

A novel small molecular weight compound with a carbazole structure that demonstrates potent human immunodeficiency virus type-1 integrase inhibitory activity

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The integration of reverse transcribed proviral DNA into a host genome is an essential event in the human immunodeficiency virus type 1 (HIV-1) replication life cycle. Therefore, the viral enzyme integrase (IN), which plays a crucial role in the integration event, has been an attractive target of anti-retroviral drugs. Several IN inhibitory compounds have been reported previously, yet none has been successful in clinical use. To find a new, more successful IN inhibitor, we screened a diverse library of 12 000 small molecular weight compounds randomly by *in vitro* strand-transfer assay. We identified a series of substituted carbazoles that exhibit strand-transfer inhibitory activity at low micromolar concentrations. Of these, the most potent compound exhibited an IC₅₀ of 5.00 ± 3.31 μM (CA-0). To analyse the structural determinants of strand-transfer inhibitory activity

of the carbazole derivatives, we selected 23 such derivatives from our compound library and performed further analyses. Of these 23 compounds, six showed strong strand-transfer inhibition. The inhibition kinetics analyses and ethidium bromide displacement assays indicated that the carbazole derivatives are competitive inhibitors and not intercalators. An HeLa4.5/LTR-nEGFP cell line was employed to evaluate *in vitro* virus replication inhibition of the carbazole derivatives, and IC₅₀ levels ranged from 0.48–1.52 μM. Thus, it is possible that carbazole derivatives, which possess structures different from previously-reported IN inhibitors, may become novel lead compounds in the development of IN inhibitors.

Keywords: integrase inhibitor, carbazole, HIV-1, antiretroviral drug

Introduction

Human immunodeficiency virus type 1 (HIV-1), causative agent of acquired immunodeficiency syndrome (AIDS), possesses three critical enzymes for replication. These are protease (PR), reverse transcriptase (RT), and integrase (IN) (Ruscetti, 1985; Kohl *et al.*, 1988; LaFemina *et al.*, 1992). As inactivating any of these enzymes may negate the infectivity of HIV-1, the enzymes have been targets of anti-retroviral drug development. Indeed, great progress in anti-retroviral drug discovery has been achieved in recent decades, and today 10 RT inhibitors and eight PR inhibitors (De Clercq, 1992; Tronchet & Seman, 2003; Balzarini, 2004; Imamichi, 2004) are available for anti-retroviral treatments. The third enzyme, IN, has also been a major target of inhibitor development. L-708,906 and L-731,988, which possess diketo acid moieties within their

structures, were the first IN-specific inhibitors discovered (Pommier *et al.*, 2000; Dayam & Neamati, 2003; Pluymers *et al.*, 2002; Hazuda *et al.*, 2000). S-1360 and L-870,810, which also have diketo acid moieties, are IN inhibitors that have reached clinical Phase I/II trials for the first time (Johnson *et al.*, 2004; Hazuda *et al.*, 2004). However, although there have been large advances in the development of IN inhibitors, further research and analysis is required to develop clinically usable compounds.

Integrase (IN), the leading target of novel anti-retroviral inhibitor development, is the enzyme responsible for integration, wherein reverse transcribed HIV-DNA is inserted into a host genome, and is critical for viral replication, which in turn establishes latency and chronic infection (Chun *et al.*, 1995). IN is composed of three distinct

domains – the N-terminal domain (amino acids 1–50) with a zinc-binding motif (Schauer & Billich, 1992; Burke *et al.*, 1992), the catalytic core domain (amino acids 50–212) with polynucleotidyl transfer activity and sequence-specific endonuclease activity (Engelman & Craigie, 1992; Engelman *et al.*, 1994) and the C-terminal domain (amino acids 212–288), which has been thought to relate to nonspecific DNA binding (Khan *et al.*, 1991; Woerner & Marcus-Sekura, 1993).

At present, the function and structure of each domain has not been fully understood. The most well-analysed domain is the catalytic core domain, and its active site has highly conserved amino acidic residues Asp64, Asp116 and Glu152, which are critical for polynucleotidyl transfer activity (LaFemina *et al.*, 1992; Engelman *et al.*, 1995). Previously reported potent IN inhibitors L-708,906, L-731,988, L-801,810, S-1360 and 5-CITEP are all targeted to this domain. These inhibitors bind to the active site, displace divalent metal ion Mg^{2+} from the active site and inactivate the catalytic activity of IN (Grobler *et al.*, 2002; Dayam & Neamati, 2003; Goldgur *et al.*, 1999; Johnson *et al.*, 2004). No specific inhibitors have been reported for the N-terminal and C-terminal domains.

In the present study we attempted to identify novel IN inhibitory compounds, and therefore we conducted a random screening of a library of small molecular weight compounds. As a result, we discovered a series of novel IN inhibitory compounds with carbazole structures, that are quite different from previously reported inhibitory compounds.

Materials and methods

Preparation of integrase

The sequence coding the NL4-3 integrase (IN) was cloned into pET28b(+) (Novagen, Madison, WI, USA), generating pET-IN that codes NL4-3 IN with a hexa-histidine tag at the N-terminus. *Escherichia coli* strain Rosetta (DE3) (Novagen) transformed with pET-IN was grown in 1 l of Super Broth (Biofluids, Camarillo, CA, USA) containing 100 µg/ml kanamycin at 30°C until the optical density of the culture had reached between 0.5 and 0.7 at 600 nm. The recombinant protein expression was induced by isopropyl-1-thio-D-galactopyranoside. After incubation for 3 h, the cells were harvested and resuspended in 100 ml of preparation buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl) and disrupted by sonication. Following high-speed centrifugation at 40 000×g for 45 min at 4°C, the pellet was homogenized in GBB buffer (50 mM Tris-HCl, pH 8.0, 6 M Guanidine HCl and 2 mM 2-ME). The residual pellet was again sonicated and centrifuged at 40 000×g for 30 min at 4°C.

