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Mechanism of resistance to S138A substituted enfuvirtide and its application to peptide design

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ABSTRACT

T-20 (enfuvirtide) resistance is caused by the N43D primary resistance mutation at its presumed binding site at the N-terminal heptad repeat (N-HR) of gp41, accompanied by the S138A secondary mutation at the C-terminal HR of gp41 (C-HR). We have discovered that modifying T-20 to include S138A (T-20_{S138A}) allows it to efficiently block wild-type and T20-resistant viruses, by a mechanism that involves improved binding of T-20_{S138A} to the N-HR that contains the N43D primary mutation. To determine how HIV-1 in turn escapes T-20_{S138A} we used a dose escalation method to select T-20_{S138A}-resistant HIV-1 starting with either wild-type (HIV-1_{WT}) or T-20-resistant (HIV-$1_{N43D/S138A}$) virus. We found that when starting with WT background, I37N and L44M emerged in the N-HR of gp41, and N126K in the C-HR. However, when starting with HIV-$1_{N43D/S138A}$, L33S and I69L emerged in N-HR, and E137K in C-HR. T-20_{S138A}-resistant recombinant HIV-1 showed cross-resistance to other T-20 derivatives, but not to C34 derivatives, suggesting that T-20_{S138A} suppressed HIV-1 replication by a similar mechanism to T-20. Furthermore, E137K enhanced viral replication kinetics and restored binding affinity with N-HR containing N43D, indicating that it acts as a secondary, compensatory mutation. We therefore introduced E137K into T-20_{S138A} (T-$20_{E137K/S138A}$) and revealed that T-$20_{E137K/S138A}$ moderately suppressed replication of T-20_{S138A}-resistant HIV-1. T-$20_{E137K/S138A}$ retained activity to HIV-1 without L33S, which seems to be a key mutation for T-20 derivatives.

Our data demonstrate that secondary mutations can be consistently used for the design of peptide inhibitors that block replication of HIV resistant to fusion inhibitors.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) fusion to host cell membrane is mediated by formation of a six-helix bundle of the transmembrane subunit gp41 (Chan et al., 1997). Peptides corresponding to amino acid sequences of the gp41 carboxyl-terminal heptad repeat (C-HR) inhibit the HIV-1 fusion by acting as decoys and interfering with the formation of the six-helix bundle (Chan et al., 1998; Malashkevich et al., 1998). Although modified peptides such as SC34EK (Nishikawa et al., 2009), T-2635 (Dwyer et al., 2008), and D-peptides (Welch et al., 2007), and small molecules (Debnath et al., 1999) have been developed, T-20 (enfuvirtide) is the only fusion inhibitor approved for HIV therapy. It is a 36 amino acid peptide derived from the sequence of C-HR of gp41. It is thought to bind at the N-HR domain of gp41and interfere with the C-HR-N-HR interactions required for membrane fusion and injection of virus into the host cell. T-20 has potent anti-HIV-1 activity and effectively suppresses replication of HIV-1 *in vivo* (Kilby et al., 1998; Lalezari et al., 2003; Lazzarin et al., 2003). However, HIV-1 rapidly develops resistance through mutations in the amino-terminal HR (N-HR) of gp41, especially in the region between L33 and L45, which

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is thought to be the binding site of T-20 (Aquaro et al., 2006; Cardoso et al., 2007; He et al., 2008). Among these residues, N43D in the N-HR is one of the representative mutations for resistance to T-20 (Bai et al., 2008; Cabrera et al., 2006; Oliveira et al., 2009; Izumi et al., 2009; Ueno et al., 2009). Interestingly, most variants show impaired replication fitness, and thus often go on to acquire secondary mutations, such as S138A (Xu et al., 2005), in the C-HR region of gp41 that corresponds to the sequence of T-20. We and others have recently demonstrated that S138A functions as secondary resistance mutation and enhances resistance to T-20 by restoring impaired replication kinetics of T-20-resistant variants that contain primary mutations in the N-HR region, most notably N43D (Izumi et al., 2009; Watabe et al., 2009).

