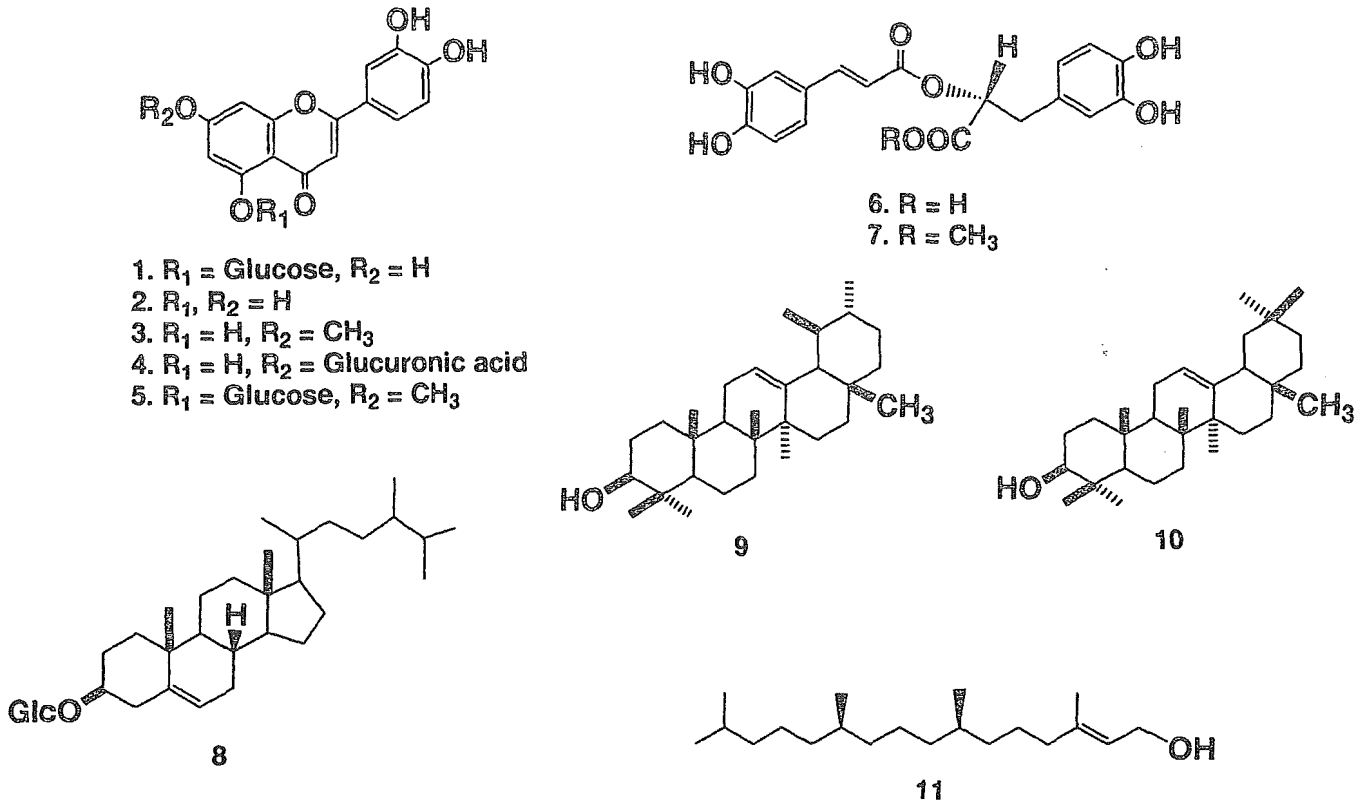

Figure 2. Chemical constituents of *C. parvifolius*

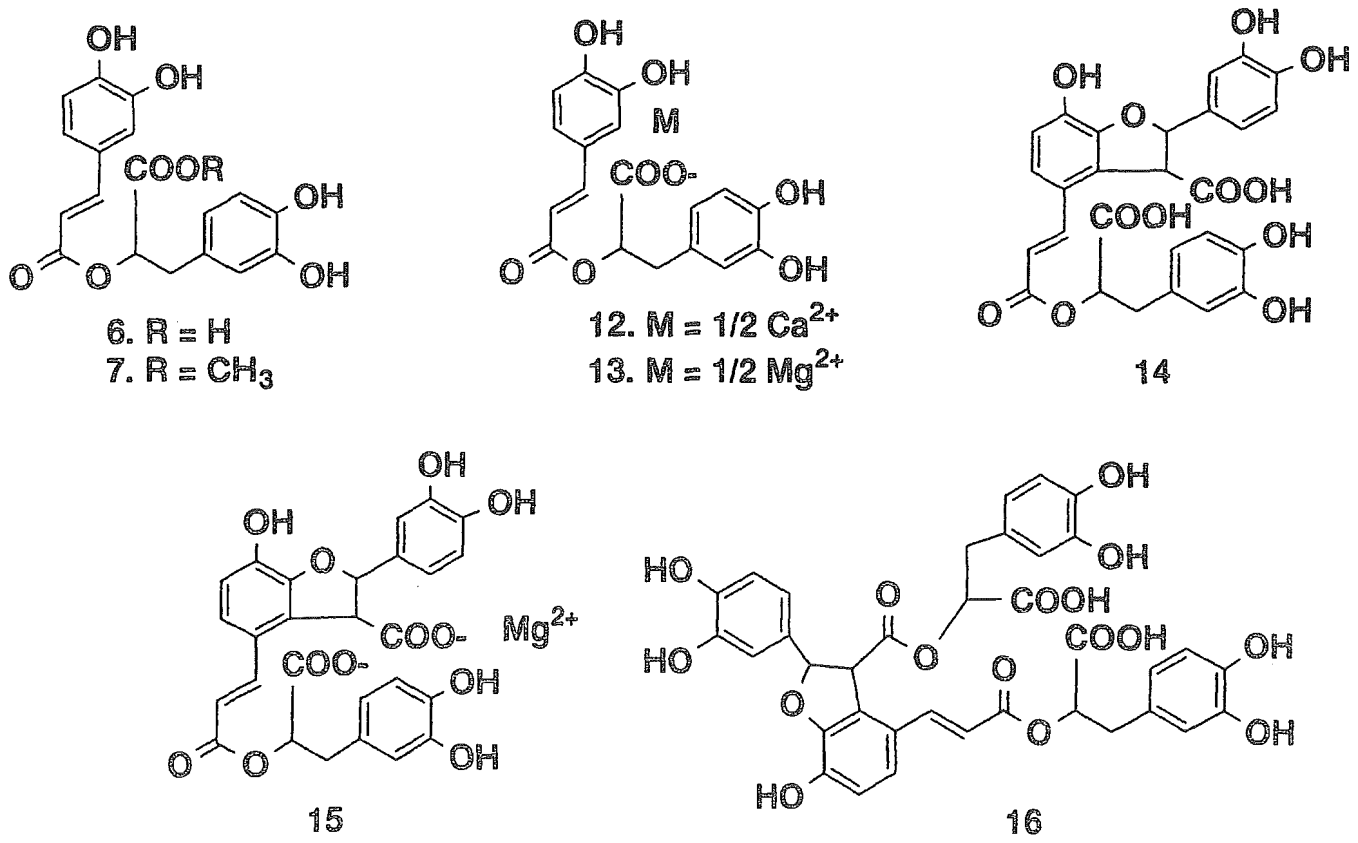

Figure 3. Rosmarinic acid and related compounds

3.1 and 5.0 μM, respectively. Robinson *et al.* (1996) reported that caffeic acid was inactive against HIV-1 IN (IC_{50} > 100 μM). Therefore, it may be concluded that HIV-1 IN inhibitory effects of rosmarinic acid derivatives increase in order of monomers, dimers, trimers and tetramers, and that the metal-chelated derivatives are more potent than the non-binding ones. Lim *et al.* (1997) have reported the HIV-1 RT inhibitory effect of

Table 3. Inhibitory activity of rosmarinic acid and its related compounds on HIV-1 IN

Compound	IC_{50} (μM)
Dimers	
Rosmarinic acid (**6**)	5.0 ± 0.9
Rosmarinic acid methyl ester (**7**)	3.1 ± 0.8
Calcium rosmarinate (**12**)	0.8 ± 0.3
Magnesium rosmarinate (**13**)	1.0 ± 0.2
Trimers	
Lithospermic acid (**14**)	1.4 ± 0.2
Magnesium lithospermate (**15**)	0.7 ± 0.1
Tetramer	
Lithospermic acid B (**16**)	1.0 ± 0.2

The results are the mean ± SD (n = 4).

caffeic acid and its derivatives isolated from *Cordia spinescens*. Their results showed that RT inhibitory activity increased in order of monomers, dimers, and trimers, while lithospermate B, a tetramer, showed low activity. The mode of inhibition on RT by calcium rosmarinate, magnesium rosmarinate and magnesium lithospermate was determined to be non-competitive with respect to methyl-^{3}H-thymidine 5′-triphosphate (dTTP) (Lim *et al.*, 1997).

