2.3. Viruses and construction of recombinant HIV-1 clones

Two laboratory strains, HIV-1_{IIIB} and HIV-2_{EHO}, were used. Multi-drug resistant clinical HIV-1 strains, which had been exposed to over 10 anti-HIV-1 drugs for at least 3 years, were passaged in PHA-stimulated PBMCs (PHA-PBMCs) and stored at −80 °C until further use. Recombinant infectious HIV-1 clones carrying various mutations in the *pol* gene were generated using pNL101 (Jeang et al., 1993). Briefly, desired mutations were introduced into the XmaI–NheI region (759 bp) of pTZNX1, which encoded Gly-15 to Ala-267 of HIV-1 RT (strain BH 10) by a site-directed mutagenesis method (Weiner et al., 1994). The XmaI–NheI fragment was inserted into a pNL101-based plasmid, pNL-RT, generating various molecular clones with the desired mutations. To generate pNL-RT, we first introduced a silent mutation at NheI site of the pNL101, GCTAGC to GCCAGC (underlined; 7251 n.t. from the 5′-LTR) by site-directed mutagenesis. Then, the ApaI–SalI fragment of pNL101 without the NheI site was replaced with that of pSUM9 (Shirasaka et al., 1995), to introduce XmaI and NheI site in the RT coding region. The presence of intended substitutions and the absence of unintended substitutions in the molecular clones were confirmed by sequencing. Each molecular clone (2 μg/ml as DNA) was transfected into 293T cells (4×10^5 cells/6-well plate) by FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). After 24 h, MT-2 cells (10^6 cells/well) were added and co-cultured with 293T cells for an additional 24 h. When an extensive cytopathic effect was observed, the cell supernatants were harvested, and the virus was further propagated in MT-4 cells. The culture supernatant was harvested and stored at −80 °C until further use.

2.4. Determination of drug susceptibility

The inhibitory effect of test compounds on viral replication for 5 days was evaluated in MT-4 cells by the MTT method as described previously (Kodama et al., 2001). The sensitivity of NRTI-resistant infectious clones to test compounds was determined by the MAGI assay as described (Nameki et al., 2005). The drug susceptibility of HIV-1 clinical isolates was determined on day 7 by a commercially available p24 antigen assay (Kodama et al., 2001). Briefly, PHA-PBMCs (10^6 cells/ml) were exposed to each viral preparation at $TCID_{50}$ of 50 and cultivated in 200 μl of culture medium containing various concentration of the drug in 96-well culture plates. All assays were performed in triplicate, and the amounts of p24 antigen produced by the cells into the culture medium were determined. A 2′-deoxynucleoside competition assay was performed by the same way as the MAGI assay. An adenosine deaminase (ADA) inhibitor, dCF, was added for preventing conversion of 2′-deoxyadenosine (dA) to 2′-deoxyinosine (dI) (final concentration 0.4 μM). The effect of dCK expression on activities of test compounds was examined by measurement of SEAP activity in the supernatant. At first, the target cells (HT-1080, HT-1080/Ara-C^r, and HT-1080/Ara-C^r/dCK) were suspended in 96-well plates (5.0×10^3 cells/well). On the following day, the cells were inoculated with HIV-1_{IIIB} (500 MAGI unit/well, giving 500 blue cells in MAGI cells) in the presence of serially diluted compounds. After 48 h incubation, supernatant was collected and SEAP activity in the supernatant was measured using BD Great EscAPe SEAP chemiluminescence detection kit (BD Biosciences Clontech., Palo Alto, CA) and Wallac 1450 MicroBeta Jet Luminometer (PerkinElmer, Wellesley, MA).

2.5. The effect of ADA

The effect of ADA on EdA or EFdA was examined by high performance liquid chromatography (HPLC). ADA (0.01 U) derived from bovine intestinal tract was added into 0.5 ml of 0.5 mM EFdA in 50 mM Tris–HCl buffer (pH 7.5), and incubated at 25 °C. Samples were collected each 15 min and analyzed by HPLC.

2.6. HIV-1 replication assays

MT-2 cells (2.5×10^5 cells/5 ml) were infected with each virus preparation (500 MAGI units) for 4 h. The infected cells were then washed and cultured in a final volume of 5 ml. Culture supernatants (200 μl) were harvested from days 1 to 7 after infection, and the p24 antigen amounts were quantified (Nameki et al., 2005).

For competitive HIV-1 replication assay (CHRA), two titrated infectious clones to be examined were mixed and added to MT-2 cells (10^5 cells/3 ml) as described previously (Kosalaraksa et al., 1999; Nameki et al., 2005). To ensure that the two infectious clones being compared were of approximately equal infectivity, a fixed amount (500 MAGI units) of one infectious clone was mixed with three different amounts (250, 500 and 1000 MAGI units) of the other infectious clone. On day 1, one-third of the infected MT-2 cells were harvested, washed twice with phosphate-buffered saline, and the cellular DNA was extracted. The purified DNA was subjected to nested PCR and then direct DNA sequencing. The HIV-1 coculture which best approximated a 50:50 mixture on day 1 was further propagated. Every 4–6 days, the cell-free supernatant of the virus coculture (1 ml) was transmitted to new uninfected MT-2 cells. The cells harvested at the end of each passage were subjected to direct sequencing, and the viral population change was determined by the relative peak height in the sequencing chromatogram. The persistence of the original amino acid substitution was confirmed in all infectious clones used in this assay.

2.7. Molecular modeling studies

The programs SYBYL and O were used to prepare models of the complexes of wild-type, M184V, and insertion mutant HIV-1 RT with DNA and the triphosphates of 3TC and EFdA. The starting atomic coordinates of HIV-1 RT were from the structure described by Huang et al. (PDB code 1RTD) (Huang et al., 1998). The side-chain mutations were manually modeled using mostly conformations encountered in RT structures that carry such mutations. The local structures of mutants were optimized using energy minimization protocols in SYBYL. The triphosphates of the inhibitors were built based on the structures of dTTP in

Table 1
Antiviral activity against HIV-1 and HIV-2 strains in MT-4 cells

Compound (abbreviation)	EC_{50} (μM)[a]		Selectivity[b]	
	$HIV\text{-}1_{IIIB}$	$HIV\text{-}2_{EHO}$	CC_{50} (μM)[c]	index
2′-Deoxy-4′-C-ethynyl-adenosine (EdA)	0.0095 ± 0.0027[d]	0.006 ± 0.0015	104 ± 6.2	11,000
2′-Deoxy-4′-C-ethynyl-2-fluoroadenosine (EFdA)	0.000073 ± 0.000017	0.000098 ± 0.000022	9.8 ± 3.4	134,000
2′,3′-Dideoxy-4′-C-ethynyl-2-fluoroadenosine (EFddA)	1.17 ± 0.29	1.07 ± 0.23	230 ± 33	196
2′,3′-Didehydro-3′-deoxy-4′-C-ethynyl-2-fluoroadenosine (EFd4A)	0.11 ± 0.033	0.089 ± 0.0007	98 ± 26	899
2′-Deoxy-4′-C-cyano-2-fluoroadenosine (CNFdA)	0.1 ± 0.034	0.09 ± 0.0087	>340	>3,300
2′-Deoxy-4′-C-ethynyl-2-chloroadenosine (ECldA)	0.00069 ± 0.00018	0.0006 ± 0.0000028	230 ± 16	339,000
2′,3′-Dideoxyinosine (ddI)	27 ± 12	24 ± 4.4	>100	>4
3′-Azido-3′-deoxythymidine (AZT)	0.0028 ± 0.00062	0.0022 ± 0.00073	30 ± 7.2	10,800

Anti-HIV activity was determined by the MTT method.

[a] EC_{50} represents the concentration that blocks HIV-1 replication by 50%.

[b] Selectivity index is calculated by the CC_{50}/EC_{50} for $HIV\text{-}1_{IIIB}$.

[c] CC_{50} represents the concentration that suppress the viability of HIV-1-unexposed cells by 50%.

[d] Data shown are mean values with standard deviations for at least three independent experiments.

1RTD, or of tenofovir diphosphate in the ternary complex of HIV-1 RT/DNA/TFV-DP (Tuske et al., 2004).

3. Results

3.1. Antiviral activity of 4′- and 2-substituted deoxyadenosine analogs

We evaluated the activity of 4′- and 2-substituted deoxyadenosine analogs against HIV-1 with the MTT assay using MT-4 cells. The 2′-deoxy-4′-C-ethynyl nucleoside with adenine as the base (EdA) exerted comparable activity to AZT (Table 1). 2-Fluoro substituted EdA, EFdA, was the most potent against HIV-1 with a sub-nanomolar EC_{50} of 0.073 nM. Selectivity of EFdA and ECldA was much increased compared to parental EdA or AZT. However, EFdA was also relatively cytotoxic compared to other inhibitors of this series. The 2-chloro (Cl) substitution also provided enhanced activity but with a decreased toxicity. Further modifications of the sugar ring from 2′-deoxyribose to 2′,3′-dideoxy- or 2′,3′-didehydro-2′,3′-dideoxy-ribose (EFddA or EFd4A) resulted in a drastic decrease of inhibitory potential. Substitution of the 4′-E group with a structurally similar 4′-cyano group also resulted in markedly decreased inhibitory activity. These results indicate that the 3′-OH and 4′-E moieties in the sugar ring are indispensable for high efficacy, and that antiviral activities are augmented by the modification with F- or Cl-moiety at the adenine 2-position. These compounds suppressed the replication of HIV-2 at comparable levels as HIV-1, consistent with the hypothesis that they act as nucleoside reverse transcriptase inhibitors (De Clercq, 1998).

3.2. Antiviral activity against HIV-1 variants resistant to NRTIs

To assess the effect of 4′- and 2-substituted adenosine analogs against drug resistant HIV-1, we generated recombinant infectious clones carrying various NRTI resistant mutations and tested them using the MAGI assay. We found that EdA, EFdA, and ECldA efficiently suppressed many of the viruses resistant to approved NRTI including the multi-drug resistant (MDR) virus, although the 3TC-resistant variant $HIV\text{-}1_{M184V}$ and the multi-drug resistant variant $HIV\text{-}1_{M41L/T69SSG/T215Y}$ (Winters et al., 1998) showed modestly increased EC_{50} values to these compounds (Table 2). Interestingly, highly active 4′-E analogs, which have 3′-OH such as EFdA or ECldA, were even more effective against the dideoxy-type NRTI resistant variants K65R, L74V, and Q151M complex than they were against WT RT (Table 2). In contrast, 4′-E analogs without 3′-OH (EFddA and EFd4A) were similar or less effective with these resistant variants compared to WT, although the effect seems to be minimal. EFd4A and 2′-deoxy-4′-C-cyano-2-fluoroadenosine (CNFdA) were moderately active against $HIV\text{-}1_{WT}$ and $HIV\text{-}1_{MDR}$ (Shirasaka et al., 1995), but less active against $HIV\text{-}1_{M184V}$. Susceptibility of even the least active EFddA was still in the low micromolar range, but decreased against both $HIV\text{-}1_{M184V}$ and $HIV\text{-}1_{MDR}$, by 84- and 13-fold, respectively.

3.3. Antiviral activity of EFdA against multi-drug resistant clinical isolates

We went on to further characterize EFdA, the most potent compound of the series, against clinical isolates from patients exposed to many anti-AIDS drugs. Five multi-drug resistant strains ($HIV\text{-}1_{IVR405}$, $HIV\text{-}1_{IVR406}$, $HIV\text{-}1_{IVR412}$, $HIV\text{-}1_{IVR413}$, and $HIV\text{-}1_{A03}$), which contained various drug-resistance mutations in HIV-1 genes (Table 3), were used. These clinical isolates showed high resistance to AZT, 3TC ($HIV\text{-}1_{IVR406}$), and ddI ($HIV\text{-}1_{IVR412}$). $HIV\text{-}1_{IVR415}$ was also a drug-experienced virus but did not have NRTI-resistance mutations and showed no resistance, or less resistance to the NRTIs tested. Hence, it was used as a drug-sensitive HIV-1. Although antiviral activity of EFdA was slightly reduced against $HIV\text{-}1_{IVR405, IVR406, IVR412}$ (5.7- to 7.6-fold) compared to $HIV\text{-}1_{IVR415}$, the activity was high enough to suppress viral replication. It should be emphasized that EFdA was active against $HIV\text{-}1_{IVR406}$, which had the 3TC-resistant M184I substitution.