To preempt this escape strategy, we have previously designed a peptide analog of T-20 with the S138A change incorporated in it (T-20_{S138A}; Fig. 1A) and showed that this peptide significantly suppresses replication of T-20-resistant HIV-1 through enhancement of binding affinity to mutated N-HR, such as N-HR_{N43D} (Izumi et al., 2009). Using circular dichroism (CD) and structural analyses, we also demonstrated that the S138A change provided increased stability to the six-helix bundle (Watabe et al., 2009). In subsequent studies, we validated our approach on another peptide-based fusion inhibitor, C34. In this case, we designed a variant of C34 carrying a secondary escape mutation, N126K, selected for the induction of C34 resistance (Nameki et al., 2005) and also present in HIV-1 isolates from T-20 experienced patients (Baldwin et al., 2004; Cabrera et al., 2006; Svicher et al., 2008). We showed that this C34 variant can effectively inhibit replication of C34-resistant HIV-1. These studies provided the proof of principle that it is possible to design improved peptide-based fusion inhibitors that are efficient against a major mechanism of drug resistance through introduction of resistance-associated mutation(s).

It remains unknown to this date how HIV-1 develops further resistance to T-20_{S138A}. Moreover, it is not known whether we can expand our strategy and modify T-20_{S138A} to include the secondary mutation(s) that emerge during the selection of T-20_{S138A}-resistant HIV, resulting in a strategy that is applicable to the design of peptides customized to address viral resistance mutations. Hence, in the current study we selected T-20_{S138A}-resistant HIV-1 *in vitro* by a dose-escalating method. We revealed that the resistance mutations that emerged during selection experiments with wild-type or T-20-resistant HIV-1 are located in both the N-HR and the C-HR regions. Furthermore, the I37N and L33S mutations appeared to act as primary mutations for wild-type and T-20-resistant HIV-1, respectively. E137K, a C-HR mutation located in the T-20 sequence, improved replication kinetics and enhanced affinity to N-HR, indicating that E137K acts as a secondary mutation. Introducing the E137K change into the T-20_{S138A} (T-$20_{E137K/S138A}$) resulted into a peptide inhibitor effective against T-20_{S138A}-resistant variants, suggesting that secondary or compensatory mutations can be widely applicable to the design of next generation peptide-based inhibitors that are active against HIV-1 resistant to earlier generation fusion-targeting drugs.

2. Materials and methods

2.1. Cells and viruses

MT-2 and 293T cells were grown in RPMI 1640 medium and Dulbecco's modified Eagle medium-based culture medium, respectively. HeLa-CD4-LTR-β-gal cells were kindly provided by Dr. M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease (Bethesda, MD), and used for the drug susceptibility assay, as previously described (Nameki et al., 2005; Nishikawa et al., 2009). Recombinant infectious HIV-1 clones carrying various mutations were generated through site-directed mutagenesis of the pNL4-3 plasmid, as previously described (Nameki et al., 2005; Nishikawa et al., 2009). Each molecular clone was transfected into 293T cells with *Trans*IT (Madison, WI). After 48 h, the supernatants were harvested and stored at −80 °C.

2.2. Antiviral agents

The peptides used in this study (Fig. 1A) were chemically synthesized using standard Fmoc-based solid-phase techniques, as previously described (Oishi et al., 2008; Otaka et al., 2002). An HIV-1 reverse transcriptase inhibitor, 2′,3′-dideoxycytidine (ddC) was purchased from Sigma–Aldrich Japan (Tokyo, Japan) and used as a control.

2.3. Determination of drug susceptibility

Peptide sensitivity of infectious clones was determined by the multinuclear activation of galactosidase indicator (MAGI) assay as previously described (Nameki et al., 2005; Nishikawa et al., 2009). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 cells/well) were plated in flat 96-well microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 clones (60 MAGI units/well, resulting into 60 blue cells after 48 h incubation) and cultured in the presence of various concentrations of drugs in fresh medium. Forty-eight hours after virus exposure, all the blue cells stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that reduced HIV-1 infection by 50% (50% effective concentration [EC_{50}]).

2.4. Induction of HIV-1 variants resistant to T-20_{S138A}

MT-2 cells were exposed to HIV-1 and cultured in the presence of T-20_{S138A}. Cultures were incubated at 37 °C until an extensive cytopathic effect (CPE) was observed. The culture supernatants were used for further passages in MT-2 cells in the presence of two-fold increasing concentrations of T-20_{S138A} when massive CPEs were seen in the earlier periods. Each passage usually took 5–7 days. The timing is highly dependent on the type of specific mutations introduced, as previously reported (Nameki et al., 2005; Shimura et al., 2010). For example, a passage that follows introduction of novel mutation(s) should shorten the passage period to perhaps 4–5 days. However, there will be longer delays for passages where there are no novel mutations or when there is appearance of only secondary mutations. The dose-escalation process was repeated until resistant variants were obtained. This selection was carried out for a total of 60 passages (approximately 1 year). At the indicated passages (Fig. 1B and C), the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from the infected MT-2 cells.