The supernatant was filtered through a 0.22 µm filter and mixed with 1 ml of nickel-affinity resin (Sigma, St. Louis, MO, USA), and incubated overnight at 4°C. The resin was washed twice by mixing with 20 ml of GBB containing 5 mM imidazole (Sigma). The protein was eluted with GBB containing 1 M imidazole. The fractions containing integrase were pooled and 0.5 M EDTA was added to a final concentration of 5 mM. This eluted protein was then sequentially dialysed against (i) 6 M guanidine HCl, 50 mM Tris-HCl (pH 8.0), 2 mM 2-ME, 1 mM EDTA for 2 h at room temperature, (ii) 6 M guanidine HCl, 50 mM Tris-HCl (pH 8.0), 10 mM DTT, 1 mM EDTA for 16 h at room temperature, (iii) 4 M urea, 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM DTT, 0.1 mM EDTA for 16 h at 4°C, (iv) 2 M urea, 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM DTT, 0.1 mM EDTA, 20% (w/v) glycerol for 16 h at 4°C, (v) 1 M urea, 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM DTT, 0.1 mM EDTA, 15 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 20% (w/v) glycerol for 16 h at 4°C, and (vi) 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM DTT, 0.1 mM EDTA, 15 mM CHAPS, 20% (w/v) glycerol for 16 h at 4°C. The final preparation was stored at –80°C.

The purified enzyme activity was confirmed and evaluated by strand-transfer assay using M8 apparatus (IGEN, Gaithersburg, MD, USA).

Preparation of test compounds

A diverse library of 12 000 small-molecule compounds was supplied by Toyama Chemicals Co. Ltd. (Toyama, Japan). All test compounds were dissolved in DMSO and adjusted to 2 mM concentration. S-1360 was synthesized as positive control for strand transfer assay.

Construction of strand-transfer assay

Two different strand-transfer assay systems were employed in the IN inhibitor screening trial. For the first screening step, an M8 apparatus and strand-transfer assay kit, ORIGEN HIV integrase assay (IGEN), was used. In brief, magnetic beads coated with 29 mer donor double-stranded DNA (dsDNA) were mixed with purified IN (15 pmol), followed by adding the test compound and 20 mer target dsDNA tagged with ruthenium, conducting electronically inducible fluorescence chemistry, and incubating for 1 h at 37°C. Subsequently, the entire reaction solution was applied to the M8 apparatus, and then strand-transfer products were captured by a magnet in the flow-circuit of the equipment. The amount of the strand-transfer product was measured by ruthenium fluorescence activity. For the second and later screening steps, in-house strand-transfer assay was employed. The in-house assay was designed in 96-well plate format to achieve high-throughput screening.

The following donor and target DNA oligonucleotides were designed and used:

Donor-1 (D1): 5'-ACTGCTAGAGATTTTCCA-CACTGACTAAAAG-3'

Donor-2 (D2): Biotin-5'-CTTTTAGTCAGTGTGGA-AAATCTCTAGCA-3'

Target-1 (T1): 5'-CTAGAGATTTTCCACACTGACT-AAAAG-3'-Digoxigenin (DIG),

Target-2 (T2): 5'-CTTTTAGTCAGTGTGGAAAA-TCTCTAG-3'-DIG

To form dsDNA, the D1–D2 pair and the T1–T2 pair were mixed in the presence of 0.1 M NaCl and denatured for 10 min at 95°C, followed by an annealing process, gradual cooling down to room temperature. One pmol biotinylated donor dsDNA (D1–D2), 15 pmol IN protein and 5 µl test compounds (100 µM in DMSO) were mixed together in assay buffer (25 mM 3-(N-morpholino)-propanesulfonic acid, pH 7.2, 25 mM NaCl, 10 mM MgCl₂, 10 mM DTT, 5% PEG, 10% DMSO), followed by the addition of 0.75 pmol target dsDNA (T1–T2), and adjusted to a final volume of 100 µl and incubated for 1 h at 37°C. After the incubation, the mixture was adjusted to a final volume of 200 µl with ELISA buffer (20 mM Tris [pH 8.0], 0.4 M NaCl, 10 mM EDTA, 0.1 mg/ml sonicated DNA). To harvest the strand-transfer product, the mixture was transferred into a 96-well micro titre plate coated with streptavidin (PIERCE, Rockford, IL, USA), followed by adding an alkaline phosphatase conjugated anti-DIG antibody (Roche Diagnostics, Mannheim, Germany) and a disodium 3-(4-methoxy Spiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) substrate (Roche). The lumino-intensity was quantified with a Luminous CT-9000D luminometer (DIA-IATRON, Tokyo, Japan).

In addition to the above two different strand-transfer assays, a strand-transfer assay with radioisotope labelled target DNA and SDS-PAGE was employed in order to visually confirm the strand-transfer inhibition (Craigie *et al.*, 1995). By use of T4 polynucleotide kinase (TAKARA BIO, Osaka, Japan), the 5' end of 20 mer target oligonucleotide-A (5'-TGTGGAAAATCTCTAGCAGT-3') was labelled with [γ -³²P] ATP (370 MBq/µl, Amersham Bioscience, Tokyo, Japan). After the labelling reaction was terminated by adding EDTA, complementary oligonucleotide-B (5'-ACTGCTAGAGATTTTCCACA-3') was added, and dsDNA was formed by heat denaturation and gradual cooling to room temperature. Unincorporated [γ -³²P]ATP was removed by G-25 Column (Amersham Bioscience, Piscataway, NJ). The reaction products were applied to 20% denatured polyacrylamide gel electrophoresis (300V/25A). The result of the electrophoresis was analysed by BAS-2500 (Fuji film, Tokyo, Japan).