To evaluate antiviral activity of EFdA to M184V containing isolates in detail, two isolates harboring M184V were used. We used the MAGI assay that directly determines inhibition on a single replication cycle of HIV-1, so that we could eliminate the possible effects of multiple repli-

Table 2
Anti HIV-1 activity against drug-resistant infectious clones

	EC_{50} (μM)[a]								
	EdA	EFdA	EFddA	EFd4A	CNFdA	ECldA	ddI	AZT	3TC
WT	0.021	0.0011	1.2	0.35	0.21	0.0064	4.1	0.015	0.71
K65R	0.0082	0.00023 (×0.2)	1.56	0.2	ND[b]	0.0017 (×0.3)	ND	0.0039 (×0.3)	ND
L74V	0.01	0.00048 (×0.4)	2.52	0.54	ND	0.0015 (×0.2)	14.6	0.019 (×1.3)	ND
V75T	0.0075	0.00067 (×0.6)	9.13	0.95	ND	0.005 (×0.8)	ND	0.047 (×3.1)	ND
M41L/T215Y	0.062	0.0016 (×1.5)	6.7	1.67	ND	0.0065 (×0.1)	ND	0.12 (×8)	ND
M41L/T69SSG/T215Y[c]	0.18	0.0065 (×6)	ND	ND	ND	0.025 (×4)	21	0.20 (×13)	9.9
MDR[d]	0.011	0.00074 (×0.7)	16	0.46	0.69	0.0057 (×0.9)	40	18 (×1200)	1.1
P119S	0.018	0.00067 (×0.6)	ND	ND	ND	0.0062 (×1)	ND	0.0033 (×0.2)	0.6
T165A	0.045	0.001 (×0.9)	ND	ND	ND	0.0082 (×1.3)	ND	ND	0.66
I142V	0.077	0.001 (×0.9)	ND	ND	ND	0.0062 (×1)	ND	0.016 (×1)	0.36
T165R	0.088	0.0016 (×1.5)	ND	ND	ND	0.012 (×1.9)	ND	0.011 (×0.7)	0.28
M184V	0.088	0.0083 (×7.5)	101	6.41	1.76	0.084 (×13)	ND	0.0021 (×0.1)	>100
T165R/M184V	0.6	0.014 (×13)	ND	ND	ND	0.17 (×27)	ND	0.0053 (×0.4)	>100
I142V/T165R/M184V	0.81	0.023 (×22)	ND	ND	ND	0.41 (×65)	ND	0.0076 (×0.5)	>100
T165A/M184V[e]	0.43	0.015 (×14)	ND	ND	ND	0.16 (×25)	ND	0.0049 (×0.3)	>100
P119S/T165A/M184V[e]	0.5	0.015 (×14)	ND	ND	ND	0.20 (×32)	ND	0.0043 (×0.3)	>100

Anti-HIV activity was determined with the MAGI assay.

[a] The data shown are mean values obtained from the results of at least three independent experiments.

[b] ND: not determined.

[c] HIV-1 variant contains T69S substitution and 6-base pair insertions between codons for 69 and 70 (Ser-Gly) with AZT resistant mutations M41L/T215Y (Winters et al., 1998).

[d] Multi-dideoxynucleoside resistant HIV-1 contains mutations (AGT-GGT, SG) in the *pol* region: A62V/V75I/F77L/F116Y/Q151M (Shirasaka et al., 1995).

[e] These variants were reported by Nitanda et al. during induction of Ed4T resistant variants.

cation cycles on measured antiviral activity. In this assay, EFdA effectively suppressed both replication of HIV-1_{IVR443} and HIV-1_{IVR463}. Compared to the EC_{50} value for HIV-1_{WT} in Table 2, reduction of the activity was less than 3-fold, suggesting that EFdA suppresses relatively efficiently 3TC-resistant variants with either M184I or M184V mutations.

3.4. ADA stability of EFdA

Cellular ADA is known to convert dA to dI through deamination. Phosphorylation of the deaminated dA analogs, *e.g.*, dI, is less efficient, resulting in low conversion of the active triphosphate (TP) form. In order to assess if the activation of these compounds to their TP forms would be affected by the activity of ADA, we tested whether ADA can degrade EdA or EFdA. While EdA was almost completely deaminated after 90 min exposure to ADA, EFdA was not deaminated for up to at least 90 min (Fig. 2). These results indicate that the 2-halo-substitution in EdA confers significant resistance to degradation by ADA.

3.5. Phosphorylation of EFdA

Currently available NRTIs need to be converted to the TP form by host cellular kinases before incorporation into newly synthesized proviral DNA. It has been shown that the antiviral effect of NRTIs was reversed by the addition of their physiological counterpart 2′-deoxynucleosides (Bhalla et al., 1990; Mitsuya et al., 1985). To identify the phosphorylation pathway, we examined whether the antiviral activity of EFdA was reversed by the addition of 2′-deoxynucleosides. Surprisingly, the addition of dC decreased the antiviral activity of EFdA in a dose-dependent manner (Fig. 3). In contrast, dT and dG had no effect on the

Table 3
Antiviral activity of EFdA against clinical isolates

Clinical isolates	Amino acid substitutions in the reverse transcriptation	EC_{50} (μM)			
		AZT	ddI	3TC	EFdA
PBMCs[a]					
IVR405	M41L/E44D/D67G/V118I/Q151M/L210W/T215Y	1.76	2.45	0.55	0.0012
IVR406	M41L/E44D/D67N/V118I/M184I/L210W/T215Y/K219R	0.64	1.46	>10	0.0011
IVR412	M41L/E44D/V75L/A98S/L210W/T215F	3.97	9.11	0.83	0.0016
IVR413	M41L/E44D/D67N/V75L/A98S/V118I/L210W/T215Y/K219R	1.0	2.22	1.46	0.00021
A03	M41L/E44D/D67N/L74V/L100I/K103N/V118I/L210W/T215Y	0.53	2.15	0.49	0.0001
IVR415	None	0.0028	0.33	0.078	0.00021
MAGI cells[b]					
IVR443	I135T/Y181C/M184V	0.027	3.6	>100	0.0031
IVR463	M41L/E44D/D67N/M184V/H208Y/L210W/T215Y	0.31	7.5	>100	0.0032

All assays were performed in triplicate. AZT, ddI, and 3TC were served as a control.

[a] Antiviral activity was determined by the inhibition of p24 antigen production in the culture supernatant.

[b] HeLa-CD4/CCR5-LTR/β-gal cells was used for the MAGI assay.

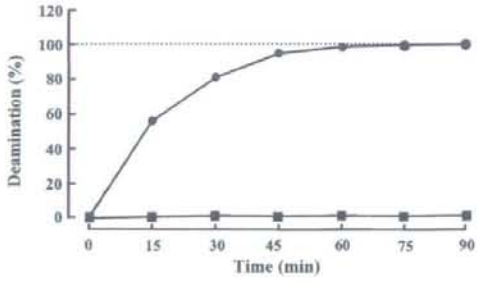

Fig. 2. Stability of EFdA following exposure to ADA. EdA or EFdA was incubated with ADA as described in Section 2. The deamination of adenine to inosine was analyzed by HPLC at indicated time points. The data represent the percent of starting compound (EdA: circle, EFdA: box) that was not deaminated by ADA.

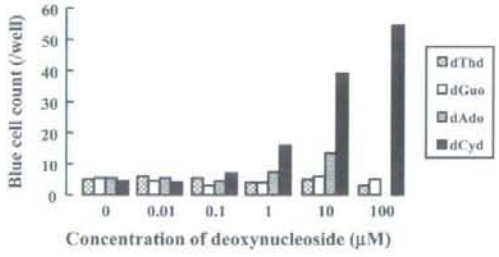

Fig. 3. Reversal of the antiviral activity of EFdA in the presence of 2′-deoxynucleosides. Each 2′-deoxynucleoside was added to the medium with serial dilution in the presence of EFdA (3.5 nM). The effect on EFdA activity was determined by the MAGI assay. An ADA inhibitor, dCF, was used during dA competition.

activity of EFdA. We could observe a slight reversal of the antiviral effect by addition of 10 μM dA with dCF. Effect of 100 μM dA could not be examined because of its cytotoxicity. It should be noted that all other tested analogs, including EFddA and EFd4A, were also reversed by the addition of dC (data not shown).

To confirm that the cellular dCK mediates the phosphorylation of EFdA, we examined the antiviral activity of EFdA in the HT-1080, dCK-deficient HT-1080/Ara-C^r (Obata et al., 2001), and dCK-transduced HT-1080/Ara-C^r cell lines. As expected, the antiviral activity of EFdA was markedly reduced in HT-1080/Ara-C^r cells (677-fold), but restored in dCK-transfected cells (Table 4). The same activity profile was observed with ddC, which is also phosphorylated by dCK (Starnes and Cheng, 1987). In contrast, AZT showed comparable activity among three cell lines, since it is known to be phophorylated mainly by thymidine kinase (Furman et al., 1986). Although dCK appears to be the main enzyme responsible for mono-phosphorylation of EFdA, other kinases, such as adenosine/deoxyadenosine kinases, may be partially involved in mono-phosphorylation of EFdA, especially since weak reduction in antiviral activity of EFdA was observed in addition of dA in high concentrations. Moreover, even in dCK-deficient HT-1080/Ara-C^r cells, EFdA exerted moderate antiviral activity. Hence, while it is possible that other kinases may be contributing to a smaller extent to the phosphorylation of EFdA, it appears that dCK is the enzyme that primarily phosphorylates this inhibitor.

3.6. Resistance to EFdA

In order to elucidate the mechanism of drug resistance to 4′-E analogs, we selected variants resistant to EdA, a parental compound of EFdA with the dose escalating methods (Nameki et al., 2005). After 58 passages in the presence of EdA, the resistant variants were obtained. Sequence analysis of the entire RT region revealed that a novel combination of mutations, I142V/T165R/M184V was introduced. Similar mutations (I119S/T165A/M184V) were observed in a Ed4T-resistant variant (Nitanda et al., 2005). Hence, we generated infectious clones containing these mutations and tested the antiviral activity of 4′-E analogs against them (Table 2). Mutation in T165, either Arg or Ala, enhanced the resistance against EdA, EFdA, and ECldA in the presence of the M184V mutation. Similar resistance profiles were observed for the I142V/M184V mutations. Furthermore, the triple mutant HIV-$1_{I142V/T165R/M184V}$ had the highest resistance among all tested variants. On the other hand, I142V or T165R alone did not affect the antiviral activity of EFdA or ECldA, although EdA or EFddA showed slightly decreased susceptibility. These results suggest that M184V appears to be the main mutation responsible for 4′-E analog resistance, and the addition of I142V and/or T165R augments the effect of M184V.