2.5. Viral replication kinetics assay

MT-2 cells (10^5 cells/1 mL) were infected with each virus preparation (500 MAGI units) for 16 h. Infected cells were then washed and cultured in a final volume of 3 mL. The culture supernatants were collected on day 2 through day 5 post-infection, and amounts of p24 antigen were determined.

2.6. CD spectroscopy

Each peptide was incubated at 37 °C for 30 min (the final concentrations of peptides were 10 μM in phosphate buffered saline [PBS];

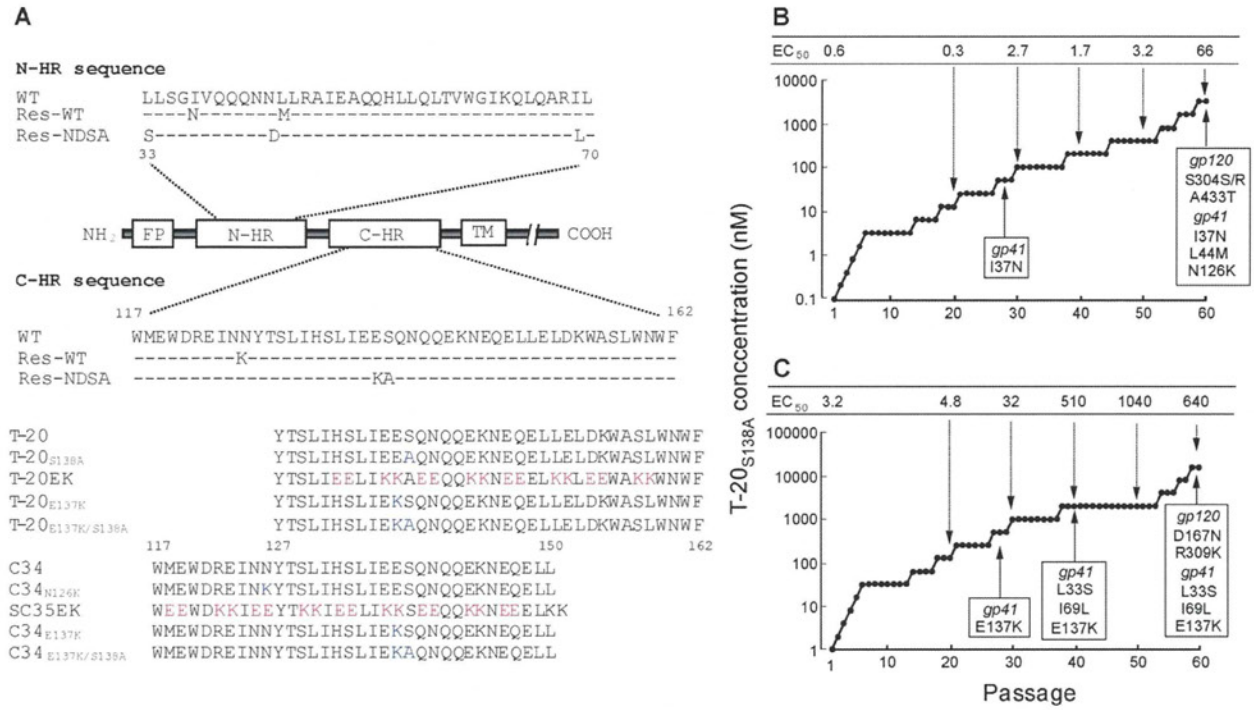


Fig. 1. Domains of gp41 and induction of T-20_{S138A}-resistant HIV-1. (A) Domains of gp41, substitutions observed during *in vitro* passage with T-20_{S138A}, and amino acid sequences of T-20- and C34-based peptides used in this study. The locations of the fusion peptide (FP), amino-terminal heptad region (N-HR), carboxyl-terminal heptad region (C-HR), transmembrane domain (TM), and C-HR-derived peptides are shown. The residue numbers of T-20 and C34 correspond to their positions in gp41. Substitutions of N- and C-HR in gp41 of wild-type (WT) and T-20_{S138A}-resistant HIV-1 are shown. Res.-WT and Res.-NDSA indicate resistant HIV-1 that were initially selected from wild-type and HIV-$1_{N43D/S138A}$, respectively. (B and C) Induction of T-20_{S138A}-resistant HIV-1 by dose-escalating selection in MT-2 cells. Induction of resistant HIV-1 was carried out for a total of 60 passages of HIV-1_{WT} (B) and HIV-$1_{N43D/S138A}$ (C), in 0.1 nM and 1 nM of T-20_{S138A}, respectively. At the indicated passages, proviral DNA was sequenced, and the EC_{50} values of the HIV-1 variants were determined using the MAGI assay. To improve the replication kinetics, substitution of D36G was introduced into the NL4-3 background used in this study (wild-type virus) (Izumi et al., 2009; Mink et al., 2005).