Inhibition kinetics of IN

To analyse the strand-transfer inhibition mechanism of the test compounds, whether the action is competitive inhibition or non-competitive inhibition, Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were evaluated. Strand-transfer inhibition was evaluated on eight different time points (0, 1, 3, 5, 7.5, 10, 15, and 20 min) with four different compound concentrations (0, 1, 5, 10 µM) and target DNA concentrations (0.167, 0.25, 0.5, and 1 pmol). The initial reaction rate constants of IN were determined by linear regression using linear data points of product concentration–time plots. K_m and V_{max} were calculated from the Y-axis intercept in a plot of the slopes of Lineweaver-Burk analysis.

Intercalative activity evaluation

To clarify the possibility of intercalative activity of test compounds, ethidium bromide (EtBr) displacement assay was carried out following the protocol reported previously (Cain *et al.*, 1978). In brief, 1 µM calf thymus DNA (Invitrogen, Carlsbad, CA, USA) was mixed with EtBr (final concentration at 1.26 µM) and reaction buffer (2 mM HEPES, 10 µM EDTA, 9.4 mM NaCl, pH 7.0), and incubated for 10 min at room temperature. After the incubation, test compounds were added into the calf thymus DNA–EtBr mixture at different concentrations (final concentrations of 0.01–1000 µM). Fluorescence intensity of each mixture was determined by Fluoroskan Ascent FL (Helsinki, Finland. Excited at 544 nm, emitted at 590 nm). Actinomycin D (ICN Biomedical, Aurora, OH, USA), which is known as an intercalator, was employed as the positive control of the assay.

Molecular modelling studies

Molecular modelling studies were carried out using SYBYL software Version 6.9.1 (Tripos, St. Louis, MO, USA) running on an SGI Fuel workstation equipped with 600-MHz R14000 processor (SGI, Mountain View, CA, USA).

Evaluation of *in vitro* antiviral activity

To evaluate HIV-1 replication inhibition by selected test compounds, *in vitro* antiviral assays were performed using a HeLa4.5/nEGFP reporter cell line. The HeLa4.5/nEGFP reporter cell line was established by transfection of CD4 and LTR driven EGFP reporter protein into the HeLa cell line. HeLa4.5/nEGFP reporter cells were maintained with D-MEM (Sigma) containing 5% FCS (Hyclone, Logan, UT, USA), 500 µg/ml G418, 1 µg/ml blasticidin and 2 µg/ml puromycin.

One day before conducting the assay, 1x10⁴ HeLa4.5/nEGFP cells were seeded into clear bottom black 96-well plates (NUNC, Rochester, NY, USA) with

200 μl /well medium and incubated at 37°C, 5% CO₂. The next day, 1250 TCID₅₀ HXB2 were added in each well, followed by addition of the test compounds in final concentrations of 5, 1, 0.2, 0.04, 0.008, 0.0016, 0.00032, and 0.000064 μM . Forty-eight hours after infection, the cells were fixed by 3.2% formaldehyde and the nuclei of cells were stained by 10 $\mu\text{g}/\text{ml}$ Hoechst33342 (Molecular Probes, Engene, OR, USA). EGFP positive cell number (EGFP⁺) and Hoechst33342 positive cell number (hoechst33342⁺) were determined by Cellomics Array Scan, HSC Systems (Beckman Coulter, Tokyo, Japan).

Inhibitory activity of each compound was determined by the following formula:

$$\% \text{ inhibition} = 1 - \left\{ \frac{(\text{EGFP}^+ \text{ cell number with drug} / \text{hoechst33342}^+ \text{ cell number with drug}) - (\text{EGFP}^+ \text{ cell number without infection} / \text{hoechst33342}^+ \text{ cell number without infection})}{(\text{EGFP}^+ \text{ cell number without drug} / \text{hoechst33342}^+ \text{ cell number without drug}) - (\text{EGFP}^+ \text{ cell number without infection} / \text{hoechst33342}^+ \text{ cell number without infection})} \right\}$$

Results

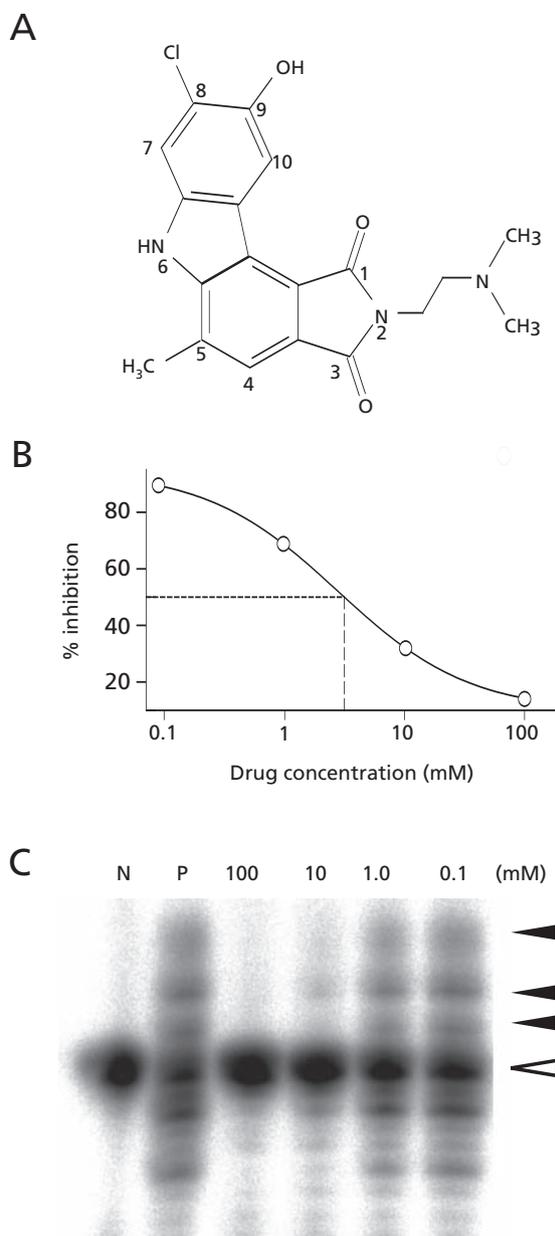
A small molecule bearing a carbazole moiety demonstrated strand-transfer inhibitory activity