3.7. Replication of resistant HIV-1

For acquisition of high-level resistance to EFdA as well as EdA, three mutations, I142V, T165R, and M184V were required as described above. To examine the effect of the mutations on the viral replication kinetics we performed an assay that follows production of p24 gag antigen. All clones with M184V showed reduced replication kinetics (Fig. 4A), consistent with the reports that introduction of M184V markedly impairs replication kinetics (Wainberg et al., 1996; Yoshimura et al., 1999). Introduc-

Table 4
The effect of dCK expression on the EFdA antiviral activity[a]

Cell	EC_{50} (μM)[b]		
	AZT	ddC	EFdA
HT-1080	0.0032 ± 0.001	0.75 ± 0.22	0.00031 ± 0.0001
HT-1080/Ara-C^r	0.0027 ± 0.0005 (0.84)	84 ± 15 (112)	0.21 ± 0.03 (677)
HT-1080/Ara-C^r/dCK	0.0025 ± 0.00074 (0.78)	0.51 ± 0.16 (0.68)	0.000098 ± 0.000034 (0.32)

[a] SEAP activity in the culture supernatants were determined on day 2 after virus infection.
[b] The data shown are mean ± S.D. and fold increase in EC_{50} compared to HT-1080 is shown in parentheses.

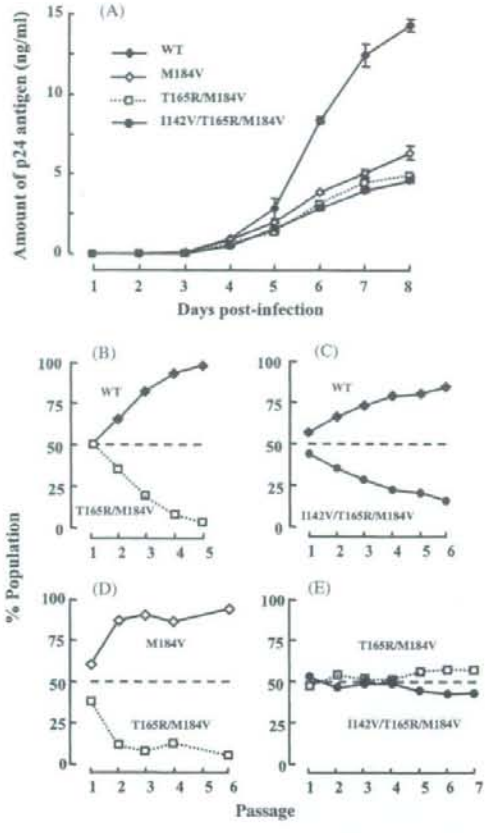

Fig. 4. Replication kinetics of HIV-1 clones with mutations. Production of p24 antigen in culture supernatant was determined with a commercially available p24 antigen kit. Profiles of replication kinetics (p24 production) of HIV-1_{WT} (closed diamonds), HIV-1_{M184V} (open diamonds), HIV-$1_{T165R/M184V}$ (open squares with broken line) and HIV-$1_{I142V/T165R/M184V}$ (open circles) were determined with MT-2 (A). Representative results from three independent triplicate determinations of p24 production with newly titrated viruses are shown. MT-2 cells were infected simultaneously with equal amounts of two HIV-1 clones to be compared. At each passage (5–6 days) the proviral sequences were determined and the percent population of each clone is reported at different passage; competition of WT and T165R/M184V (B); competition of WT with I142V/T165R/M184V (C); competition of M184V with T165R/M184V (D), and competition of T165R/M184V with I142V/T165R/M184V (E). At least two independent CHRAs were performed and are shown the representative results.

tion of T165R or I142V/T165R mutations in an M184V background (HIV-$1_{T165R/M184V}$ or HIV-$1_{I142V/T165R/M184V}$, respectively) further impaired HIV replication compared to HIV-1_{M184V}. I142V which enhanced EFdA resistance of HIV-$1_{T165R/M184V}$ (Table 2) conferred no replication rescue of HIV-$1_{T165R/M184V}$. To determine detailed replication kinetics, we performed CHRA which compares qualitatively viral replication. As shown in Fig. 4A, HIV-$1_{T165R/M184V}$ and HIV-$1_{I142V/T165R/M184V}$ showed reduced replication kinetics compared to HIV-1_{WT} (Fig. 4B and C). Replication kinetics ofHIV-$1_{I142V/T165R/M184V}$ was comparable to that of HIV-$1_{T165R/M184V}$, which showed further reduced replication kinetics compared to HIV-1_{M184V} (Fig. 4D and E). In another experiment, replication of HIV-$1_{I142V/T165R/M184V}$ was slightly decreased compared to HIV-$1_{T165R/M184V}$ (data not shown). These results suggest that introduction of three EdA mutations that also confers EFdA resistance impaired replication of HIV-1 in much greater extent compared to that of M184V.

4. Discussion

At present, HIV-1 variants containing NRTI-resistance mutations are widely observed not only in NRTI-experienced but also in NRTI-naïve patients. In such cases treatment failure is sometimes observed within short periods. The NRTI tenofovir, appears to be more effective against drug-experienced HIV-1 strains (Srinivas and Fridland, 1998). Unlike the other clinically available NRTIs, tenofovir has highly flexible acyclic ribose ring without a 3′-OH. Structural studies have suggested that the compact size of this inhibitor may contribute to the absence of highly resistant mutant strains against tenofovir (Tuske et al., 2004). Despite its unique structural profile, tenofovir is similar to other NRTIs in that it also lacks a 3′-OH group.

In contrast, the highly active 4′-E analogs such as EFdA retain the 3′-OH group of the canonical dNTP substrate. Similar to other NRTIs, they are also phosphorylated by cellular enzymes to their TP active form, which in turn serves as a substrate for HIV RT that incorporates them in an elongating primer during DNA synthesis. Following incorporation, replication is further inhibited by chain termination, although the specific mechanism of chain termination remains to be elucidated. Despite the fact that the 4′-E analogs have a 3′-OH like canonical dNTP substrates, cellular polymerases are likely to discriminate against these analogs, and not incorporate them during cellular DNA polymerization, as suggested by *in vitro* experiments with mitochondrial polymerase γ (Nakata et al., 2007). Alternatively, it is also possible that cellular proofreading systems excise the 4′-E analogs after their incorporation into cellular DNA.

The 3′-OH also plays an important role in phosphorylation of EdA analogs. Based on crystallographic results Sabini et al. reported that the interaction between 3′-OH of nucleosides and catalytic site of dCK was important for efficient nucleoside phosphorylation (Sabini et al., 2003). Alternatively, it is possible that the EFddA and EFd4A nucleosides that lack 3′-OH are poor substrates for HIV RT. The substitution at the 2-position of the purine base is also likely to contribute to highly potent *in vivo* activity of EF- or ECldA, possibly by preventing deamination of the inhibitor by ADA. ADA deaminates the adenine base into inosine, which is a poor substrate for cellular kinases. Similar ADA resistance has been reported for 2′-deoxy-2-chloroadenosine, a chemotherapeutic agent against hairy cell leukemia and chronic lymphocytic leukemia (Carson et al., 1980). ADA resistance may contribute to a longer intracellular half-life for EFdA-TP as compared to that of AZT (Nakata et al., 2007), indicating that substitution of 2-position plays an important role in sustained activity. When CEM cells were exposed to AZT or EFdA at concen-

tration of 0.1 μM, amounts of corresponding intracellular TP-forms were comparable (Nakata et al., 2007). However, inhibitory effect of EFdA in MT-4 and MAGI cells was approximately 40- and 15-fold superior compared to that of AZT (Tables 1 and 2). Taken together, HIV-1 RT appears to preferentially incorporate EFdA-TP compared to AZT-TP, although detailed enzymatic confirmation is needed. The parental EdA also seems to be a good substrate for HIV-1 RT; however, it may be subjected to deamination, resulting in comparable activity to AZT.

There are at least two mechanisms by which HIV RT can become resistant to NRTIs: first, HIV RT can acquire mutations at, or close to the dNTP-binding site, such that help it discriminate against NRTI-triphosphates, while it retains its ability to recognize the normal dNTP substrates (Huang et al., 1998). In the case of M184V/I the discrimination is based on steric conflict between a part of the inhibitor (the sulfur atom of the thioxolane ring in the case of 3TC), and the β-branched side chain of Val or Ile at the mutation site (Sarafianos et al., 1999). Mutations at other residues of the dNTP-binding site are responsible for discrimination of dideoxynucleosides from dNTPs during both the substrate-recognition (Martin et al., 1993) and the catalytic steps (Deval et al., 2002; Selmi et al., 2001). The other mechanism of NRTI resistance is based on an excision reaction (phosphorolysis) that unblocks NRTI-terminated primers using a molecule of ATP as the pyrophosphate donor (Meyer et al., 1999). The product of this reaction is a dinucleoside tetraphosphate and an unblocked primer that can continue viral DNA synthesis. In this case, the role of resistance mutations is to optimize binding of an ATP molecule that is used for the nucleophilic attack at the primer terminus. Most of AZT resistance as well as multi-NRTI resistance of RT with insertions at the fingers subdomain are thought to be based on an ATP-based unblocking mechanism. The insertions in RT destabilize the normally stable ternary complex (RT/template-primer/dNTP) and facilitate the ATP-mediated pyrophospholysis (Boyer et al., 2002). The fingers insertion mutant can excise all nucleotide analogs, with various degrees of efficiency.

Our molecular modeling studies are consistent with a mechanism of resistance to 4′-E analogs that involves steric hindrance between the 4′-E group of the inhibitors and the side chain of V184, reminiscent of the resistance mechanism to 3TC. While a single M184V mutation confers strong resistance to 3TC (>100-fold), it causes only moderate (8- to 13-fold) resistance to EFdA and ECldA (Table 2). This is consistent with our molecular modeling analysis where the bulky and rigid 4′-E moiety appears to cause some steric hindrance with the Val or Ile side chain at position 184 during incorporation of the 4′-E nucleotides by the M184V enzyme (Fig. 5). The steric interaction appears to be stronger during incorporation of 3TC-TP (Fig. 5E) than EFdA-TP (Fig. 5C). Interestingly, the M184V mutation appears to confer stronger resistance to 4′-methyl substituted nucleotides, than to the 4′-ethynyl substituted nucleotides (Kodama et al., 2001). The decreased resistance of 4′-ethynyl substituted compounds may be in part the result of compensatory favorable interactions of the longer ethynyl group with residues of the dNTP binding site, including Y115 and D185. Such interactions may moderate the effect of the steric interactions of the 4′-ethynyl with residue V184 in the M184V mutant. Resistance of M184V to dideoxy-derivatives such as EFddA was unexpectedly high (84-fold). Although we cannot explain the detailed mechanism of the difference in resistance, it is possible that the presence of a 3′-OH in the EFdA (but not in the EFddA and EFd4A) results in more stabilizing interactions with residues such as Q151 that compensate for the steric hindrance by M184V (Fig. 5D). Hence, EFddA and EFd4A may be easier to push out of the binding pocket than analogs with a 3′-OH. For stronger resistance to 4′-E-2-halo-dAs, other mutations at positions 142 and 165 in addition to the M184V are required (Table 2) to substantially decrease inhibitor binding. It should be noted that the T165R/M184V mutations were also observed during induction of resistant variants to parental compound EdA. Nitanda et al. also reported that resistant variants for 4′-Ed4T contain M184V with T165A (Nitanda et al., 2005). As shown in Fig. 5A, the effect of the T165R mutation seems to be through the loss of a hydrogen bond between the side chain of Q182 and the side chain OH moiety of T165. Instead, there may be a hydrogen bond between C=O of the main chain of 184 and Q182 in the case of T165R/A, which would affect the positioning of the residue in position 184. Interestingly, HIV-2 has an Arg residue at position 165, whereas HIV-1 has Thr. We could not find decreased susceptibility in HIV-1_{T165R} or HIV-2, indicating that R165 becomes relevant only when Val is at the 184 position. When this residue is Met (T165R in M184 background), resistance is not affected substantially (1.5- to 2-fold resistance, Table 2) because of the flexibility of the Met side chain. However, when the 184 residue is Val (T165R/M184V), the position of 184 may be affected in a way that exacerbates the steric interactions between the ethynyl group of the incoming EFdA and the side chain of V184, resulting in resistance to EFdA and the other 4′-E analogs (13–27-fold resistance, Table 2). At this point it is not clear why the I142V mutation further augmented the effects of M184V and T165R. Finally, as shown in Fig. 5B, there are no apparent substantial steric problems for binding of EFdA to HIV-$1_{M41L/T69SSG/T215Y}$ RT, and the enzyme–inhibitor interactions are likely to be similar to those with dNTP and consistent with the relatively low resistance observed with this variant (Table 2) that is known to cause strong excision-based NRTI resistance. Crystallographic studies with the RT resistant variants complexed with the inhibitors should provide more insights into the mechanism of resistance.