pH 7.4). CD spectra were recorded on an AVIV model 202 spectropolarimeter (Aviv Instruments, Proterion Corporation, Piscataway, NJ) with a 1 mm path-length cuvette at 25 °C as the average of eight scans. The thermal stability was assessed by monitoring the change in the CD signal at 222 nm. The midpoint of the thermal unfolding transition (melting temperature [T_m]) of each complex was determined as previously described (Izumi et al., 2009).

3. Results

3.1. Selection of HIV-1 resistant to T-20_{S138A}

An HIV-1_{NL4-3} strain containing a D36G substitution, which improves replication kinetics, was used as a wild-type virus (HIV-1_{WT}) and for the construction of various mutants, as described (Izumi et al., 2009; Mink et al., 2005). HIV-1_{WT} or T-20-resistant HIV-$1_{N43D/S138A}$ were used for selection of T-20_{S138A}-resistant HIV-1. MT-2 cells were infected with HIV-1_{WT} and HIV-$1_{N43D/S138A}$, and incubated in the presence of T-20_{S138A} at the initial concentrations of 0.1 nM and 1 nM, respectively. At the indicated passages, the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from the infected MT-2 cells. During the selection, mutations in the gp41 were observed and are shown in Fig. 1B and C.

In the selection with HIV-1_{WT} (Fig. 1B), at passage 28 (P-28), when T-20_{S138A} concentration was 51.2 nM (P-28, 51.2 nM), isoleucine at position 37 in the gp41 was substituted to asparagine (I37N). At P-60 (3.3 μM), L44M and N126K in the gp41 further emerged. On the other hand, in the selection with T-20-resistant HIV-$1_{N43D/S138A}$ (Fig. 1C), at P-28 (512 nM) and at P-40 (2 μM), E137K in the gp41, and L33S and I69L in the gp41 emerged, respectively. The emergence of the I69L mutation in diverse HIV-1 strains has been previously reported (Eshleman et al., 2007). At P-60, the resistance of selected viruses from HIV-1_{WT} and HIV-$1_{N43D/S138A}$ to T-20_{S138A}, reached approximately 110- and 200-fold, respectively. These results indicate that even though T-20_{S138A} was active against T-20 resistant variants, resistant HIV-1 emerged relatively rapidly compared with the next generation fusion inhibitors, such as SC34EK, which required 120 passages to acquire the resistance (Shimura et al., 2010).

3.2. Susceptibility of T-20_{S138A}-resistant HIV-1 to T-20 and C34 derivatives

To validate our resistance data we used site-directed mutagenesis to prepare recombinant HIV-1 with the T-20_{S138A}-resistance mutations and examined its susceptibility to T-20 and C34 derivatives with MAGI assay (Table 1). We also used as controls the modified α-helix T-20- and C34-peptide inhibitors, T-20EK (Oishi et al., 2008) and SC35EK (Nishikawa et al., 2009; Shimura et al., 2010), respectively, which are more efficient *in vitro* replication inhibitors of T-20-resistant HIV-1 than T-20 or C34. Finally, we also used as a control $C34_{N126K}$, a modified version of C34 that includes the resistance-associated N126K substitution that effectively suppress replication of C34-resistant HIV-1 *in vitro* (Izumi et al., 2009).

Selected mutations I37N and L33S provided various levels of resistance to T-20 and its derivatives, T-20_{S138A} and T-20EK, apparently acting as primary mutations to peptides with a T-20 backbone (Table 1). Other mutations, L44M, I69L, and E137K, which were

Table 1
Antiviral activity of C-HR-derived peptides against gp41 recombinant viruses.