A diverse library of 12 000 small-molecule compounds was screened for strand-transfer inhibitory activity at 100 μM concentration by M8 apparatus. Seventy-two compounds that demonstrated more than 80% strand-transfer-inhibition were selected and applied to the second screening using in-house strand-transfer assay. In the second screening, to confirm dose-dependent inhibition of the test compounds, each compound was tested at four different concentrations. Of the 72 compounds, a compound bearing a carbazole moiety, 8-chloro-2-[2-(dimethylamino)ethyl]-9-hydroxy-5-methylpyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (coded as **CA-0**), was found to demonstrate potent strand-transfer inhibitory activity (Figure 1A). As shown in Figure 1B, **CA-0** demonstrated clear dose-dependent inhibition of the strand-transfer reaction with an IC₅₀ of 5.00 \pm 3.31 μM . The dose-dependent inhibition was also confirmed by SDS-PAGE with [γ -³²P] labelled target DNA. As demonstrated in Figure 1C, strand-transferred product bands diminished along with increased concentration of the inhibitor. IC₅₀ value determined from intensities of the bands was 1.24 \pm 0.09 μM , which was consistent with that evaluated via the plate assay.

Strand-transfer inhibition of 23 carbazole derivatives, and the relationship between their structures and inhibitory activity

To understand the relationship between structure and strand-transfer inhibition activity, we selected 23 carbazole

Figure 1. Structure and strand transfer inhibitory activity of 8-chloro-2-[2-(dimethylamino) ethyl]-9-hydroxy-5-methylpyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**CA-0**)

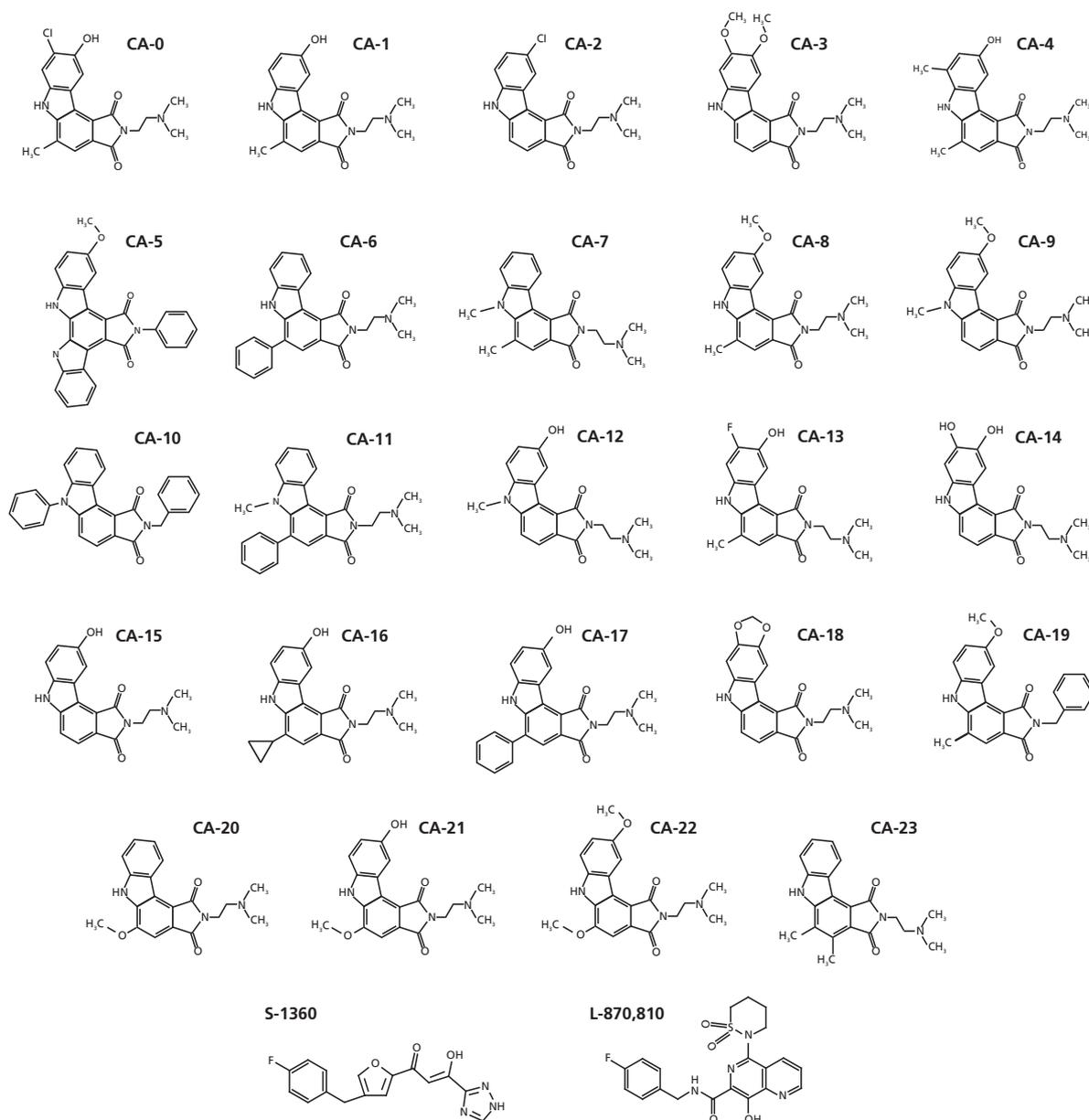


(A) The structure of **CA-0**, a strand transfer inhibitory compound identified from among a library of 12 000 small molecular weight compounds. It has a carbazole structure as a scaffold. The small numbers written beside the structure indicate the residue number of the compound. **(B)** A dose-response curve of **CA-0**. The dotted line indicates the IC₅₀ point of the chemical, which was 5.00 \pm 3.31 μM . **(C)** A strand transfer assay by radioisotope-labelled oligonucleotide. Lane 1 "N" stands for the negative control, with only a radioisotope-labelled nucleotide. Lane 2 "P" stands for positive control, with radioisotope-labelled nucleotide and recombinant integrase. Lanes 3 to 6 were with inhibitor. The open triangle and solid triangle indicate labelled oligonucleotide and strand transfer products, respectively.