The M184V, one of three mutations associated with EFdA resistance, develops rapidly under therapy with 3TC and has been reported to alter several profiles of RT function, including decreased RT processivity (Back et al., 1996), reduced nucleotide-dependent primer unblocking (Gotte et al., 2000), and increased fidelity (Wainberg et al., 1996). These profiles result in impaired viral fitness, hypersensitivity to other NRTIs, especially AZT (Larder et al., 1995), and delayed appearance of mutations, respectively. Our results show that modest resistance to EdA comes at a significant cost for the virus: The I142V and T165R mutations reduced even further viral replication kinetics of M184V-containing virus. Furthermore, the virus containing these mutations retains the AZT hyper-susceptibility which is induced by

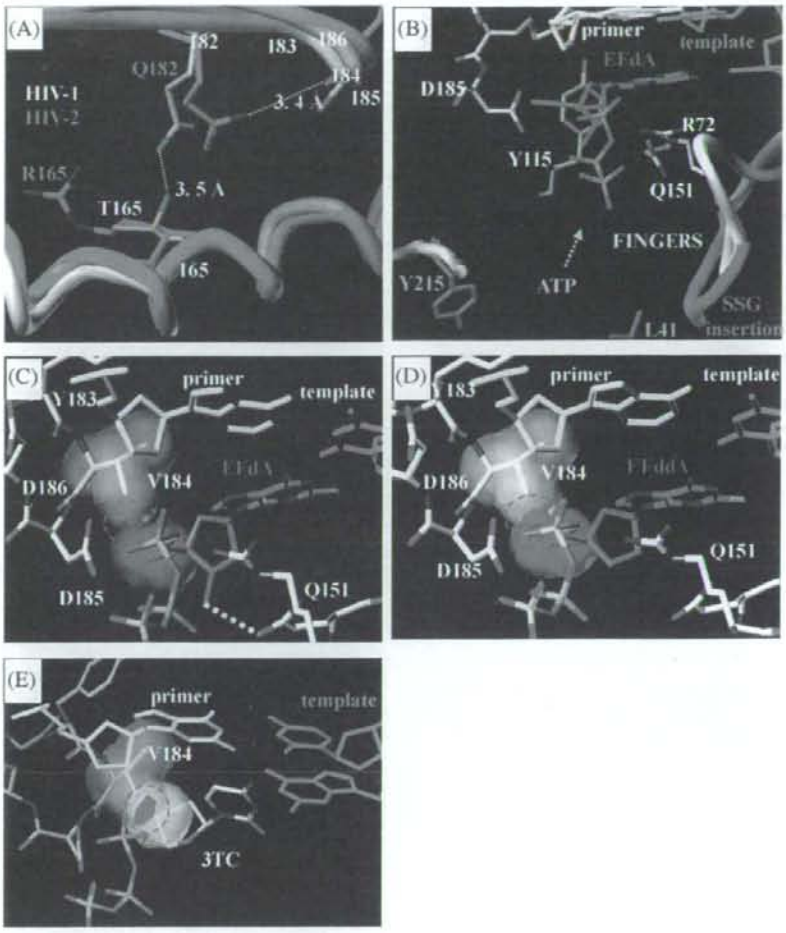

Fig. 5. Structural modeling of reverse transcriptase and compounds. (A) Superposition of the polymerase active sites of HIV-1 (gray) and HIV-2 (magenta) reverse transcriptases. Q182 makes a hydrogen bond with T165 in HIV-1. In the T165R mutant of HIV-1, the arginine side chain is expected to have a conformation similar to the one observed for R165 in HIV-2. In this context, Arg does not make a hydrogen bond with the side chain of residue 182 that may now interact with the main chain carbonyl of M184, which is at the immediate vicinity of the inhibitor-binding site. Such interaction may explain how the T165R mutation exacerbates the role of the M184V mutation in resistance to EFdA. (B) Proposed interactions of EFdA-triphosphate (TP) at the polymerase active site of the "fingers-insertion" NRTI-resistant HIV-1 RT, carrying the M41L/T69SSG/T215Y mutations. Possible steric interactions between the 4′-E group of EFdA-TP (C) or EFddA-TP (D) and the sulfur (S) of the pseudosugar ring of 3TC-TP (E). Van der Waals surfaces of 4′-E group (C and D) and S at sugar ring (E) are indicated in green and yellow. Possible steric interactions are shown as overalap of Van der Waal volumes of interacting atoms (in red).

M184V (Table 2), although further experiments are needed. These results suggest that the I142V and T165R mutations simply enhance resistance of M184V RT to EdA rather than optimize the viral fitness of the M184V virus. The increased cost for the virus to overcome inhibition pressure by EdA may have significant clinical benefits in the treatment of HIV infections.

Since EFdA is initially phosphorylated mainly by dCK and its activity was attenuated by addition of dC (data not shown), it is likely that dC analogs, such as 3TC and emtricitabine (FTC) that are mainly phosphorylated by dCK would act as a competitor of EFdA phosphorylation. However, one of dC analogs, apricitabine (ATC) showed little competition for the intracellular phosphorylation of 3TC and FTC (Bethell et al., 2007). Thus, interaction of NRTIs using identical phosphorylation enzymes should be carefully examined.

In conclusion, we have shown that the 2-halogen substituted EdAs have exceptionally potent subnanomolar antiviral activities. The 2-F substituted analog exhibited the highest potency and had a selectivity index significantly improved over that of approved NRTIs. In fact, results from our parallel studies with mice show no toxicity of EFdA (data not shown). The earlier studies also showed that a

parental nucleoside, EdA was not toxic in mice (Kohgo et al., 2004). The half-life of the intracellular TP form of EFdA is substantially extended (~17 h) compared to that of AZT (~3 h) (Nakata et al., 2007), suggesting that it may be possible to administer these inhibitors once a day. Further investigation may lead to their development as potential therapeutics against HIV infections.

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Broad Antiretroviral Activity and Resistance Profile of the Novel Human Immunodeficiency Virus Integrase Inhibitor Elvitegravir (JTK-303/GS-9137)▽†

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Integrase (IN), an essential enzyme of human immunodeficiency virus (HIV), is an attractive antiretroviral drug target. The antiviral activity and resistance profile in vitro of a novel IN inhibitor, elvitegravir (EVG) (also known as JTK-303/GS-9137), currently being developed for the treatment of HIV-1 infection are described. EVG blocked the integration of HIV-1 cDNA through the inhibition of DNA strand transfer. EVG inhibited the replication of HIV-1, including various subtypes and multiple-drug-resistant clinical isolates, and HIV-2 strains with a 50% effective concentration in the subnanomolar to nanomolar range. EVG-resistant variants were selected in two independent inductions, and a total of 8 amino acid substitutions in the catalytic core domain of IN were observed. Among the observed IN mutations, T66I and E92Q substitutions mainly contributed to EVG resistance. These two primary resistance mutations are located in the active site, and other secondary mutations identified are proximal to these primary mutations. The EVG-selected IN mutations, some of which represent novel IN inhibitor resistance mutations, conferred reduced susceptibility to other IN inhibitors, suggesting that a common mechanism is involved in resistance and potential cross-resistance. The replication capacity of EVG-resistant variants was significantly reduced relative to both wild-type virus and other IN inhibitor-resistant variants selected by L-870,810. EVG and L-870,810 both inhibited the replication of murine leukemia virus and simian immunodeficiency virus, suggesting that IN inhibitors bind to a conformationally conserved region of various retroviral IN enzymes and are an ideal drug for a range of retroviral infections.

Three unique and essential HIV enzymes, protease (PR), reverse transcriptase with RNase H (RT), and integrase (IN), appear to be ideal targets for the development of inhibitors of human immunodeficiency virus (HIV) replication. Anti-HIV drugs targeting PR (PR inhibitors [PIs]) and RT (nucleoside/nucleotide RT inhibitors [NRTIs] and nonnucleoside RT inhibitors [NNRTIs]) have been approved for use in the treatment of HIV infection. Combinations of these drugs used in highly active antiretroviral therapy can effectively suppress HIV replication in vivo to undetectable levels and have led to significant declines in HIV-associated mortality (28, 40). However, the emergence of drug-resistant HIV variants can attenuate the efficacy of antiretroviral treatment. Some primary infections also result from the transmission of HIV strains that possess drug-resistant genotypes and phenotypes (9). To suppress these drug-resistant variants, new anti-HIV drugs that block new targets are urgently needed.

IN, a 32-kDa protein resulting from the proteolytic cleavage of the *gag-pol* precursor, plays an essential role in the integration of proviral DNA into the host genome. As LaFemina et al. previously reported that there is no human homologue of HIV IN (31), it is an attractive target for the development of new antiretroviral therapeutic agents without adverse effects. IN consists of three domains: an N-terminal zinc finger domain and a C-terminal DNA-binding domain flank a central catalytic core domain (CCD) that plays a critical role in its enzymatic activity (13, 14). Following reverse transcription, IN exerts at least two functions: the cleavage of two conserved nucleotides from the 3′ ends of both strands of the viral cDNA (3′ processing) (1) and, subsequently, the ligation of the viral cDNA into the host genome (strand transfer) (14). Gap filling of the interfaces between the viral and host genomic DNA is then completed using the host DNA repair machinery via a mechanism that is not yet fully understood. The completion of integration results in a fully functional provirus, which can then be used to initiate viral DNA transcription.

Several compounds that inhibit IN activity have been described, including diketo acid (DKA) derivatives such as L-731,988 (24) and S-1360 (16), both of which have potent

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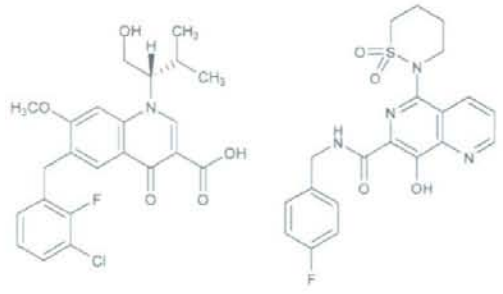

elvitegravir (EVG) L-870,810

FIG. 1. Structure of EVG and L-870,810. A dihydroquinoline carboxylic acid derivative, EVG, and a naphthyridine carboxamide derivative, L-870,810 (a representative IN inhibitor), are shown.

antiviral activity. Crystal structure analysis has indicated that 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2*H*-tetrazol-5-yl)-propenone, an S-1360 derivative, binds to the CCD, the putative active site of IN (19). In vitro resistance selection experiments with several IN inhibitors demonstrated that mutations in the CCD of IN play a significant role in the generation of IN inhibitor-resistant viral variants. In vitro selection of HIV-1 in the presence of the DKA IN inhibitors L-731,988 and S-1360 resulted in the emergence of viral variants carrying IN mutations associated with resistance. These mutations, including T66I, S153Y, and M154I, are located in close proximity to the catalytic triad residues (D64, D116, and E152) in the CCD of IN (16, 24). In contrast, L-870,810 (Fig. 1), which has previously demonstrated potent antiviral activity in HIV-1-infected patients in a monotherapy study (33), induced unique IN mutations, including V72I, F121Y, T125K, and V151I, when HIV was selected with the compound in vitro (23). These mutations are also located in the active site of IN, suggesting that a common mechanism may be involved in the acquisition of resistance to IN inhibitors.