	EC_{50} (nM)					
	T-20	T-20_{S138A}	T-20EK	C34	$C34_{N126K}$	SC35EK
HIV-1_{WT} [a]	2.4 ± 0.6	0.6 ± 0.1	1.9 ± 0.5	2.1 ± 0.7	1.6 ± 0.5	2.4 ± 0.9
HIV-1_{I37N}	47 ± 6.9 (**20**)	4.3 ± 1.3 (7.2)	21 ± 2.4 (**11**)	3.3 ± 1.1(1.6)	1.9 ± 0.1 (1.2)	1.0 ± 0.4(0.4)
HIV-1_{L44M}	4.1 ± 1.2 (1.7)	0.7 ± 0.2 (1.2)	2.2 ± 0.6 (1.2)	1.1 ± 0.3(0.5)	0.8 ± 0.2 (0.5)	0.6 ± 0.2(0.3)
HIV-1_{N126K}	4.4 ± 1.3 (1.8)	1.2 ± 0.4 (2.0)	2.8 ± 0.2 (1.5)	6.3 ± 1.2(3.0)	1.5 ± 0.2 (0.9)	3.3 ± 0.2(1.4)
HIV-$1_{I37N/N126K}$	660 ± 180(**275**)	16 ± 4.8 (**27**)	14 ± 5.1 (7.4)	20 ± 4.5(9.5)	3.4 ± 0.4 (2.1)	2.9 ± 0.3(1.2)
HIV-$1_{I37N/L44M/N126K}$	>1000 (>417)	130 ± 40(220)	240 ± 95(126)	66 ± 23 (31)	4.0 ± 0.8 (2.5)	1.1 ± 0.1(0.5)
HIV-1_{L33S}	23 ± 5.5 (9.6)	3.1 ± 0.6 (5.2)	13 ± 2.6 (6.8)	3.2 ± 1.1(1.5)	2.1 ± 0.1 (1.3)	3.0 ± 0.8(1.2)
HIV-1_{N43D}	49 ± 10 (**20**)	3.5 ± 0.9 (5.8)	4.1 ± 1.2 (2.2)	4.4 ± 0.4(2.1)	1.4 ± 0.1 (0.8)	0.4 ± 0.2(0.2)
HIV-1_{I69L}	2.1 ± 0.5 (0.9)	0.5 ± 0.2 (0.8)	2.2 ± 0.4 (1.2)	2.7 ± 0.2(1.3)	2.2 ± 0.5 (1.4)	2.7 ± 0.5(1.1)
HIV-1_{E137K}	2.0 ± 0.3 (0.8)	0.7 ± 0.1 (1.2)	2.5 ± 0.4 (1.3)	2.6 ± 0.2(1.2)	2.3 ± 0.7 (1.4)	3.1 ± 0.8(1.3)
HIV-$1_{N43D/S138A}$	84 ± 16 (**35**)	3.2 ± 1.0 (5.3)	3.4 ± 1.1 (1.8)	2.7 ± 0.2(1.3)	1.6 ± 0.5 (1.0)	0.3 ± 0.1(0.1)
HIV-$1_{L33S/N43D/S138A}$	>1000 (**>417**)	550 ± 72(**174**)	330 ± 94 (14)	30 ± 9.2(2.6)	4.2 ± 1.2 (0.4)	0.9 ± 0.3(0.4)
HIV-$1_{N43D/E137K/S138A}$	110 ± 31 (**46**)	14 ± 4.7 (**23**)	7.0 ± 2.4 (3.7)	7.4 ± 1.9(3.5)	2.1 ± 0.7 (1.3)	1.9 ± 0.6(0.8)
HIV-$1_{L33S/N43D/E137K/S138A}$	>1000 (**>417**)	>1000(**>1667**)	>1000 (**>526**)	31 ± 5.0 (**15**)	6.7 ± 1.7 (4.2)	1.2 ± 0.2(0.5)
HIV-$1_{L33S/N43D/I69L/E137K/S138A}$	>1000 (**>417**)	>1000(**>1667**)	>1000 (**>526**)	50 ± 12 (**24**)	28 ± 7.1(**17.5**)	1.0 ± 0.9(0.4)

Anti-HIV activity was determined using the MAGI assay. Fifty percent effective concentration (EC_{50}) values and SD were obtained from the results of at least three independent experiments. Shown in parentheses are the fold-increases in resistance (increase in EC_{50} value) calculated by comparison to a wild-type virus (HIV-1_{WT}). Increases of over 10-fold are indicated in bold.