In terms of the bioactivities of constituents of *C. parvifolius*, rosmarinic acid has been reported to show antiviral (Mazumder *et al.*, 1997; Arda *et al.*, 1997), antithyrotropic (Auf'mkolk *et al.*, 1985), anti-inflammatory (Englberger *et al.*, 1988), antibacterial (Kuhnt *et al.*, 1995) and antioxidative activities (Lamaison *et al.*, 1990). Luteolin, a flavonoid, was reported to exhibit a smooth muscle relaxant property (Sanchez *et al*, 1996), an immunomodulatory effect (Liang *et al.*, 1997), and anti-inflammatory (Pieroni and Pachaly, 2000), anti-tumor (Makino *et al.*, 1998) and antiviral activities (Fesen *et al.*, 1994). Of the constituents of *C. parvifolius* obtained in the present investigation, it was shown that rosmarinic acid (**6**), rosmarinic acid methyl ester (**7**), together with luteolin (**2**) and luteolin 7-methyl ether (**3**), were the most active components against HIV-1 IN. Lithospermate B, a tetramer of caffeic acid, was reported to show antihepatotoxic (Hase *et al.*, 1996), anti-HIV-1 RT (Lim *et al.*, 1997), anti-aldose reductase (Kasimu *et al.*, 1998), antioxidative (Chen *et al.*, 1999), and antihypertensive activities (Kamata *et al.*, 1994). However, this compound was not found in the aerial parts of *C. parvifolius* in our study.