derivatives with different substituents. As demonstrated in Figure 2, all compounds had pyrrolo[3,4-c]carbazole structures as scaffolds, and all except **CA-5**, **CA-10** and **CA-19** had 2-dimethylaminoethyl group at position R2. Six of the 23 compounds demonstrated potent strand-transfer inhibition comparable to that of **CA-0**. These compounds were **CA-1**, **CA-4**, **CA-8**, **CA-9**, **CA-12** and **CA-13**. IC_{50}

values of these test compounds were similar with positive control **S-1360**. Moderate inhibitory activities were observed in twelve compounds, **CA-2**, **CA-3**, **CA-7**, **CA-11**, **CA-14**, **CA-15**, **CA-16**, **CA-17**, **CA-18**, **CA-21**, **CA-22** and **CA-23**. Five compounds, **CA-5**, **CA-6**, **CA-10**, **CA-19** and **CA-20**, did not show significant inhibition, even at the highest concentration tested

Figure 2. Structures of **CA-0** and 23 carbazole derivatives evaluated for strand transfer inhibitory activity



CA-0 and 23 related compounds with carbazole scaffold tested for strand-transfer inhibitory activities are depicted. **S-1360** and **L-870,810**, which have previously been reported as potent IN inhibitors, are also shown.

(100 μM). The compounds that demonstrated potent strand-transfer inhibitory activity were also confirmed by gel-based assay, and IC_{50} values determined from the gel-based assay were consistent with the values determined via in-house plate assay (Table 1).

Carbazole derivatives are competitive inhibitors of integrase

To investigate the strand-transfer inhibitory mechanisms and kinetics of the compounds, we determined V_{max} and K_m of the inhibition by Lineweaver–Burke plot analyses. We selected two compounds, **CA-0** and **CA-13**, for the analyses. As summarized in Table 2, larger K_m values (nM)

were observed with higher inhibitory concentration, whereas V_{max} values (RU/min) did not change and remained consistent at any inhibitory concentration (Figure 3). As shown in Figure 3A and 3B, data-fitted lines of different time points converged on the Y axis, indicating that **CA-0** and **CA-13** inhibited strand-transfer in a competitive manner.

Carbazole derivatives have not shown intercalative activity

Due to their planar structure and their manner of competitive inhibition, we were concerned that the compounds might have the intercalative activity to destroy substrate dsDNA, rather than binding to the IN to block its enzyme activity. To clear the possibility of the intercalation, EtBr displacement assay was carried out. Since EtBr intercalates into dsDNA and makes visualization possible by growing fluorescence under UV light, intercalative activity of the test compounds can be evaluated by whether the test compounds displace incorporated EtBr out from dsDNA. As shown in Figure 4, fluorescence intensity diminished in a dose-dependent manner by actinomycin D, a compound known as a potent intercalator. In contrast, our two test compounds **CA-0** and **CA-13** did not affect fluorescence intensity, even at the highest concentration of 1 mM, suggesting that **CA-0** and **CA-13** were not intercalators.

Antiviral activity

We employed a single replication infectivity assay using HeLa4.5/EGFP cells to investigate the potency of antiviral activity. IC_{50} values of **CA-0** and the six compounds were 0.48, 0.92, 1.52, 0.79, 0.8, 0.69, 0.51 μM , respectively. The IC_{50} values of all seven compounds were 5.5 to 10.4-fold lower than that of the strand transfer assay (Table 1A). The discrepancy in IC_{50} between the two assays can be explained by stoichiometry of the inhibitor and the target enzyme in the two assays, and the estimated amount of IN in-strand transfer assay was higher than in the

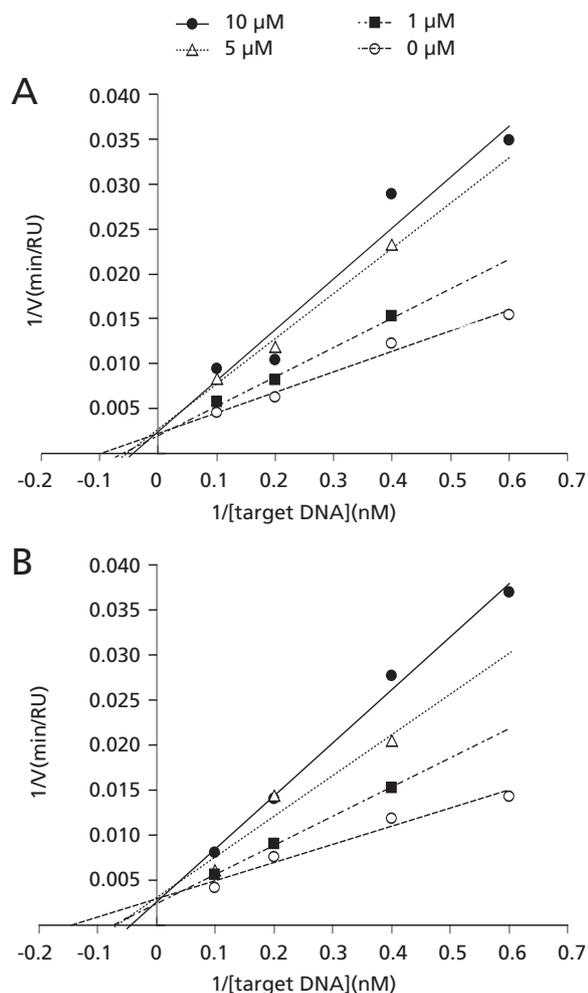
Table 1. Strand transfer and *in vitro* viral replication inhibitory activities of carbazole derivatives