[a] To improve the replication kinetics, substitution of D36G, observed in majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (wild-type virus; HIV-1_{WT}) (Izumi et al., 2009; Mink et al., 2005).

observed in wild-type HIV-1 as polymorphisms (Kuiken et al., 2010; Loutfy et al., 2007), conferred little resistance to all peptide fusion inhibitors tested. However, introduction of L44M to HIV-$1_{I37N/N126K}$ (HIV-$1_{I37N/L44M/N126K}$) remarkably enhanced resistance to T-20 derivatives. This was consistent with previous studies that also reported a resistance enhancement (1.8-fold) by L44M to T-20 (Loutfy et al., 2007). Collectively, these data suggest that L44M has as a role in HIV-1 resistance as a secondary mutation. All peptides sufficiently suppressed HIV-1_{I69L}, suggesting that I69L may be a secondary mutation or a polymorphism. N126K conferred only marginal resistance (<3-fold) to all peptide fusion inhibitors, but in the background of I37N (HIV-$1_{I37N/N126K}$) it enhanced resistance to T-20, T-20_{S138A}, and C34. L33S, which was originally reported as a C34 resistance associated mutation (Armand-Ugon et al., 2003), significantly enhanced resistance in the background of N43D/S138A mutations (HIV-$1_{L33S/N43D/S138A}$). Similar to the N126K mutation, E137K also enhanced resistance by N43D/S138A (HIV-$1_{N43D/E137K/S138A}$) and L33S/N43D/S138A (HIV-$1_{L33S/N43D/E137K/S138A}$) to T-$20_{S138A}$, T-20, and T-20EK. These results indicate that L33S and I37N appear to be primary mutations for T-20 derivatives.

3.3. Effect of substitutions in the gp120 on peptide susceptibility

Polymorphisms in the gp120 that influence co-receptor usage may influence T-20 susceptibility (Labrosse et al., 2003; Reeves et al., 2002). Meanwhile, others reported that T-20 susceptibility was not influenced by co-receptor usage (Cilliers et al., 2004; Melby et al., 2006). Resistance induction experiments performed in this study revealed that most laboratory strains with *in vitro* resistance to fusion inhibitors acquired substitutions in both the gp120 and the gp41 (Armand-Ugon et al., 2003; Eggink et al., 2011; Fikkert et al., 2002; Izumi et al., 2010; Nameki et al., 2005; Shimura et al., 2010). However, most substitutions showed little impact on resistance, and only contributed to a small enhancement of replication capacity (Eggink et al., 2011; Izumi et al., 2010; Nameki et al., 2005; Shimura et al., 2010). In the present study, we examined peptide susceptibility of cloned viruses that contain all Env substitutions observed in the selection (both gp120 and gp41). Most substitutions in the gp120 attenuated resistance to fusion inhibitors (Table 3). Therefore, *in vitro* experiments showed that substitutions in the gp120 are not likely associated with resistance.

3.4. Influence of mutations in the gp41 on HIV-1 replication

To address the effects of mutations on HIV-1 replication, we examined the replication kinetics of T-20_{S138A}-resistant HIV-$1_{N43D/S138A}$ variants. Consistent with a previous report (Lohrengel et al., 2005), the L33S mutation did not significantly affect the replication kinetics and infectivity compared with those of HIV-1_{WT} (Fig. 2A). The S138A mutation restored the replication

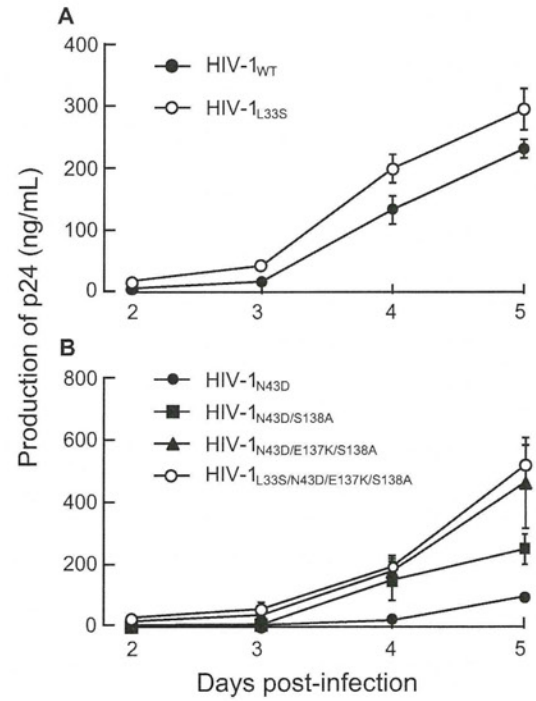


Fig. 2. Replication kinetics of T-20_{S138A}-resistant variants. Replication kinetics of T-20_{S138A}-resistant recombinant variants that introduced L33S mutation (A), or combinations of L33S, E137K, and S138A mutations in HIV-1_{N43D} (B). To improve replication kinetics, the D36G polymorphism was introduced into the NL4-3 background used in this study (HIV-1_{WT}). Supernatants from infected MT-2 cells were collected on days 2–7 and the amount of p24 produced was determined. Representative results are shown as mean values with standard deviations estimated from three independent experiments.