Although no IN inhibitors are currently approved for clinical use (41), two IN inhibitors, elvitegravir (EVG) (formerly known as JTK-303/GS-9137, being codeveloped by Gilead Sciences and Japan Tobacco) (Fig. 1) (43, 56) and raltegravir (MK-0518, developed by Merck) (22), are currently being investigated in clinical studies of HIV-1-infected patients. In a phase II study, antiretroviral treatment-experienced patients using 125 mg EVG (boosted with ritonavir) along with an active optimized background regimen showed >2-$\log_{10}$ declines in their viral loads that were durable through week 24 (56).

Here, we describe the antiviral activity, mechanism of action, and resistance profile of EVG in vitro. EVG exerted potent anti-HIV activity against not only wild-type strains but also drug-resistant clinical isolates. Interestingly, EVG also showed antiviral activity against murine leukemia virus (MLV) and simian immunodeficiency virus (SIV). These results imply that IN inhibitors are ideal agents for the treatment of a range of retroviral infections. During the selection of EVG-resistant viral variants, novel IN mutations emerged. Combinations of these mutations conferred resistance to EVG and reduced susceptibility to other IN inhibitors, suggesting that there is a common mechanism underlying the resistance to IN inhibitors. One such mechanism may be conformational changes induced by multiple mutations located in the active site of IN.

MATERIALS AND METHODS

Antiviral agents. Zidovudine (AZT) and dextran sulfate (DS5000) (average molecular weight, 5,000) were purchased from Sigma (St. Louis, MO). Efavirenz (EFV) (NNRTI) and nelfinavir (NFV) (PI) were used for the control inhibitor. EVG (43), L-731,988 (42), L-870,810 (23), and S-1360 (16) were synthesized as described previously. The structures of EVG and L-870,810 are depicted in Fig. 1.

Cells and viruses. MT-2 and MT-4 cells were grown in RPMI 1640 medium. 293T cells were grown in Dulbecco's modified Eagle's medium. These media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin. HeLa-CD4/CCR5-LTR/β-gal cells (5) were kindly provided by J. Overbaugh through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (Bethesda, MD), and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 200 μg/ml hygromycin B, 10 μg/ml puromycin, and 200 μg/ml geneticin. Peripheral blood mononuclear cells (PBMC) were obtained from healthy HIV-1-seronegative donors by centrifugation through Ficoll-Hypaque density gradients. PBMC were stimulated with 20 U/ml interleukin-2 (Shionogi, Osaka, Japan) and 0.5 μg/ml phytohemagglutinin (Sigma) for 3 days and then used for assays as described previously (30).

Three laboratory strains, HIV-1_{IIIB}, HIV-2_{EHO}, and HIV-2_{ROD}, were used in this study. Various subtypes of drug-naïve clinical isolates of HIV-1 (four isolates of subtype B and seven isolates of non-B subtypes) were employed. Four drug-resistant clinical isolates of HIV-1, including IVR401, IVR409, IVR411, and IVR415, were kindly provided by S. Oka (AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan).

Determination of HIV drug susceptibility. Inhibitory effects of compounds on HIV infection were determined using multinuclear activation of a galactosidase indicator (MAGI) assay, as previously described (37). Inhibitory effects on HIV-1 clinical isolates were measured by p24 production, and cytotoxicity was measured by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, as described previously (30). Antiviral activities and cytotoxicities of inhibitors are presented as the concentrations that block viral replication by 50% (50% effective concentration [EC_{50}]) and that suppress the viability of target cells by 50%, respectively.

Quantification of HIV-1 DNA species. MT-2 cells (5×10^5 cells) were infected with HIV-1_{IIIB} at a multiplicity of infection (MOI) of 0.1 in the absence or presence of various inhibitors. Infected cells were washed after incubation for 2 h at 37°C. At 24 h postinfection, DNA was extracted using DNAzol reagent (Invitrogen, Carlsbad, CA).

Quantification of integrated HIV-1 DNA and the two-long-terminal-repeat (2-LTR) circle was performed by real-time quantitative PCR as described previously (4). To normalize DNA species among inhibitors, β-globin amplification was used as an internal control (51). Reactions were analyzed by using the ABI Prism 7500 sequence detector (PE Applied Biosystems, Foster City, CA), and results were then normalized and expressed as relative HIV-1 DNA species compared to a "no-inhibitor" control.

In vitro strand transfer assay. An oligonucleotide-based strand transfer assay was performed as previously described (8), with some modifications. Briefly, preprocessed oligonucleotide H-U5V1-2 (5′-ATGTGGAAAATCTCTAGCA-3′), derived from the U5 end of the HIV-1 LTR, was labeled at the 5′ end with [γ-^{32}P]ATP. Radiolabeled H-U5V1-2 was annealed to H-U5V2 (5′-ACTGCTA GAGATTTTCCACAT-3′) and then used for assays. Recombinant HIV-1 IN derived from HIV-1 NL4-3 (wild type) or EVG-selected mutants was prepared using an *Escherichia coli* expression system. The strand transfer assay was performed with 1 μM IN and 150 nM substrate DNA in 20 mM MOPS (morpholinepropanesulfonic acid) buffer with 30 mM $MgCl_2$ incubated in either the presence or absence of IN inhibitors at 37°C for 60 min. Reaction products were analyzed by electrophoresis on 25% polyacrylamide gels and quantified using a BAS-2500 imaging system (Fuji Photo Film, Tokyo, Japan). The concentration of IN inhibitor that inhibited the production of strand transfer products by 50% (50% inhibitory concentration [IC_{50}]) compared to the control was determined.

Selection of EVG-resistant HIV-1 variants in vitro. MT-2 cells (2×10^5 cells) were infected with HIV-1_{IIIB} and then cultured in the presence of 0.5 nM (see Fig. 3A) or 0.1 nM (see Fig. 3B) EVG. Cultures were incubated at 37°C until an

TABLE 1. Antiviral activities against laboratory HIV strains[a]

Strain	Mean EC_{50} (nM) ± SD		
	AZT	EVG	L-870,810
$HIV\text{-}1_{IIIB}$	7.1 ± 1.3	0.7 ± 0.3	6.3 ± 0.3
$HIV\text{-}2_{EHO}$	22 ± 9.1	2.8 ± 0.8	11 ± 1.9
$HIV\text{-}2_{ROD}$	19 ± 4.7	1.4 ± 0.7	8.6 ± 0.4

[a] Antiviral activity was determined using the MAGI assay. Data shown are means and standard deviations obtained from at least three independent experiments.

extensive cytopathic effect (CPE) was observed, and the culture supernatant was then harvested for further passage in fresh MT-2 cells. The concentration of EVG was increased when a significant CPE was observed. At the indicated passages (see Fig. 3A and B), proviral DNA was extracted from infected MT-2 cells and then subjected to PCR, followed by direct population-based sequencing. Susceptibility to EVG at the indicated passages was determined using the MAGI assay (see Fig. 3A) or p24 production (see Fig. 3B).

Recombinant HIV-1 clones. An HIV-1 infectious clone, pNL101 (38), kindly provided by K.-T. Jeang (NIH, Bethesda, MD), was used to generate recombinant HIV-1 clones. Wild-type HIV-1 ($HIV\text{-}1_{WT}$) was constructed by replacing the *pol* coding region (nucleotide positions 2006 of the ApaI site to 5122 of the NdeI site of pNL101) with HIV-1 strain BH10. The *pol* coding region contains a silent mutation at nucleotide 4232 (TTTAGA to TCTAGA) resulting in the generation of a unique XbaI site. Recombinant HIV-1 IN infectious clones were generated using a modified pNL101-based vector, $pNLRT_{WT}$. In brief, mutations were introduced into the XbaI-NdeI region (891 bp) of $pSLInt_{WT}$, which encodes nucleotides 4232 to 5122 of pNL101, using an oligonucleotide-based site-directed mutagenesis method (54). Next, the XbaI-NdeI fragments were inserted into pBNΔInt, which encodes nucleotides 5122 (NdeI) to 5785 (SalI) of pNL101. Finally, the XbaI-SalI region (1,554 bp) was inserted into pNL101. Each infectious clone was transfected into 293T cells. The following day, MT-2 cells were added, and the supernatants were harvested when an extensive CPE was observed.

Replication kinetics of resistant HIV-1 variants. MT-2 cells (10^5 cells) were infected with each virus preparation (500 MAGI units) for 4 h. The infected cells were then washed and cultured in the presence or absence of EVG. The culture supernatants were harvested on day 5 after infection, and p24 levels were quantified using a Retro-Tek HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix, Buffalo, NY).

Evaluation of antiretroviral activities of IN inhibitors. The MLV-based retroviral vector pRCV/LIG (15) and plasmid pcDNA-VSVG, encoding the vesicular stomatitis virus envelope glycoprotein (a generous gift from H. Miyoshi, RIKEN Bioresource Center, Tsukuba, Japan), were employed to generate viral particles. These plasmids were cotransfected into an MLV-derived Gag-Pol-expressing packaging cell line, GP293 (Clontech, Palo Alto, CA). After 48 h of transfection, culture supernatants were filtered through a 0.45-μm membrane and stored at −80°C until use.

An HIV-1-based luciferase expression vector, pBC-LIG; pCMVΔ8/9, encoding the HIV-1 viral proteins including IN; and pcDNA-VSVG were transfected into 293T cells to generate pseudotyped HIV-1. The viruses were used to infect 293T cells (10^5 cells per well in 12-well plates) at an MOI of 0.02 in the absence or presence of inhibitors. After 48 h of transduction, luciferase activity was determined using a luciferase assay system (Promega, Madison, WI) and an LB 9507 luminometer (Berthold, Bad Wildbad, Germany).

An SIV molecular clone, pMA239 (46), containing the full SIVmac239 genome, was a kind gift from E. Ido, Institute for Virus Research, Kyoto University. pMA239 was used to generate viral stocks as previously described (6). Antiviral activities of IN inhibitors against SIVmac239 were determined using the MAGI assay as described above.

Molecular modeling studies. A three-dimensional model of EVG in complex with HIV-1 IN CCD was prepared by PyMOL software, version 0.97, using previously reported data (44). Amino acid residues involved in resistance to EVG were displayed within this model.

RESULTS

Anti-HIV activities of IN inhibitors. The antiviral activity of EVG against $HIV\text{-}1_{IIIB}$, $HIV\text{-}2_{EHO}$, and $HIV\text{-}2_{ROD}$ was first evaluated by the MAGI assay. EVG showed potent antiviral activity against three laboratory strains of HIV, with EC_{50} values in the subnanomolar to nanomolar range (Table 1). Next, we evaluated the activity of EVG against wild-type clinical isolates representing various subtypes of HIV-1. EVG suppressed the replication of all HIV-1 subtypes tested, with an antiviral EC_{50} ranging from 0.10 to 1.26 nM (Table 2). Moreover, EVG suppressed the replication of HIV-1 clinical isolates carrying NRTI, NNRTI, and PI resistance-associated genotypes, as did a control IN inhibitor, the compound L-870,810 (see Table S1 in the supplemental material). The cytotoxicities of these inhibitors were also determined using an MTT colorimetric assay. Mean values for the concentration that suppresses the viability of target cells by 50% for EVG and L-870,810 in PBMC obtained from three independent donors were 4.6 ± 0.5 μM and 2.7 ± 0.6 μM, respectively. Thus, EVG can suppress various HIV strains, including diverse HIV-1 subtypes and clinical isolates carrying multiple mutations associated with resistance to currently approved antiretroviral drugs.

TABLE 2. Antiviral activities of EVG against various subtypes of HIV-1[a]

Subtype	Isolate	EC_{50} (nM)	
		AZT	EVG
A	RW/92/016	7.91	0.41
B	96USHIPS7	8.41	0.26
	BR/92/021	2.13	0.76
	BR/93/017	1.10	0.18
	BR/93/022	11.7	1.13
C	BR/92/025	2.84	0.10
D	UG/92/046	7.26	0.50
E	CMU02	9.07	1.26
F	BR/93/020	25.3	0.74
G	JV1083	11.1	0.35
O	BCF01	1.52	1.17

[a] Antiviral activity was determined using p24 ELISA.