	IC_{50} in strand transfer assay		Anti-HIV activity
	Plate assay (μM)	Gel assay (μM)	IC_{50} (μM)
<i>(A) High-inhibitory group</i>			
CA-0	5.00 \pm 3.31	1.24 \pm 0.09	0.48 \pm 0.06
CA-13	4.38 \pm 2.78	1.13 \pm 0.21	0.51 \pm 0.12
CA-1	7.94 \pm 4.12	2.97 \pm 0.21	0.92 \pm 0.15
CA-4	8.99 \pm 3.39	6.34 \pm 0.89	1.52 \pm 0.46
CA-8	6.61 \pm 4.17	6.38 \pm 0.32	0.79 \pm 0.07
CA-9	4.42 \pm 1.87	4.10 \pm 0.46	0.80 \pm 0.11
CA-12	5.93 \pm 3.53	3.14 \pm 0.04	0.69 \pm 0.15
<i>(B) Intermediate-inhibitory group</i>			
CA-2	22.50 \pm 2.27	ND	ND
CA-3	72.69 \pm 5.44	ND	ND
CA-7	11.88 \pm 7.66	ND	ND
CA-11	57.00 \pm 3.13	ND	ND
CA-14	17.37 \pm 1.79	ND	ND
CA-15	27.28 \pm 9.10	ND	ND
CA-16	20.51 \pm 15.11	ND	ND
CA-17	50.64 \pm 19.02	ND	ND
CA-18	10.68 \pm 8.88	ND	ND
CA-21	25.01 \pm 10.60	ND	ND
CA-22	16.92 \pm 7.32	ND	ND
CA-23	16.94 \pm 7.82	ND	ND
<i>(C) Intermediate-inhibitory group</i>			
CA-5	>100	ND	ND
CA-6	>100	ND	ND
CA-10	>100	ND	ND
CA-19	>100	ND	ND
CA-20	>100	ND	ND
<i>(D) Previously reported inhibitor</i>			
S-1360	4.67 \pm 1.89	ND	ND

Underline, indicates original compound; IC_{50} , 50% inhibition concentration; ND, not done.

Table 2. Inhibition kinetics of representative carbazole compounds **CA-0** and **CA-13**

Chemical	Concentration	V_{max} (RU/min)	K_m (nM)
CA-0	10 μM	463.16 \pm 63.16	30.40 \pm 7.80
	5 μM	402.58 \pm 32.21	26.21 \pm 7.40
	1 μM	370.14 \pm 84.42	12.71 \pm 2.02
	0 μM	454.55 \pm 0.02	9.18 \pm 1.18
CA-13	10 μM	409.70 \pm 35.47	19.31 \pm 4.68
	5 μM	439.07 \pm 164.74	14.83 \pm 0.24
	1 μM	438.08 \pm 53.85	11.09 \pm 2.42
	0 μM	429.83 \pm 136.46	7.08 \pm 0.64

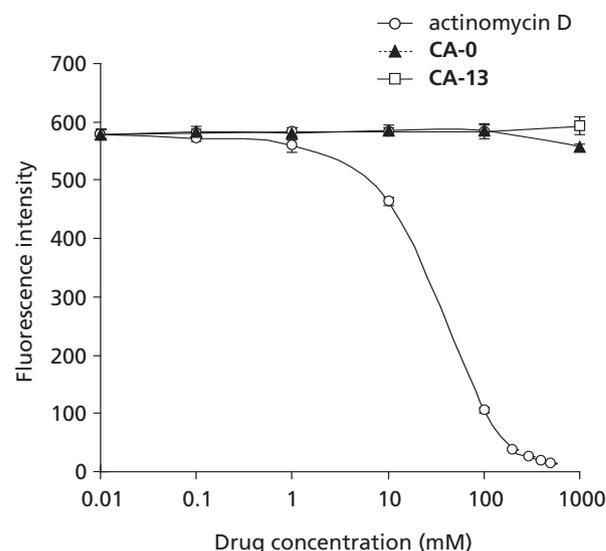
Figure 3. Inhibition kinetics assays of two representative carbazole derivatives, CA-0 and CA-13

Lineweaver-Burke plot analyses of (A) CA-0 and (B) CA-13 are depicted.

HeLa4.5/EGFP assay. Seven compounds exhibited considerable toxicity, suggesting that efforts toward decreasing toxicity are necessary for the further development of carbazole-based inhibitors.

Discussion

Carbazole, a fused phenyl-ring structure with hydrophobicity, has provided an interesting scaffold for the development of novel drugs. Staurosporine, discovered among microbial alkaloids, was the first carbazole derivative reported to demonstrate biological activity (Omura *et al.*, 1977; Furusaki *et al.*, 1978; Furusaki *et al.*, 1982), which was protein kinase C inhibition (Tamaoki *et al.*, 1986).

Figure 4. Ethidium bromide displacement assays of two representative carbazole derivatives, CA-0 and CA-13

To evaluate intercalative activities of carbazole derivatives, ethidium bromide displacement assays were carried out for two representative compounds, CA-0 and CA-13.

Other carbazole derivatives have demonstrated various other activities, such as topoisomerase inhibition (Marotto *et al.*, 2002; Facompre *et al.*, 2002; Carrasco *et al.*, 2001), hypotensive activity (Furusaki *et al.*, 1982), platelet aggregation inhibition (Oka *et al.*, 1986), and anti-fungal activity (Sunthitikawinsakul *et al.*, 2003). In this report we present another possible activity of carbazole derivatives, that of HIV-1 integrase inhibitor.