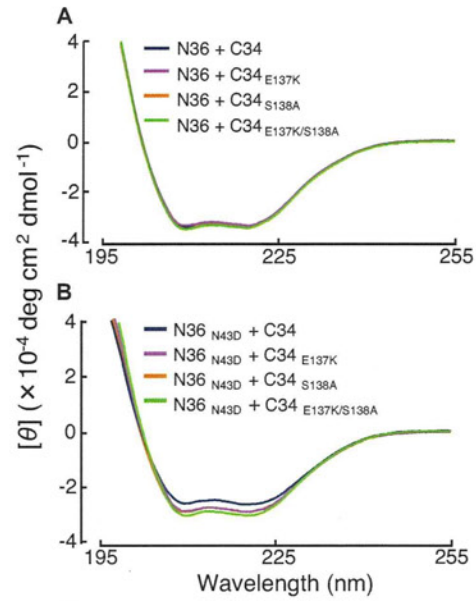
kinetics of HIV-1_{N43D} (Fig. 2B), as previously described (Izumi et al., 2009). E137K was also associated with N43D mutation *in vivo* (Svicher et al., 2008), and restored infectivity impaired by N43D (Tolstrup et al., 2007). Introduction of E137K into N43D/S138A enhanced the replication kinetics, and further addition of L33S to N43D/E137K/S138A resulted in equivalent replication kinetics compared with HIV-$1_{N43D/E137K/S138A}$ (Fig. 2B) as observed in HIV-1_{WT} based mutants. During the passage of HIV-$1_{N43D/S138A}$, a synonymous mutation at amino acid position L44, TTG to CTG, was observed. Interestingly, $L_{TTG}44L_{CTG}$ enhanced viral replication kinetics through enhanced stability of the Rev-responsive element (RRE) secondary structure (Ueno et al., 2009). Therefore, we examined the viral replication kinetics of mutants with $L_{TTG}44L_{CTG}$, and compared HIV-1_{WT}, with HIV-$1_{L44L\text{-}CTG}$, and HIV-$1_{L33S/N43D/L44L\text{-}CTG/E137K/S138A}$ with HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$. As expected, the presence of $L_{TTG}44L_{CTG}$ enhanced replication in all viruses. Surprisingly, mutants with resistance mutations showed enhanced replication kinetics as determined by the p24 production assay of culture supernatants (Fig. 4A). Therefore, we further examined infectivity using the MAGI assay and determined that the infectivity of resistance variants containing $L_{TTG}44L_{CTG}$ was reduced compared with HIV-1_{WT} (Fig. 4B). These results indicate that the primary mutation, L33S, possesses less ability to attenuate HIV-1 replication, while I69L, S138A, and E137K enhance replication kinetics of T-20-resistant HIV-1 to a comparable level of HIV-1_{WT}.

3.5. Circular dichroism

To clarify the effect of E137K substitutions on peptide binding, we examined the binding affinities of E137K-containing C-HR peptides to N-HR using CD analysis. CD spectra reveal the presence of stable α-helical structures of six-helix bundles that are required for biological activity and are thought to mechanistically and thermodynamically correlate with HIV-1 fusion (Bianchi et al., 2005). Since *in vitro* T-20 does not interact with the N36 peptide (amino acid positions 35–70 of the N-HR), we used instead peptide C34 with E137K and/or S138A substitutions (Fig. 1A). We found that mixtures of $C34_{E137K}$, $C34_{S138A}$, or $C34_{E137K/S138A}$ with N36 or $N36_{N43D}$ showed sufficient and comparable α-helicity at 25 °C (Fig. 3A and B). We also determined the thermal stability of the helical complexes formed by the N36 and C34 peptides, which is also an indication of the binding affinity of these peptides. Hence, we measured and compared the melting temperatures (T_m) of various complexes, which indicates the 50% disruption of the six-helix bundle (Fig. 3C). Complexes of N36 and C34 containing the S138A and E137K/S138A substitutions (N36/$C34_{S138A}$ and N36/$C34_{E137K/S138A}$, respectively), showed higher thermal stability than N36/C34. Similarly, S138A and E137K/S138A restored the binding affinity of C34 to $N36_{N43D}$. These results indicate that E137K acts as a compensatory mutation for the T-20_{S138A}-resistance primary mutation, causing enhancement of replication kinetics.