Recently, there have been many reports on HIV-1 IN inhibitory assays using isotope-labelled substrates and denaturing gel separation of reaction products (Fesen *et al.*, 1994; Burke *et al.*, 1992; Mazumder *et al.*, 1996; Mazumder *et al.*, 1997; Neamati *et al.*, 1997; Reddy *et al.*, 1999). These *in vitro* methods are referred as standard integration assays and give clear results. However, they are inconvenient and time consuming, especially when screening inhibitors from many samples. Recently, an assay for HIV-1 IN activity using DNA-coated plates has been described (Hazuda *et al.*, 1994; Vink *et al.*, 1994; Chang *et al.*, 1996). It is a non-radioisotopic technique and can be used for screening the inhibitory activity of plant extracts or any compounds against HIV-1 IN. In this case, the 96 well-plates were used for the screening test. It is simple, convenient and accurate and does not require centrifugation, electrophoretic or DNA denaturation steps. This assay screens for both 3′-processing and 3′-strand transfer and can be used without any exposure to radioisotopes. We therefore used this assay method for screening for HIV-1 IN inhibitory substances in the present study.

The screening of medicinal plants for HIV-1 IN inhibitory activity represents a promising approach to discover compounds that interfere with retroviral replication. Until now, many available antiviral agents as HIV-1 PR inhibitors have become available such as saquinavir (SQV), nelfinavir (NFV) and amprenavir (APV), while HIV-1 RT inhibitors include zidovudine (AZT), didanosine (DDI) and abacavir (1592U89) (Hirsch *et al.*, 1998). However, no HIV-1 IN inhibitors have yet been developed as anti-HIV drugs. Therefore, searching for HIV-1 IN inhibitory substances from medicinal plants, is promising in this regard, and the multiplate integration assay developed by our group seems to be an appropriate method for screening a large number of samples for HIV-1 IN inhibitory activity.

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Anti-HIV-1 activity and mode of action of mirror image oligodeoxynucleotide analogue of zintevir

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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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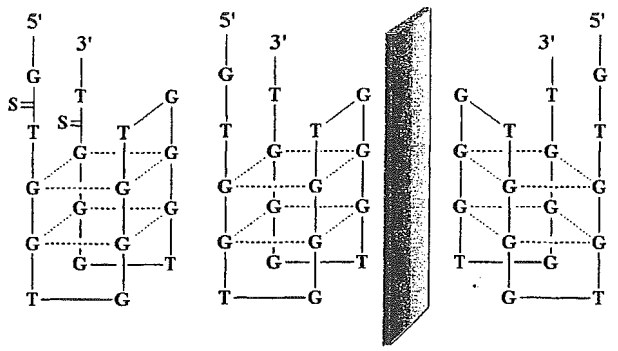

Fig. 1. Structures of Zintevir, D-17mer, and L-17mer.

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.

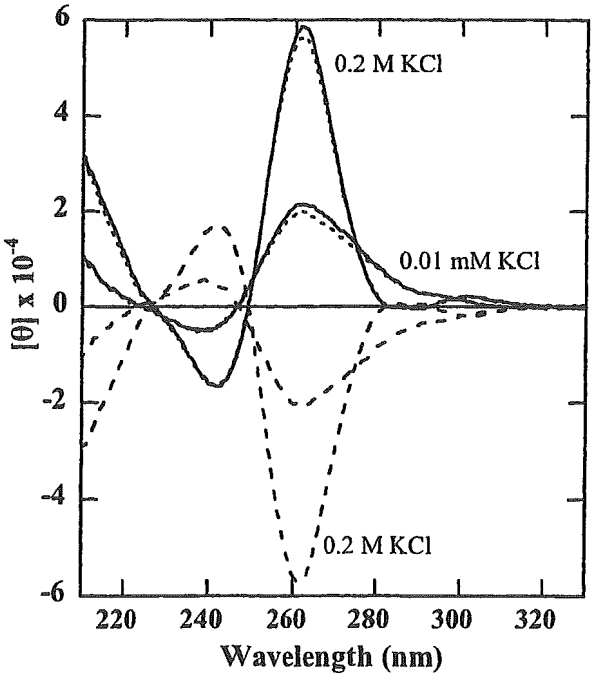

Fig. 2. CD spectra of Zintevir (solid lines), D-17mer (dotted lines), and L-17mer (broken lines) in 20 mM lithium phosphate (pH 7.0) containing 0.2 M or 0.01 mM KCl at 25 °C.

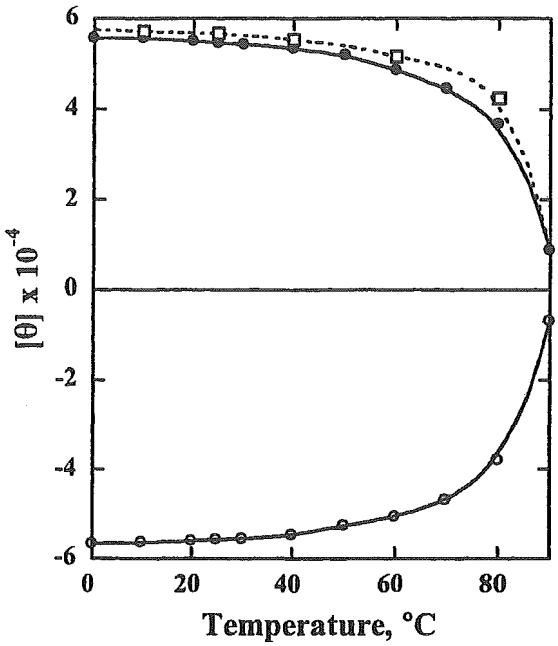

Fig. 3. Temperature dependence of molar ellipticity at 260 nm of Zintevir (open square), D-17mer (closed circle), and L-17mer (open circle) in 20 mM lithium phosphate (pH 7.0) containing 0.2 M KCl.