As compounds with three or four fused aromatic ring structures have been reported to demonstrate intercalative activity (Fukui & Tanaka, 1996; Dziegielewski *et al.*, 2002), we initially suspected that our carbazole derivatives also have intercalative activities, penetrating and disturbing target dsDNA, resulting in pseudo strand-transfer inhibition. Indeed, several carbazole derivatives have been recognized to demonstrate intercalative activity (Facompre *et al.*, 2002; Long *et al.*, 2002). We confirmed that actinomycin D, which is a well-known intercalator (Ross *et al.*, 1979; Wilson & Jones, 1982), demonstrated strand-transfer inhibition in our assay (data not shown). However, taking into consideration the data that our carbazole derivatives inhibited strand-transfer in a competitive manner, and also that the compounds could not displace EtBr out from dsDNA, we assume that our derivatives bind to part of the IN molecule, to the region responsible for DNA target

binding or to the catalytic site responsible for strand-transfer activity.

To understand in greater detail the substituents responsible for strand-transfer inhibitory activity, we analysed 23 carbazole derivatives, and classified them into three categories according to their levels of inhibition (Table 1). Six compounds were classified as the high-inhibition group, which demonstrated IC_{50} of less than 10 μM , 12 compounds were classified as the intermediate group, which demonstrated IC_{50} of greater than 10 μM and less than 100 μM , and five compounds were classified as the non-inhibition group, in which we did not observe significant inhibition even at the highest concentration tested (100 μM).

Comparing the compounds between and within these three categories, we recognized three factors responsible for strand-transfer inhibition. The first and most important factor is the incidence of a 2-dimethylaminoethyl group at position R2 (Figure 1A).

CA-8, which possesses a 2-dimethylaminoethyl group at position R2, demonstrated high inhibitory activity (IC_{50} : $6.61 \pm 4.17 \mu M$), but **CA-19** (IC_{50} : $>100 \mu M$), which possesses a phenyl ring structure at the same R2 position, did not demonstrate inhibitory activity. Thus, it is clear that the incidence of a 2-dimethylaminoethyl group, which has a basic property, is critical for strand-transfer inhibition activity. Indeed, we recognized that all compounds in the “high-inhibitory group” and “intermediate-inhibitory group” had this basic substituent at position R2 (Table 1A, 1B, Figure 2). In contrast, three of five compounds in the “non-inhibitory group” had the phenyl ring structure at R2 position. It is thought that these compounds might bind to the acidic region on the IN molecule and compete with the target dsDNA.

The second factor is the incidence of a methyl (Me) group at position R5, R6 or R7. We recognized that compounds in the high inhibitory group had at least one Me group at the R5, R6 or R7 position (Table 1A, Figure 2). Comparing **CA-1** (IC_{50} : $7.94 \pm 4.12 \mu M$), **CA-4** (IC_{50} : $8.99 \pm 3.39 \mu M$), and **CA-12** (IC_{50} : $5.93 \pm 3.53 \mu M$) with **CA-15** (IC_{50} : $27.28 \pm 9.10 \mu M$), it is clear that the incidence of an Me group within the R5 to R7 positions was an important factor for enhanced inhibitory activity. It seems that the position of the substituent may not be critical between R5 and R6, as we did not see significant differences between **CA-1** (IC_{50} : $7.94 \pm 4.12 \mu M$) and **CA-12** (IC_{50} : $5.93 \pm 3.53 \mu M$), and also between **CA-8** (IC_{50} : $6.61 \pm 4.17 \mu M$) and **CA-9** (IC_{50} : $4.42 \pm 1.87 \mu M$).

According to the IC_{50} levels of **CA-5** ($>100 \mu M$), **CA-6** ($>100 \mu M$) and **CA-11** ($>100 \mu M$), it appears that bulky substituents at the R5 position have a negative effect on inhibition (Table 1C, Figure 2). Furthermore, the inhibition potential of the three compounds **CA-1** (IC_{50} :