Mechanism of anti-HIV activity of EVG. First, we performed a "time-of-addition" experiment as described previously (30), with some modifications. MT-4 cells were infected with $HIV\text{-}1_{IIIB}$ at an MOI of 0.5. One hour after infection, infected cells were extensively washed, and compounds were added, including an NNRTI (EFV at 100 nM), a PI (NFV at 500 nM), or EVG (100 nM). Amounts of p24 antigen were determined at 31 h postinfection. The antiviral activity of EFV gradually decreased from 6 h postinfection and disappeared at 12 h postinfection, whereas the antiviral activity of EVG decreased from 10 h postinfection and was no longer detected by 12 h postinfection. On the other hand, the PI NFV effectively blocked the infection up to 12 h postinfection and still exerted approximately 20% inhibitory activity up to 24 h postinfection. These results strongly suggest that EVG inhibits the HIV replication at a step that occurs after reverse transcription but before proteolytic cleavage, consistent with the integration step.

To elucidate the mode of action of EVG on HIV-1 replication, the levels of intracellular HIV-1 DNA species were determined using real-time quantitative PCR (Fig. 2A). MT-2 cells were infected with $HIV\text{-}1_{IIIB}$ in the presence or absence

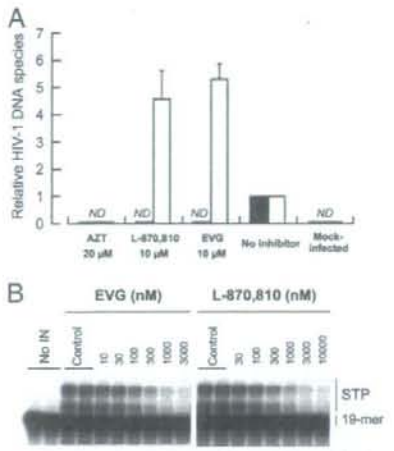

FIG. 2. Mechanism of action of EVG. (A) Quantification of HIV-1 DNA species. MT-2 cells were infected with HIV-1_{IIIB} in the presence or absence of AZT, L-870,810, and EVG. Unintegrated (2-LTR) (white bars) and integrated (black bars) forms of proviral DNA were quantified by real-time PCR and normalized to the β-globin gene after 24 h of infection. The data are represented as means and standard deviations of value relative to that of the no-inhibitor control from three independent experiments. ND means that the signals were not detected even after 40 cycles of amplification. (B) Inhibitory effect of IN inhibitors on strand transfer activity. Gel electrophoresis shows strand transfer products (STP) generated from preprocessed donor DNA substrate (19-mer) covalently bound to acceptor DNA.

of a CD4-gp120 binding inhibitor, DS5000; an NRTI, AZT; an IN inhibitor, L-870,810; and EVG. Unintegrated (2-LTR) and integrated forms of reverse-transcribed HIV-1 genomic DNA were quantified after 24 h of infection and then normalized with β-globin DNA. In the presence of 20 μM AZT, neither 2-LTR nor integrated forms were detected as expected. Similar results were also observed with 20 μM DS5000 (data not shown). In the presence of 10 μM L-870,810, integrated provirus was undetectable, while relative 2-LTR levels increased about 5-fold (4.6-fold ± 1.0-fold). Similar results were observed with 10 μM EVG (2-LTR) (5.3-fold ± 0.5-fold), indicating that EVG exerts anti-HIV activity by blocking the integration step.

To further characterize the mechanism by which EVG inhibits the integration step, the effect of EVG on strand transfer was assessed by characterizing its ability to inhibit the activity of recombinant wild-type HIV-1 IN enzyme in an oligonucleotide-based strand transfer assay (Fig. 2B). EVG and L-870,810 both inhibited the synthesis of strand transfer products with IC_{50} values of 54 nM and 118 nM, respectively. Taken together, these results indicate that like L-870,810, EVG blocks integration via the inhibition of IN-mediated strand transfer.

Selection of EVG-resistant HIV-1 variants in vitro. To determine the in vitro resistance profile of EVG, EVG-resistant viral variants were selected using a dose escalation method, and the susceptibilities of the resulting selected variants to EVG (EC_{50}) were determined. Selection of resistant HIV-1_{IIIB} was initiated with 0.5 nM EVG (Fig. 3A). At passage 12 (P-12), where the concentration of EVG was 4 nM, 2 amino acid substitutions, glutamine-to-proline at IN codon 146 (Q146P) and asparagine-to-aspartic acid at IN codon 232 (N232D), were observed (Fig. 3A). An N232D substitution was previously reported to be an IN polymorphism in HIV-1 (34). The EVG EC_{50} of a P-24 variant containing a Q146P- and N232D-substituted variant was 6.2 nM. At P-32 (32 nM EVG), a T66I IN substitution was newly observed, whereas the N232D substitution had reverted to the baseline sequence. The EVG EC_{50} against a P-36 variant was 64 nM. An S147G IN substitution was detected at P-48 (128 nM EVG), and the EVG EC_{50} further increased to 635 nM. In addition, a Q95Q/K IN substitution (mixture of Q and K) and an E138E/K IN substitution were newly identified at P-54 (256 nM EVG). These mixtures, Q95Q/K and E138E/K, fully emerged in the viral pools by P-64 and P-80, respectively. The EVG EC_{50} at P-68 (1,024 nM EVG) was greater than 1,000 nM.

An independent EVG selection experiment, again using HIV-1_{IIIB}, was performed but began at 0.1 nM EVG (Fig. 3B). An E92E/Q mixture in the IN coding region was first detected at P-30 (10 nM EVG) and was predominantly E92Q by P-38 (20 nM EVG). Additional IN substitutions, H51H/Y and S147S/G, emerged at P-60 (640 nM EVG), and an E157E/Q mixture emerged at P-70 (1,280 nM EVG); the viral pools at the terminal passage P-80 (1,280 nM EVG) had the IN sequence H51Y/E92Q/S147G/E157E/Q (Fig. 3B). The emergence of each of these mutations correlated with an increase in the EVG EC_{50} of the resulting viral pools (Fig. 3). Other than the N232D polymorphism, all of these mutations are located in the CCD of IN.

Phenotypic analysis of IN recombinant viruses. (i) EVG-selected mutations. To characterize which mutations are responsible for EVG resistance, infectious HIV-1 clones containing single IN substitutions (H51Y, T66I, E92Q, Q95K, E138K, Q146P, S147G, or E157Q) that were observed to emerge under selection with EVG were generated (Fig. 3 and Table 3). Mutations were classified into two groups based on the level of resistance: mutations that conferred more than 10-fold reduced susceptibility compared to the wild type were defined as primary mutations, and mutations conferring less than 10-fold reduced susceptibility were defined as secondary mutations. T66I and E92Q substitutions conferred significantly reduced susceptibility to EVG (37- and 36-fold reduced, respectively, relative to the wild type), whereas the Q146P and S147G substitutions conferred more moderate reductions in EVG susceptibility (11-fold reduced), indicating that these four IN mutations are primary mutations involved in resistance to EVG. In contrast, H51Y, Q95K, and E157Q substitutions all conferred smaller reductions in EVG susceptibility (each less than 6.3-fold reduced compared to the wild type), suggesting that these substitutions are secondary resistance mutations. Interestingly, the E138K mutation alone conferred no reduction in susceptibility to either EVG or L-870,810. Thus, several distinct mechanisms of resistance may be represented by these different IN mutations.

Multisubstituted clones observed during EVG selection experiments were also generated. HIV-$1_{T66I/Q146P}$ showed high-level resistance to EVG (119-fold reduced susceptibility) (Table 3). Combinations of S147G with T66I/Q146P or E92Q further enhanced resistance, 412- and 356-fold, respectively.

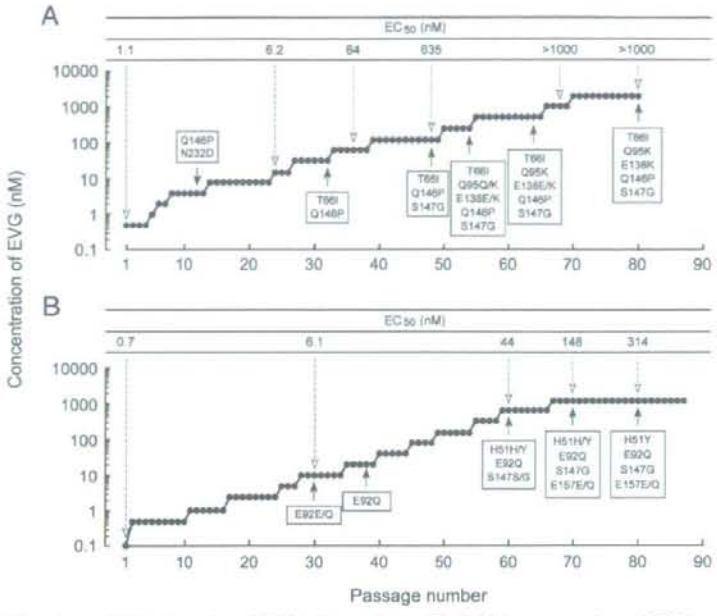

FIG. 3. Induction of EVG-resistant HIV-1. Data from MT-2 cells are shown. The initial concentrations of EVG were 0.5 nM (A) and 0.1 nM (B). Results are from two identical but independent experiments. At the indicated passage number (black arrowheads), proviral DNA extracted from infected MT-2 cells was sequenced. Amino acid substitutions are shown. The EC_{50} values of HIV-1 variants selected by EVG at the indicated passage number (white arrowheads) were determined using MAGI assay (A) or the production of p24 in MT-2 cells (B).

The triple mutant HIV-$1_{H51Y/E92Q/S147G}$ showed high-level resistance to EVG (700-fold reduced susceptibility). Interestingly, the addition of the secondary mutation H51Y, which on its own reduced EVG susceptibility only 3.6-fold, substantially enhanced resistance relative to that observed for the double mutant HIV-$1_{E92Q/S147G}$. HIV-$1_{T66I/Q95K/Q146P/S147G}$, HIV-$1_{T66I/Q95K/E138K/Q146P/S147G}$, and HIV-$1_{H51Y/E92Q/S147G/E157Q}$ mutants all showed high-level resistance to EVG, with EC_{50} values greater than 1,000 nM in all cases. These results indicate that the T66I and E92Q mutations provided the highest change (*n*-fold) in EVG susceptibility as individual resistance mutations and that the additional substitutions identified further enhance the level of resistance to EVG when combined with these primary mutations.

(ii) L-870,810-selected mutations. Infectious HIV-1 clones containing mutations (V72I, F121Y, T125K, and V151I) previously shown to be associated with resistance to L-870,810 (23) and two mutations, L74M and G163R, observed in our selection using L-870,810 (data not shown) were generated. Among these variants, HIV-1_{F121Y} and HIV-1_{V151I} demonstrated reduced susceptibility to both L-870,810 and EVG (Table 3). V151I has been observed in some HIV-1 clinical isolates and may be an IN polymorphism (34). Moreover, the effect of V151I on susceptibility to L-870,810 has been controversial (23, 29). This discrepancy might arise from the viral strain or plasmid backbone used, so further experiments to clarify the effect of V151I on IN inhibitor susceptibility are needed. HIV-$1_{F121Y/T125K}$ showed significant resistance to both L-870,810 and EVG (68-fold and 177-fold reduced susceptibility, respectively). HIV-$1_{V72I/F121Y/T125K/V151I}$ showed high-level resistance to both IN inhibitors (EC_{50} greater than 1,000 nM).