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

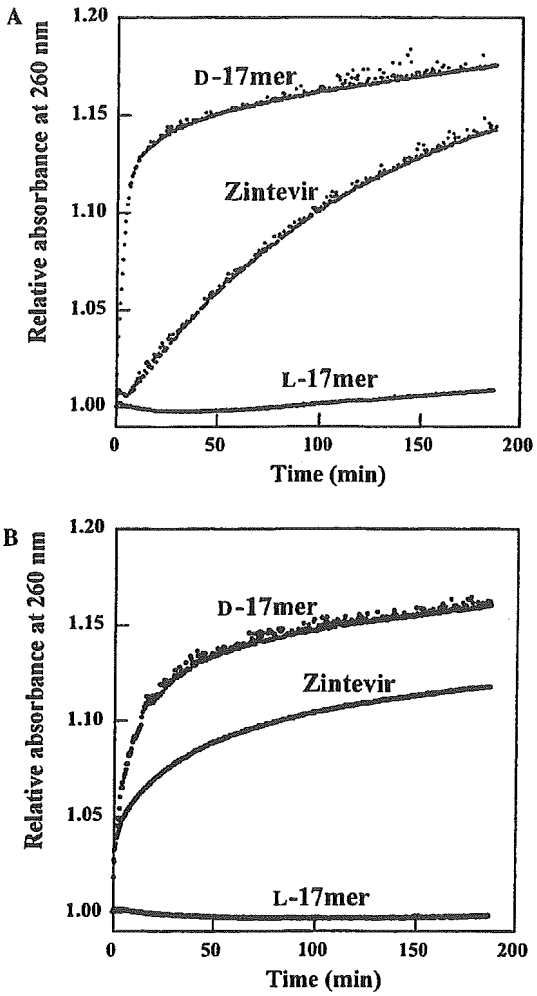

Fig. 4. Resistance of the 17mers against SVPD (A) and nuclease P1 (B). Reactions started by adding enzyme into the buffered solution of each 17mer at 37 °C. Hyperchromicity at 260 nm induced by degradation of an oligonucleotide is revealed periodically.

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

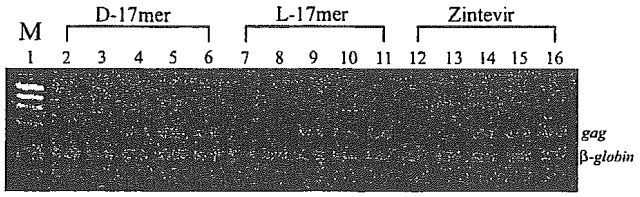

Fig. 5. Time-of-addition experiments for the 17mers. HIV-1 was adsorbed onto MT-4 cells on ice and the mixture was allowed for 1 h at this temperature in the presence or absence of each 17mer (adsorption). After free virus was washed out with ice-cold buffer, incubation was started at 37 °C and the 17mers 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.

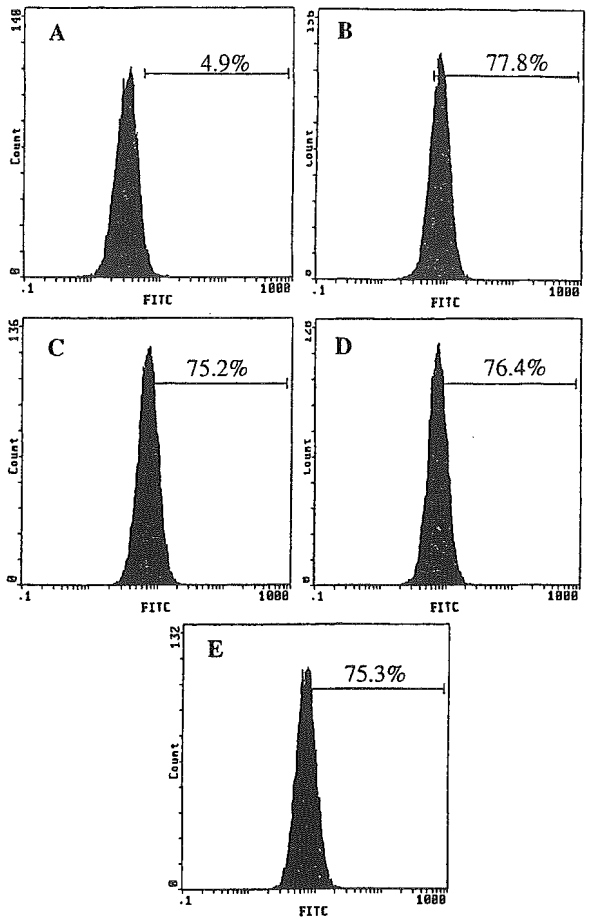

Fig. 6. Flow cytometric histograms of the binding of anti-CD4 mAb (Leu3a) on MT-4 cells: (A) fluorescence of MT-4 cells incubated only with FITC-labeled anti-mouse IgG1 antibody; (B) fluorescence of MT-4 cells incubated with anti-CD4 mAb (Leu3a), then with FITC-labeled anti-mouse IgG1 antibody; (C,D,E)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