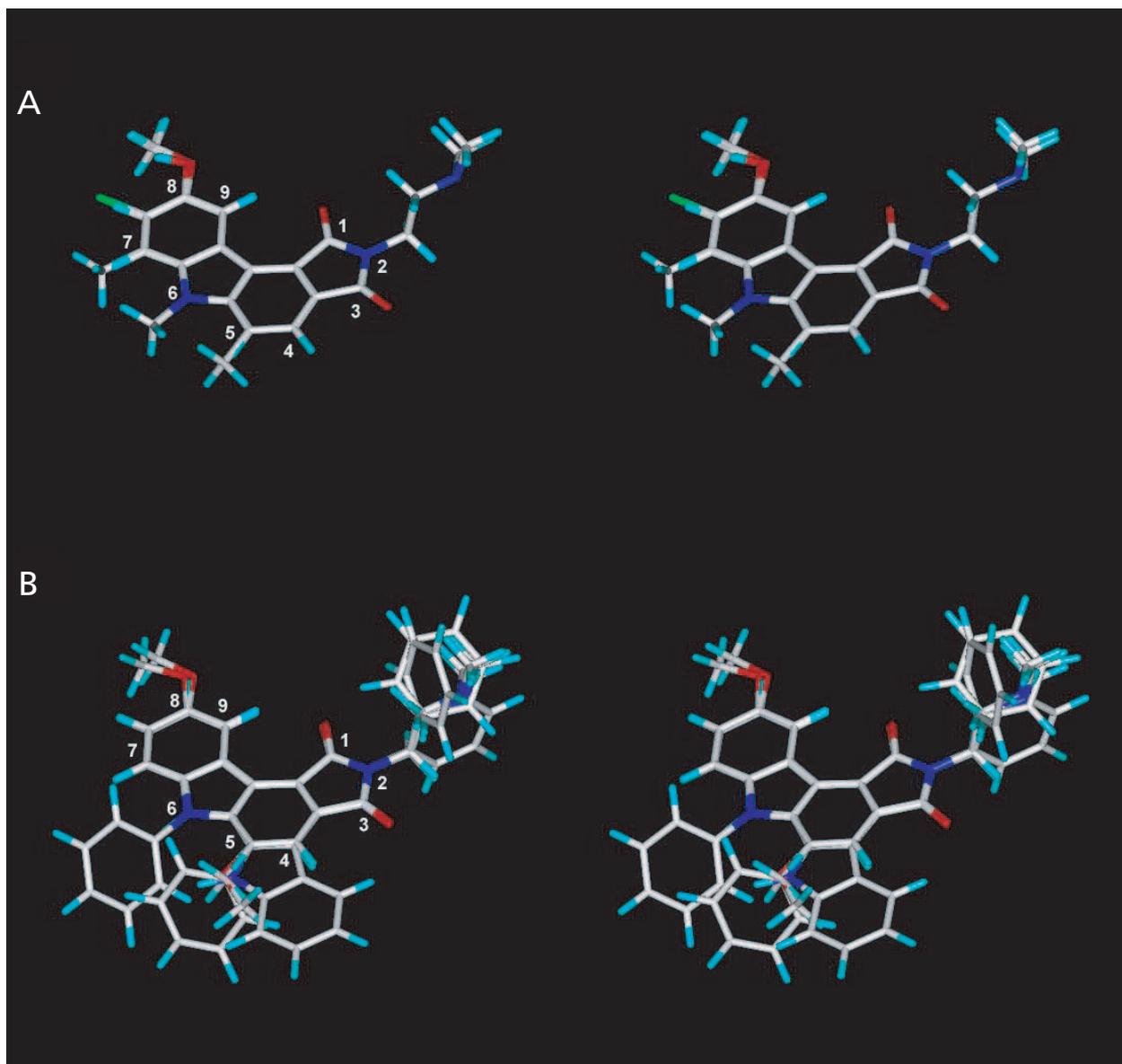
$7.94 \pm 4.12 \mu M$), **CA-16** (IC_{50} : $20.51 \pm 15.11 \mu M$) and **CA-17** (IC_{50} : $50.64 \pm 19.02 \mu M$) depended on the molecular size of their R5 substituents. It is probable that the R5 substituents of these compounds were too large and that they interfered with surrounding molecules forming the binding site (Table 1A, 1B, Figure 2). These data indicate that the binding site of carbazole might have a space limitation, and thus the size and shape of the molecules may be important factors for inhibitor activity.

The third factor is the substituent at position R9. Comparing **CA-20** (IC_{50} : $>100 \mu M$), **CA-21** (IC_{50} : $25.01 \pm 10.60 \mu M$) and **CA-22** (IC_{50} : $16.92 \pm 7.32 \mu M$), these three compounds were identical, with the exception of the substituent at position R9 (Table 1B, 1C, Figure 2). **CA-21** and **CA-22** have hydroxyl residue and a methoxy group at position R9, respectively. We noticed a significant difference in inhibitory activity between **CA-20** and **CA-21**, and between **CA-20** and **CA-22**, suggesting the possibility that both the hydroxyl group and the methoxy group at R9 formed hydrogen bonds with the amino acid molecules forming the binding sites, as these two substituents have the potential to be hydrogen bond acceptors. It appears that hydroxyl and methoxy groups have similar effects on strand-transfer inhibitory activities. In addition to the above three factors, we found that molecular interaction between R8 and R9 substituents, and their arrangement, are also important determinants for efficient inhibitory activity. **CA-3**, with two methoxy groups at R8 and R9, appears to have a bulky arrangement of the two side chains, and demonstrated an IC_{50} of $72.69 \pm 5.44 \mu M$, whereas **CA-14** and **CA-18**, which were expected to have horizontal arrangements, demonstrated lower IC_{50} values of $17.37 \pm 1.79 \mu M$ and $10.68 \pm 8.88 \mu M$, respectively (Table 1B, Figure 2).

To summarize these structural elements, and to understand the common structure of molecules that demonstrated strand-transfer inhibitory activity, we superposed inhibitor structures having significant strand-transfer inhibition (**CA-0**, **CA-1**, **CA-4**, **CA-8**, **CA-9**, **CA-12** and **CA-13**) (Figure 5A), and the structures of compounds with no inhibition (**CA-5**, **CA-6**, **CA-10**, **CA-19** and **CA-20**) (Figure 5B). In comparing these two overlapped figures, we found that the compounds with inhibitory activity share a largely identical structure and similar molecular size. In contrast, the non-inhibitory compounds had larger and more uneven-shaped side chains. Overall, the superposed structures indicate that the molecules should be planar and have basic diethylaminoethyl groups to demonstrate strand-transfer inhibitory activity.

In conclusion, we have identified a small molecular weight compound with a carbazole scaffold, which can be the lead compound for developing novel IN inhibitors. Furthermore, analysing the IN inhibitory mechanisms of

Figure 5. A structural comparison between high/intermediate inhibitory compounds and non-inhibitory compounds



Superposed structures of (A) five non-inhibitory compounds, CA-5, 6, 10, 19 and 20, and (B) seven inhibitory compounds, CA-0, 1, 4, 8, 9, 12 and 13, are demonstrated in stereo-view images. In both figures, residue numbers are indicated beside the structures. Red, dark blue and light blue indicate oxygen, nitrogen and hydrogen molecules, respectively. Green indicates chlorine or fluorine molecules. SYBYL software Version 6.9.1 running on an SGI Fuel workstation was used to construct the figures.

carbazole derivatives may yield more detailed information regarding HIV-1 IN structure and function.

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