(iii) DKA IN inhibitor-selected mutations. Highlighting the potential for related mechanisms of IN inhibitor resistance and cross-resistance, the T66I mutation has also been observed to be selected by DKA IN inhibitors such as L-708,906 and S-1360. Additional mutations, L74M and S153Y, in combination with T66I were also observed to be selected by these DKA IN inhibitors (16, 17). L74M also emerged during L-870,810 selection in our studies (data not shown) but conferred no change in susceptibility to L-870,810 when present alone and only low-level resistance (3.0-fold) to EVG (Table 3). The combination of T66I and L74M conferred slightly higher resistance to EVG (45-fold) than did T66I alone but only moderate resistance to L-870,810 (7.1-fold). Another IN mutant, HIV-$1_{T66I/S153Y}$, observed in L-708,906 selection experiments (24) showed high-level resistance to EVG (260-fold) but low-level resistance to L-870,810 (5.0-fold). These results suggest that the mechanism of EVG resistance may have some similarities to that of DKA IN inhibitors.

Taken together, these results suggest that a variety of IN mutations may be selected by EVG and other IN inhibitors. Most of the IN inhibitor resistance mutations are observed to cluster in the CCD of IN. The resulting mutations and their combinations have the capacity to confer various levels of resistance and potential cross-resistance to EVG and other IN inhibitors. Given their location in the CCD, many of these mutations may act via a common mechanism. The observed development of IN inhibitor resistance mutations resembles

TABLE 3. Susceptibilities of HIV-1 IN recombinant molecular clones[a]

Molecular clone(s)	Mean EC_{50} (nM) ± SD (fold resistance compared to wild type)				
	AZT	EVG	L-870,810	S-1360	L-731,988
$HIV\text{-}1_{WT}$	32	1.1	5.8	1,239	736
EVG mutation (expt 1)[b]					
T66I[c]	43 ± 11 (1.3)	41 ± 14 (37)	4.7 ± 2.9 (0.8)	6,403 ± 2,349 (5.2)	7,234 ± 1,210 (9.8)
Q95K	34 ± 6 (1.1)	2.9 ± 0.4 (2.6)	18 ± 2 (3.1)	ND	ND
E138K	33 ± 8 (1.0)	1.1 ± 0.4 (1.0)	3.9 ± 0.4 (0.7)	ND	ND
Q146P	26 ± 2 (0.8)	12 ± 3 (11)	5.1 ± 0.4 (0.9)	ND	ND
S147G[d]	41 ± 5 (1.3)	12 ± 5 (11)	23 ± 6 (4.0)	ND	ND
T66I/Q146P	22 ± 2 (0.7)	131 ± 12 (119)	18 ± 5 (3.1)	ND	ND
T66I/Q146P/S147G	19 ± 5 (0.6)	453 ± 62 (412)	127 ± 37 (22)	ND	ND
T66I/Q95K/Q146P/S147G	31 ± 12 (1.0)	>1,000	303 ± 76 (52)	ND	ND
T66I/Q95K/E138K/Q146P/S147G	41 ± 7 (1.3)	>1,000	306 ± 76 (53)	>10,000	>50,000
EVG mutation (expt 2)[b]					
H51Y	34 ± 8 (1.1)	4.0 ± 0.6 (3.6)	3.3 ± 0.7 (0.6)	ND	ND
E92Q	32 ± 4 (1.0)	40 ± 12 (36)	63 ± 39 (11)	ND	ND
E157Q	34 ± 8 (1.1)	6.9 ± 1.4 (6.3)	52 ± 20 (9.0)	ND	ND
E92Q/S147G	39 ± 9 (1.2)	392 ± 133 (356)	587 ± 64 (101)	ND	ND
H51Y/E92Q/S147G	54 ± 6 (1.7)	769 ± 88 (699)	374 ± 100 (64)	>10,000	22,175 ± 1,299 (30)
H51Y/E92Q/S147G/E157Q	21 ± 2 (0.7)	>1,000	340 ± 26 (59)	>10,000	18,652 ± 4,575 (25)
L-870,810 mutation					
V72I	17 ± 1 (0.5)	4.3 ± 1.1 (3.9)	9.1 ± 2.5 (1.6)	ND	ND
L74M[c]	20 ± 3 (0.6)	3.3 ± 1.1 (3.0)	4.4 ± 1.7 (0.8)	1,500 ± 302 (1.2)	4,471 ± 942 (6.1)
F121Y	15 ± 1 (0.5)	28 ± 11 (25)	51 ± 23 (8.8)	ND	ND
T125K	17 ± 3 (0.5)	2.3 ± 1.1 (2.1)	9.9 ± 3.7 (1.7)	ND	ND
V151I	21 ± 4 (0.7)	11 ± 3 (10)	104 ± 29 (18)	ND	ND
G163R	22 ± 7 (0.7)	0.8 ± 0.2 (0.7)	6.5 ± 2.6 (1.1)	ND	ND
F121Y/G163R	36 ± 5 (1.1)	60 ± 20 (55)	219 ± 20 (38)	ND	ND
F121Y/T125K	38 ± 12 (1.2)	195 ± 73 (177)	393 ± 82 (68)	ND	ND
V72I/F121Y/T125K	33 ± 7 (1.0)	143 ± 25 (130)	886 ± 79 (153)	ND	ND
V72I/F121Y/T125K/V151I	64 ± 9 (2.0)	>1,000	>1,000	>10,000	>50,000
DKA mutation					
T66I/L74M	46 ± 11 (1.4)	49 ± 5 (45)	41 ± 10 (7.1)	>10,000	23,043 ± 4,886 (31)
T66I/S153Y	26 ± 8 (0.8)	285 ± 63 (259)	29 ± 9 (5.0)	>10,000	8,478 ± 1,267 (12)

[a] Antiviral activity was determined using the MAGI assay. Data shown are means and standard deviations obtained from at least three independent experiments, and resistance (*n*-fold) of the EC_{50} of the IN recombinant molecular clone compared to that of parental $HIV\text{-}1_{WT}$ is shown in parentheses. ND, not determined.

[b] EVG selection was performed in two independent experiments, and observed mutations are separately represented.

[c] Also observed in the DKA selected mutation.

[d] Observed in two independent EVG-selected experiments.

that seen for other antiretroviral drugs such as PIs; i.e., multiple mutations are introduced in a stepwise fashion and are required for high-level resistance to the selecting inhibitors (10, 50).

Strand transfer assay. To further characterize the effect of EVG-selected resistance mutations on IN function, the effect of mutations on the enzymatic activity of recombinant IN was evaluated in an in vitro strand transfer assay (Fig. 4). IN enzymes carrying the individual mutations H51Y, S147G, and E157Q had reduced strand transfer activity relative to that of the wild type (57%, 36%, and 79% of wild-type levels, respectively). Strand transfer activities of E92Q, E92Q/S147G, and H51Y/E92Q/S147G IN enzymes decreased with the accumulation of mutations from 57% to 29 and 22% of the wild type, respectively. However, the introduction of E157Q to H51Y/E92Q/S147Q partially restored strand transfer activity to 46% of wild-type activity, suggesting that E157Q may play a role in compensating for the loss of strand transfer activity resulting from the emergence of EVG resistance mutations.

The effect of EVG-selected mutations on the inhibition of strand transfer by EVG and L-870,810 was also determined (Fig. 4). Recombinant IN enzymes carrying the individual H51Y, S147G, and E157Q substitutions remained susceptible to both EVG and L-870,810 (0.7- to 2.1-fold reduced susceptibility). E92Q IN demonstrated only moderate resistance to both IN inhibitors in the strand transfer assay (4.3-fold reduced for both inhibitors). The combination of E92Q and S147G enhanced resistance to both EVG and L-870,810 (7.6- and 8.5-fold reduced susceptibility, respectively). However, unlike the IN recombinant viruses in the antiviral assay, neither the H51Y/E92Q/S147G nor the H51Y/E92Q/S147G/E157Q IN enzymes showed further enhancement of resistance in the strand transfer assay. This difference in results from the strand transfer assay versus those from the antiviral assay may reflect differences in the recombinant IN enzyme versus the viral IN enzyme in situ. Indeed, structure-activity relationship experiments described in a previous report (43) revealed that antiviral activity and in vitro enzyme inhibition were well correlated. Nevertheless, this biochemical analysis confirmed that the E92Q IN mutation confers significantly reduced suscepti-

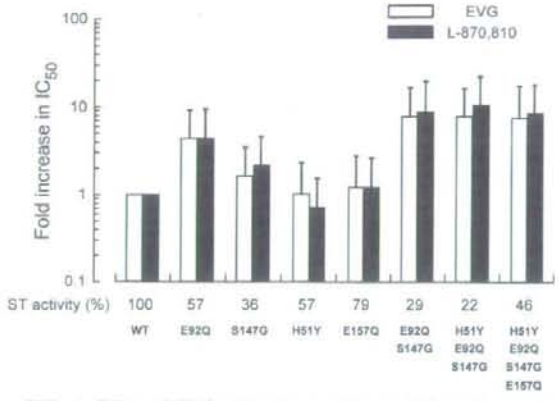

FIG. 4. Effect of EVG-selected mutations on IN strand transfer activity and on the inhibition of strand transfer by IN inhibitors. The strand transfer activities of recombinant IN enzymes carrying EVG-selected mutations were determined using an oligonucleotide-based strand transfer assay. Strand transfer (ST) activity of IN mutants was compared to that of the wild type (WT); results are shown as percentages of wild-type activity. The effect of IN inhibitors on strand transfer was also determined for wild-type and mutant IN enzymes; results are expressed as the increase (n-fold) in IC_{50} values of inhibitors relative to those of the wild type.

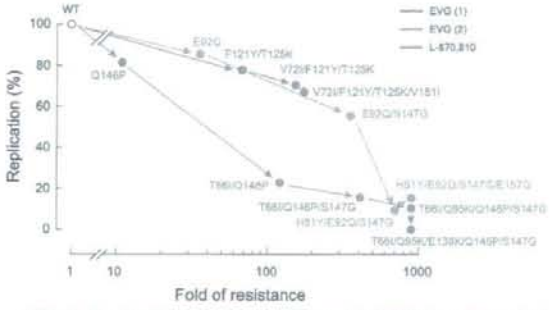

FIG. 5. Replication kinetics of EVG- and L-870,810-resistant viral variants. The replication kinetics of wild-type and IN inhibitor-resistant viral variants were determined by p24 ELISA. The relationship of replication capacity and change (n-fold) in susceptibility (shown in Table 3) is depicted. Variants are plotted according to the observed order of their emergence during selection experiments in vitro. Replication kinetics of EVG-selected mutants derived from the two independent selection experiments (shown in Fig. 3) are plotted in different colors. WT, wild type.

bility to EVG at the level of inhibition of strand transfer, consistent with its identification as a primary EVG resistance mutation in the virological analyses.

Replication kinetics of IN inhibitor-resistant variants. The effects of IN mutations on the replication kinetics of HIV-1 variants were assessed by comparing their levels of p24 production in culture supernatants to that of wild-type virus (Fig. 5). At day 5 postinfection, levels of p24 production by the HIV-1_{E92Q} and HIV-1_{Q146P} variants were 86% and 82% of HIV-1_{WT} levels, respectively. These variants showed high-level (36-fold) or moderate (11-fold) resistance to EVG (Table 3), whereas the replication levels of both were similar to those of the wild type. However, the introduction of additional EVG resistance mutations further decreased p24 production, which is indicative of a decline in the levels of viral replication. In particular, HIV-$1_{T66I/Q146P/S147G}$, HIV-$1_{T66I/Q95K/Q146P/S147G}$, HIV-$1_{T66I/Q95K/E138K/Q146P/S147G}$, HIV-$1_{H51Y/E92Q/S147G}$, and HIV-$1_{H51Y/E92Q/S147G/E157Q}$ all showed significantly reduced levels of p24 production (less than 20% of wild-type levels by day 5 in all cases). Thus, there was an inverse correlation between the levels of EVG resistance and the viral replication capacity; that is, as resistance to EVG increased, viral replication decreased. Interestingly, viral variants carrying L-870,810-selected mutations had more moderate reductions in replication capacity, even in the case of the HIV-$1_{V72I/F121Y/T125K/V151I}$ variant that had high-level resistance to both L-870,810 and EVG (68% of wild-type levels). These results indicate that mutations associated with resistance to IN inhibitors can have various effects on viral replication capacity. The reduced replication capacity of EVG-resistant variants was not rescued in the presence of the inhibitor (data not shown), as was observed previously for NFV-resistant variants in the presence of NFV (35). Thus, the reduced replication capacity of IN inhibitor-resistant variants may present a barrier to their emergence in vivo.