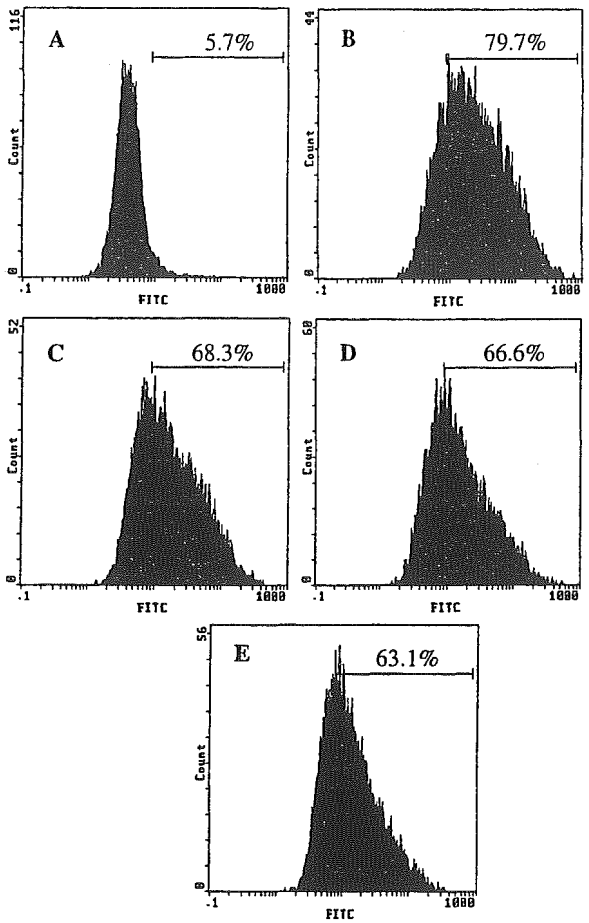

Fig. 7. Flow cytometric histograms of the binding of anti-gp120 mAb (0.5β) on Molt-4 cells persistently infected with HIV-1_{LAI}: (A) fluorescence of Molt-4 cells incubated only with FITC-labeled anti-mouse IgG1 antibody; (B) fluorescence of Molt-4 cells incubated with anti-gp120mAb (0.5β), then with 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

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Sequence Note

Genetic Diversity of HIV Type 1 in Likasi, Southeast of the Democratic Republic of Congo

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ABSTRACT

To investigate the prevalence of subtypes A and C, and the existence of recombinants of both subtypes in the southeast of the Democratic Republic of Congo (DRC), blood samples were collected from 27 HIV-infected individuals in Likasi, located in an area bordering close to Zambia, and analyzed phylogenetically. Out of the 24 strains with a positive PCR profile for *pol*-IN and *env*-C2V3, 15 (62.5%) had a discordant subtype or CRF designation: one subtype A/G (*pol/env*), four A/U (unclassified), three G/A, one G/CRF01, three H/A, one J/C, one CRF02 (G)/A, and one U/A. Nine (37.5%) strains had a concordant subtype or CRF designation: five subtype A, two C, one D, and one CRF02/G. The remaining three samples negative for PCR with *env*-C2V3 primers used in this study were further analyzed with *env*-gp41 primers and revealed the presence of two profiles: two J/J (*pol*-IN/*env*-gp41) and one C/G. These data highlight the presence of a high proportion (16/27, 59.3%) of recombinant strains and a low prevalence (4.1 and 7.4%) of subtype C based on *env*-C2V3 and *pol*-IN analyses, respectively, in Likasi. In addition, this is the first report that CRF02_AG exists in DRC, though the epidemiological significance of the existence of CRF02_AG in DRC remains unknown.

HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1) has been classified into three major phylogenetic groups, termed M (major), N (non-M, non-O), and O (outlier).[1] The vast majority of variants found worldwide and responsible for the AIDS pandemic belong to group M.[2,3] Phylogenetic analysis of group M has further led to its subclassification into nine pure subtypes. A–D, F–H, J, and K and subsubtypes A1, A2, F1, and F2.[2] Recently, it was realized that a significant fraction of HIV-1 isolates, 10–40% or more, exhibit a shift in subtypes when different regions of their genome are analyzed.[3] Currently, some of the mosaic HIV-1 genomes play a major role in the global AIDS epidemics and are designated as circulating recombinant forms (CRFs), CRF01-CRF16. Although subtypes A, C, and CRF02_AG are most prevalent in Africa, the distribution of CRF/subtype is very heterogeneous.[2–7] The proportion of CRF02_AG among subtype A strains based on *env* sequences decreases from west to central Africa, with an absence of CRF02_AG in the Democratic Republic of Congo (DRC).[2,8,9] The profile of HIV-1 endemic in DRC, such as high number of cocirculating HIV-1 subtypes, possible recombinant viruses, and unclassified strains, is consistent with that of an old and mature epidemic of HIV-1.[5]

The DRC is bordered on the southeast by Zambia. The majority (95%) of the HIV-1 strains circulating in Zambia are subtype C, although HIV-1 group M subtypes A, D, G, and J as well as group O have been identified.[2,3,10,11]

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The purpose of this study was to investigate the prevalence of subtypes A and C, and the existence of the recombinants of both subtypes in Likasi, located in the southeast of the DRC, 200 km from Lubumbashi in an area bordering Zambia.

Blood samples (10 ml) were collected from 27 HIV-1 infected individuals in February and September 2001. Plasma and buffycoat were separated and stored at −80°C until use. The plasma samples were screened for HIV antibodies with a commercial particle agglutination (PA) test kit (Serodia-HIV, Fujirebio, Tokyo, Japan).

Genomic DNA was extracted from buffycoat of the seroreactive samples using a Qiagen DNA extraction kit (Qiagen, Hilden, Germany). A part of the *pol* gene coding integrase (IN) (corresponding to 4493–4780 nt in HIV-1_{HXB2}) and *env* gene covering C2V3 (corresponding to 6975–7520 nt in HIV-1_{HXB2}) was amplified with nested polymerase chain reaction (PCR) using the primers unipol 5 (5′-TGGGTACCAGCACACAAAGGAATAGGAGGAAA-3′)/unipol 6 (5′-CCACAGCTGATCTCTGGCCTTCTCTGTAATAGACC-3′) and M5 (5′-CCAATTCCCATACATTATTGTGCCCCAGCTGG-3′)/M10 (5′-CCAATTGTCCCTCATATCTCCTCCTCCAGG-3′), respectively, in the first round, and unipol 1 (5′-AGTGGATTCATAGAAAGCAGAAGT-3′)/unipol 2 (5′-CCCCTATTCCTTCCCCTTCTTTTAAAA-3′), and M3 (5′-GTCAGCACAGTACAATGACACATGG-3′)/M8 (5′-TCCTTGGATGGGAGGGGCATACATTGC-3′), respectively, in the second round.[11] Nested PCR was performed with an AmpliTaq Gold PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Amplification was done with one cycle at 95°C for 10 min, and 35 cycles at 95°C for 30 sec, 45°C for 30 sec (for the *pol* region) or 55°C for 30 sec (for the *env* region), and 72°C for 1 min, with a final extension of 72°C for 10 min. Samples that could not be amplified with the *env*-C2V3 primers were analyzed with *env*-gp41-specific primers.[12] PCR amplification was confirmed by visualization with ethidium bromide staining of the gel. The PCR products were cloned by using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and plasmid DNA for sequencing was prepared by a plasmid miniprep kit (Sigma, Hilden, Germany). DNA sequencing was carried out using dye-deoxy terminator chemistry on an ABI 310 automatic sequencer (Applied Biosystems, Foster City, CA). We sequenced at least 12 clones to obtain a consensus sequence. Sample DNA sequences were aligned with subtype reference sequences from the Los Alamos database by CLUSTAL W (version 1.81) with subsequent inspection and manual modification. The frequency of nucleotide substitution in each base of the sequences was estimated by the Kimura two-parameter method.[13] Phylogenetic trees were constructed by the neighbor-joining method, and its reliability was estimated by 1000 bootstrap replications.[14] All alignments were gap stripped for the generation of trees. The profile of the tree was visualized by Treeview version 1.6.5.

The phylogenetic trees based on *env*-C2V3 and *pol*-integrase sequences were constructed with representative HIV-1 strains of each subtype and CRF as a reference (Fig. 1). Out of the 27 samples from Likasi, 24 (88.9%) were found to be positive for HIV-1 PCR with *pol*-IN and *env*-C2V3 primers. The remaining 3 (11.1%) samples were negative for PCR with the *env*-C2V3 primers used in this study. The phylogenetic tree based on *env*-C2V3 sequences (Fig. 1A) showed that out of the 24 samples 13 were subtype A, three C, one D, two G, one CRF01_AE, and four U (unclassified). The outcome of the phylogenetic analysis of the *pol*-IN gene is shown in Figure 1B, and revealed that 10 were subtype A, three C, one D, four G, three H, three J, two CRF02_AG, and one U. Thus, 15 (62.5%) had a discordant subtype or CRF designation: one subtype A/G (*pol/env*), four A/U, three G/A, one G/CRF01, three H/A, one J/C, and CRF02/A, and one U/A (Tables 1 and 2). Nine (37.5%) strains had a concordant subtype or CRF designation: one subtype A, two C, one D, and one CRF02/G (Tables 1 and 2). In 14 of these 15 strains, subtype A was involved in recombination events, and among the strains with a discordant subtype or CRF designations A/U (*pol/env*) was by far the most common, followed by H/A and G/A recombination. Additional PCR analyses on three samples with a *pol/env* (+/−) profile were carried out with groups M, N, and O primers for *env*-gp41. Phylogenetic analysis based on *env*-gp41 sequences revealed the presence of two profiles: two J/J (*pol*-IN/*env*-gp41) and one C/G (Tables 1 and 2).

In the current study we found that a high proportion (16/27; 59.3%) of HIV-1 strains in Likasi were intersubtype recombinants. This is higher than that reported in other regions of the country (29–44%).[5,8,15] In Lubumbashi, a city on the southeast border of the DRC with Zambia, subtype C was reported to be predominant (51.9%), followed by subtype A (22.1%).[15] Our data highlight a high prevalence of subtype A (37.0% and 52.5%) and low prevalence of subtype C (7.4% and 12.5%) in Likasi based on *env*-C2V3 and *pol*-IN analyses, respectively. The persisting civil war and population displacement from the east on the border with Ruanda and Burundi to the southern area close to Zambia could be linked to the change of HIV-1 distribution in the southeast of the DRC.[11]

Two HIV-1 strains from Likasi (00CD009 and 01CD208) significantly clustered with CRF02_AG reference strains (with 97.4% bootstrap value) (Fig. 1B). This is the first report of CRF02_AG in the DRC, suggesting that CRF02_AG is spreading into Central Africa. CRF02_AG and subtype A represent 70–80% of circulating HIV-1 strains in West and West-Central Africa.[2] However, the epidemiological significance of CRF02_AG in the DRC is yet to be investigated. The high proportion of unclassified strains (16.6%) and intersubtypic recombinants (52.3%) among HIV-1 strains circulating in the DRC indicates an old and mature epidemic of HIV-1 in the DRC.