Antiviral effect of IN inhibitors on retroviruses. The antiviral activity of EVG against other retroviruses, including MLV and SIV, was assessed. EVG and L-870,810 inhibited the integration of the HIV-based vector used as a positive control for the luciferase assay (EC_{50} values of 0.8 and 5.0 nM, respectively), as observed in the MAGI assay with HIV-1_{IIIB} (Fig. 6). EVG and L-870,810 suppressed the replication of MLV infection (EC_{50} values of 5.8 and 22 nM, respectively) as well as that of the primate retrovirus SIV (0.5 and 3.2 nM, respectively), indicating that IN inhibitors have antiviral activity against a broad range of retroviruses.

DISCUSSION

The data described here show that EVG inhibits HIV replication by specifically blocking the strand transfer reaction mediated by IN, as demonstrated by the intracellular accumulation of 2-LTR DNA products, a signature of nonproductive integration. Furthermore, EVG directly blocked the production of strand transfer products in an in vitro strand transfer assay. Confirming that EVG is a bona fide IN inhibitor, we selected EVG-resistant viral variants in vitro and demonstrated that the resulting viral variants had acquired multiple mutations in the IN coding region and had simultaneously acquired reduced phenotypic susceptibility to EVG. HIV-1 molecular clones carrying the EVG-selected IN mutations had an EVG-resistant phenotype and in many cases also had reduced susceptibility to another IN inhibitor, L-870,810. These data provide formal proof that the observed IN mutations are indeed EVG resistance mutations and that EVG is an IN inhibitor.

Among the IN mutations observed to be selected by EVG, two mutations, T66I and E92Q, appeared to provide the major contribution to EVG resistance. Both of these individual mutations resulted in >30-fold reduced susceptibility to EVG. The T66I mutation conferred cross-resistance to S-1360 and

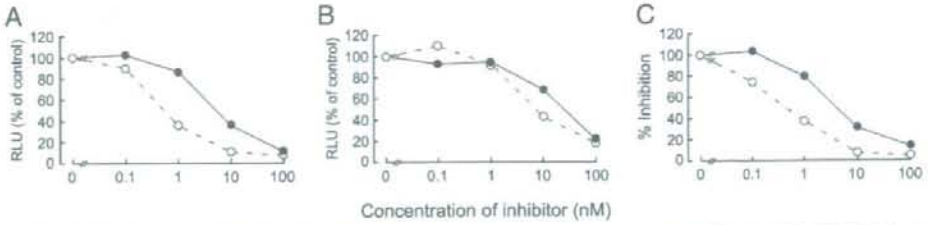

FIG. 6. Effect of IN inhibitors on retroviruses. Antiviral activities of EVG (open circles with dashed lines) and L-870,810 (closed circles with solid lines) against HIV-based (A) or MLV-based (B) vectors harboring the luciferase gene were determined by measuring luciferase activity at 48 h posttransduction. Results are expressed as percentages of relative luciferase units (RLU) compared to those of the no-inhibitor control. (C) Anti-SIV activity was determined using the MAGI assay. These results shown are one representative assay from three independent experiments.

L-731,988 (Table 3) and was also previously observed in an independent EVG selection by Jones et al. (26). The E92Q mutation, when introduced into a recombinant IN enzyme, also reduced the susceptibility of the resulting mutant IN enzyme to EVG, as measured by the reduced EVG inhibition of the in vitro strand transfer assay (Fig. 4). The other IN mutations identified, including H51Y, Q95K, E138K, Q146P, S147G, and E157Q, individually resulted in lower changes (*n*-fold) in EVG susceptibility (1.0- to 11.0-fold) but, when added to either the T66I or the E92Q mutation, further increased resistance to EVG to various degrees relative to either mutation alone. Interestingly, the accumulation of these EVG-selected IN mutations resulted in a significant attenuation of viral replication kinetics. Thus, the emergence of resistance to IN inhibitors may be associated with reductions in viral fitness, which may provide a barrier to the emergence of these mutations in vivo or be associated with lower viral loads if they do emerge.

Of the three HIV enzymes PR, RT, and IN, the structure and mechanism of IN are the least well understood, and despite extensive efforts, the structure of the complete IN enzyme remains to be determined. Only partial two-domain crystal structures of the IN apoenzyme are available, and no structure showing full-length IN bound to its viral cDNA substrate has been published. During integration in vivo, IN functions in the preintegration complex, which also includes RT and the viral DNA (2, 3). Some limited evidence suggests that RT interacts with the active site of IN (39). IN has also been proposed to function with several cellular factors including IN interactor 1 (Ini1) (27) and lens-epithelium-derived growth factor (LEDGF/p75) (7). In the context of these associated cellular factors, IN may retain a different conformation compared to that of the recombinant enzyme alone. This may be one of the reasons that only moderate EVG resistance was observed in the oligonucleotide-based strand transfer assay compared to a cell-based antiviral assay.

Alignment of several IN CCD structures deposited in the Protein Data Bank indicates that there are two regions with poorly defined or disordered structures, including residues 47 to 56 and 140 to 152 (Fig. 7; see Fig. S1 in the supplemental material). Of these two disordered regions, residues 140 to 152 have been implicated as a flexible loop involved in viral cDNA binding (20, 21, 53). Although the precise structural details are unknown, the flexible loop has been proposed to adopt different conformations in the presence or absence of the viral cDNA (12). Notably, several of the EVG-selected mutations that we observed are located on or adjacent to this proposed flexible loop, including E138K, Q146P, and S147G. The flexible loop is important for the catalytic activity of IN (21, 32), and as shown in Fig. 4, the introduction of mutations in these residues, especially S147G, drastically reduced the catalytic activity of IN. Previously published data also demonstrated that another mutation at codon 147 (S147I) resulted in HIV-1 that was highly replication defective, including effects on viral DNA synthesis (47). Indeed, S147 is highly conserved among various retroviruses (see Fig. S2 in the supplemental material), highlighting the importance of the loop for IN function. It is possible that IN inhibitor resistance mutants may have additional pleiotropic effects on processes in viral replication other

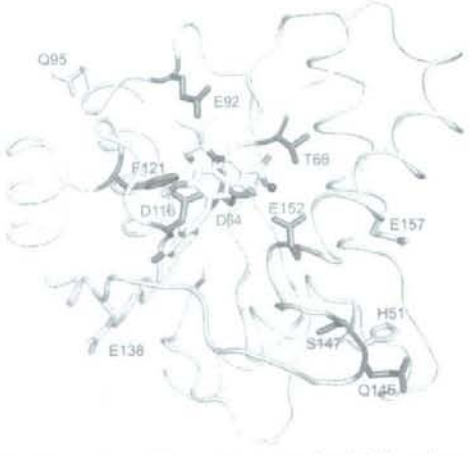

FIG. 7. Location of IN mutations associated with resistance to EVG. EVG in complex with the HIV-1 IN CCD is shown along with the catalytic triad residues (D64, D116, and E152) (green) and a magnesium ion (magenta). Amino acid residues conferring resistance to EVG as primary mutations (T66, E92, F121, Q146, and S147) or as secondary mutations (H51, Q95, E138, and E157) are shown in red and cyan, respectively. The flexible loop (residues 140 to 152) is shown in pink.

than integration; in particular, RT and IN were previously suggested to interact functionally (25).

Recently, an in silico docking simulation of HIV IN with several IN inhibitors including EVG was reported (44). Notably, that author showed that in the best-fit model for EVG docked to IN, the isobutyl substituent on the quionolone moiety of EVG orients directly towards IN residue E92. Interestingly, the hydroxyl component of the isobutyl on the quinolone replaces a water molecule that is coordinated by residue E92 between the two catalytic residues D64 and E152. This docking structure may provide insight into the mechanism of IN inhibition by EVG and provides a starting point for understanding the mechanism of EVG resistance mediated by the E92Q substitution. However, it is uncertain whether this docking simulation represents the precise binding mode of EVG with IN in vivo. Therefore, to accurately assess the binding mode of IN inhibitors with IN, available structural data need to be supplemented by a variety of other approaches. In this study, a virological approach and an enzymatic approach were integrated to characterize the mechanism of action, antiviral activity, and resistance profile of EVG in vitro.

As shown in Fig. 7, primary EVG resistance mutations are located around the catalytic triad of the CCD of IN and are surrounded by the secondary mutations. Among the residues affected by primary mutations, E92 and F121 are located close to EVG on the model and might interact with the IN inhibitor. However, the mechanism by which these mutations interact with the IN inhibitor or with the viral cDNA to mediate resistance is currently unclear. Recently, clinical isolate data from patients experiencing virologic failure in ongoing phase III studies of another IN inhibitor, raltegravir, were reported; E92Q was among the mutations noted to develop in these raltegravir failure patients, usually in combination with another IN mutation, N155H (11, 48). These preliminary clinical data and the data presented here with L-870,810, indicate that the E92Q mutation may be able to mediate resistance and potential cross-resistance to multiple IN inhibitors including EVG and raltegravir. Consistent with the data described here, site-directed mutant HIV carrying the E92Q mutation has been confirmed to show resistance to EVG and to have low-level (approximately sixfold) reduced susceptibility to raltegravir (26).

Several of the IN residues affected by primary mutations observed in EVG-selected variants including T66, E92, and S147 are absolutely conserved among the retroviruses tested (HIV-1, HIV-2, SIV, and MLV) and in retroviruses from multiple mammalian species (see Fig. S2 in the supplemental material). The significant conservation of mammalian retroviral IN CCDs at both the level of sequence homology and structure of the active site was demonstrated by the ability of EVG to inhibit HIV, SIV, and MLV IN activity. This suggests that EVG, and probably other IN inhibitors, binds to a conformationally conserved region of all retroviral INs; the binding of EVG and other IN inhibitors to IN is also likely to involve the catalytic magnesium ion. Taken together, these results suggest that several distinct mechanisms may contribute to IN inhibitor resistance, including conformational changes in the structure of IN that affect the binding of the IN inhibitor, charge effects, steric hindrance, loss of stabilizing binding interactions, or, possibly, alterations in magnesium binding.

A similar reduction in viral replication capacity as a result of drug resistance mutations was previously reported for NRTI resistance mutations (K65R, L74V, and M184V) (45, 55) and for PI resistance mutations (D30N) (49). Mutations that act to compensate for some of the loss of viral replication resulting from drug resistance, for example, GAG processing mutants, have also been described (18, 36, 52). At least one of the EVG secondary mutations, E157Q, may have an analogous role, as it partially restored strand transfer activity that was attenuated by other EVG-selected mutations and also further enhanced resistance to EVG (Fig. 4). Some secondary IN mutations might act to compensate for the altered conformation of IN resulting from the structural effects of primary resistance mutations. The E138K mutation may be such an example, as on its own, it showed no effect on susceptibility to either EVG or L-870,810. The clinical implications of the reduction in fitness resulting from the selection of EVG-resistant mutations are not yet understood.

In conclusion, EVG is a potent inhibitor of the HIV IN enzyme that acts by blocking the strand transfer reaction and is effective not only against HIV but also against other retroviruses. Moreover, the emergence of viral variants that were highly resistant to EVG was associated with significant reductions in viral replication in vitro. These results indicate that EVG should be highly effective for the treatment of HIV-1-infected patients, including those who have had virologic failure of their highly active antiretroviral therapy due to the emergence of HIV-1 drug resistance to approved antiretroviral drugs.

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