SEQUENCE DATA

The nucleotide sequences in this study were submitted to GenBank and are available under the following accession numbers: *pol*-IN (288 bp), AY661750-AY661776; *env*-C2V3 (approximately 550 bp), AY675589–AY675612; and *env*-gp41 (405 bp), AY673112–AY673114.

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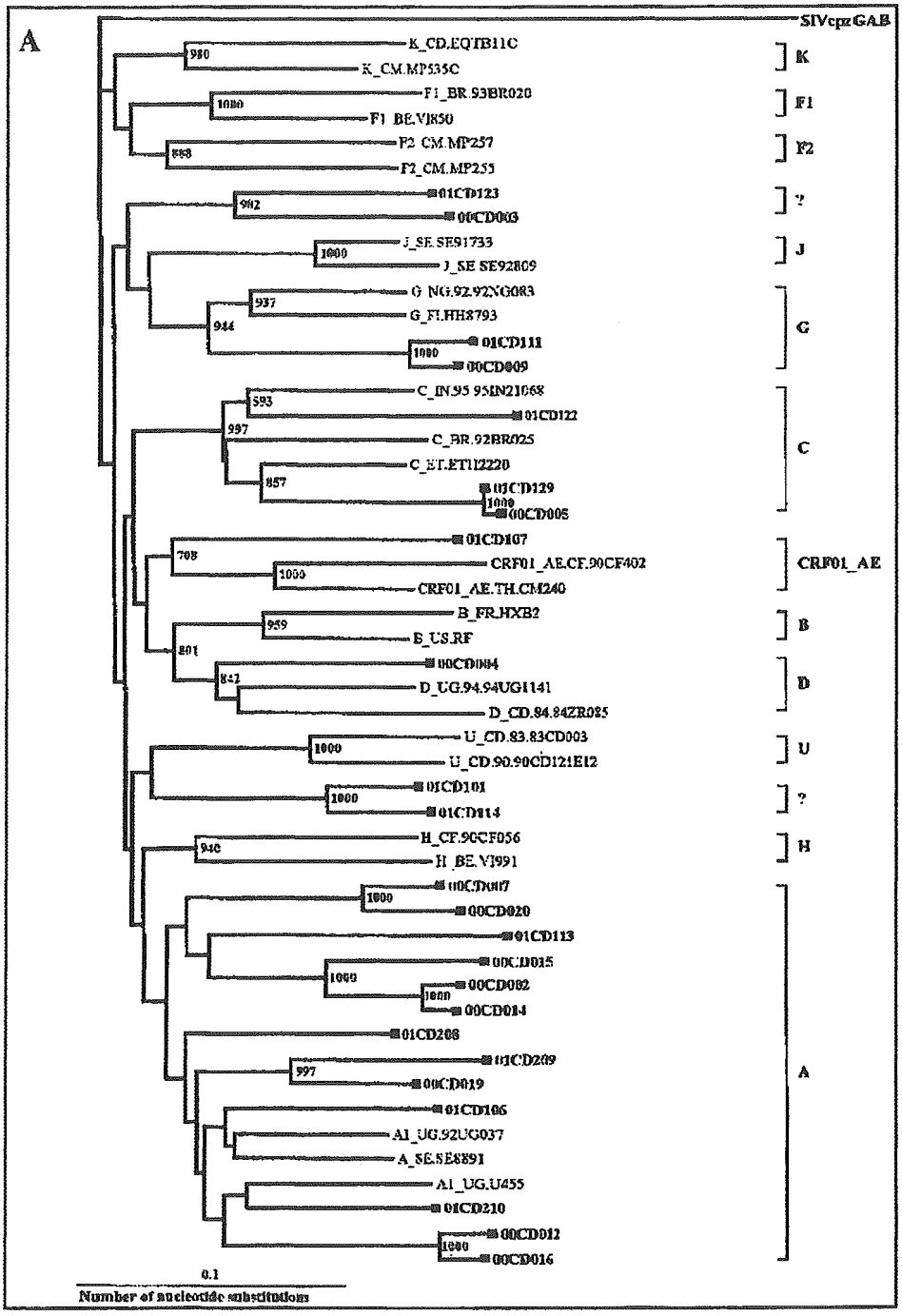

FIG. 1. Phylogenetic trees based on a part of the *env*-C2V3 gene (approximately 550 bp) (A) and *pol*-IN gene (288 bp) (B) of 24 HIV-1 strains from the southeastern Democratic Republic of Congo with reference sequences of representative subtypes/CRF. The bootstrap value at each node represents the number among 1000 bootstrap replicates that supports the branching order. Bootstrap values of 70% or higher are shown. Brackets on the right represent the major group M subtypes. Newly derived sequences (shown in bold) are marked with a filled square (■).

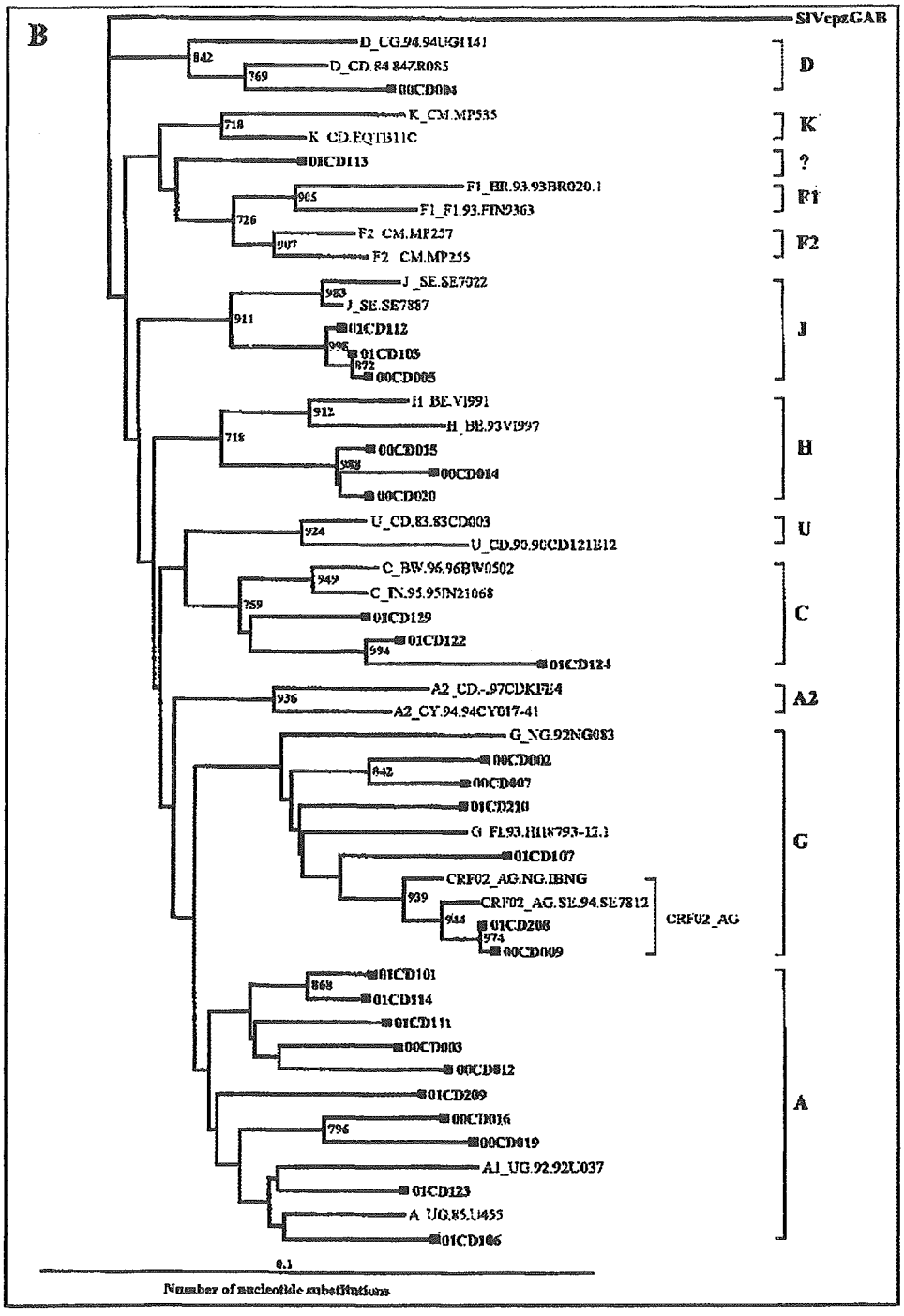

FIG. 1. *Continued*

TABLE 1. SUBTYPES IN *pol*-INTEGRASE AND *env*-(C2V3/ OR -gp41) FOR 27 HIV-1 STRAINS

ID number DRC	pol-*IN (288 bp)*	env-*C2V3 (550 bp)*	env-*gp41 (405 bp)*
00CD002	Subtype G	Subtype A	
00CD003	A	U	
00CD004	D	D	
00CD005	J	C	
00CD007	G	A	
00CD009	CRF02_AG (G)	G	
00CD012	A	A	
00CD014	H	A	
00CD015	H	A	
00CD016	A	A	
00CD019	A	A	
00CD020	H	A	
01CD101	A	A	
01CD103	J	ND[a]	J
01CD106	A	A	
01CD107	G	CRF01_AE (E)	
01CD111	A	G	
01CD112	J	ND	J
01CD113	U	A	
01CD114	A	A	
01CD122	C	C	
01CD123	A	U	
01CD124	C	ND	G
01CD129	C	C	
01CD208	CRF02_AG (G)	A	
01CD209	A	A	
01CD210	G	A	

[a]ND, not detected.

TABLE 2. SUBTYPES IN *pol*-INTEGRASE AND *env*-(C2V3) AND/OR -(gp41) GENE FOR 27 HIV-1 STRAINS

Genotypes	pol-*IN*	env-*C2V3*	n	*Total (%)*
Concordant	A	A	5	
	C	C	2	
	D	D	1	11 (40.7)
	J	J[a]	2	
	CRF02 (G)	G	1	
Discordant	A	U	4	
	A	G	1	
	C	G[a]	1	
	G	A	3	
	G	CRF01 (E)	1	
	H	A	3	16 (59.3)
	J	C	1	
	CRF02 (G)	A	1	
	U	A	1	

[a]*env*-gp41 (approximately 405 bp).

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