3.6. Antiviral activity of E137K-modified peptides

Recently, we demonstrated that introduction of the S138A secondary mutation to T-20 (T-20_{S138A}) enhanced binding to mutated N-HR and suppresses resistance of T-20-resistance variants (Izumi et al., 2009). Similarly, as shown in Fig. 3, E137K enhanced binding affinity with N-HR, suggesting that introduction of E137K to T-20 may enhance the antiviral activity of T-20. We synthesized T-20 and T-20_{S138A} variants containing the E137K change (T-20_{E137K} and T-$20_{E137K/S138A}$) (Fig. 1A) and examined their anti-HIV activity against T-20_{S138A}-resistant HIV-1 (Table 2). All peptides exhibited potent antiviral activity against HIV-1_{WT}. HIV-$1_{L33S/N43D/S138A}$ and HIV-$1_{I37N/L44M/N126K}$ showed high resistance to T-20_{E137K},



C

	T_m (°C)	
	N36	$N36_{N43D}$
C34	67.6	53.2
$C34_{E137K}$	68.9	56.7
$C34_{S138A}$	73.2	58.0
$C34_{E137K/S138A}$	74.6	63.3

Fig. 3. CD spectra (A and B) and thermal stability (C) of N36/C34 complexes. Peptide sequences used in this study are shown in FIG 1A and have also been previously described (Izumi et al., 2009). CD spectra of $C34_{E137K}$, $C34_{S138A}$, and $C34_{E137K/S138A}$ complexes with N36 (A) and $N36_{N43D}$ (B) are shown. Equimolar amounts (10 μM) of the N- and C-HR peptides were incubated at 37 °C for 30 min in PBS. The CD spectra of each mixture were then collected at 25 °C using a Jasco (Model J-710) spectropolarimeter. (C) Thermal stabilities, defined as the midpoint of the thermal unfolding transition (T_m) values, of the potential six-helix bundles of N- and C-HR peptides, were determined.

indicating that the resistance mechanism of T-20_{E137K} is similar to that of T-20_{S138A}. On the other hand, T-$20_{E137K/S138A}$ (Table 2) maintained some antiviral activity against HIV-$1_{L33S/N43D/S138A}$, HIV-$1_{L33S/N43D/E137K/S138A}$, and HIV-$1_{I37N/L44M/N126K}$ compared with other T-20 derivatives including electrostatically constrained T-20EK (Table 1 and Fig. 1). $C34_{E137K}$ and $C34_{E137K/S138A}$ significantly suppressed all HIV-1 variants tested except for HIV-$1_{I37N/L44M/N126K}$ by $C34_{E137K}$. These results indicate that peptides with resistant mutations may sustain their activity against particular resistant variants.

4. Discussion

The current study describes the introduction of resistance changes into the original and modified (T-20_{S138A}) versions of the T-20 peptide-fusion inhibitor. We analyzed the new T-20 derivatives using both wild-type and T-20-resistant strains. We also identified through dose escalation experiments, T-20_{S138A}-resistants. We found that T-20_{S138A}-resistant HIV-1 showed cross-resistance only to the T-20 derivatives, but not to C34 derivatives. Through the CD analysis, the N126K and E137K mutations in the C-HR may act as compensatory mutations for impaired interaction by a primary mutation, I37N and N43D in the N-HR, respectively. Since E137K is located within the T-20 sequence, we synthesized and characterized the activity of novel T-20-based peptides containing E137K (T-20_{E137K}). Here we demonstrate that

Table 2
Antiviral activity of E137K-induced C-HR peptides against T-20_{S138A}-resistant variants.

	EC_{50} (nM)			
	T-20_{E137K}	T-$20_{E137K/S138A}$	$C34_{E137K}$	$C34_{E137K/S138A}$
HIV-1_{WT} [a]	0.8 ± 0.2	0.5 ± 0.1	1.0 ± 0.3	0.7 ± 0.2
HIV-1_{L33S}	13 ± 3.2 (**16**)	2.2 ± 0.4 (4.5)	0.7 ± 0.2(0.7)	0.5 ± 0.1(0.7)
HIV-$1_{N43D/S138A}$	4.2 ± 0.7 (5.3)	0.7 ± 0.2 (1.4)	0.3 ± 0.1(0.3)	0.4 ± 0.1(0.6)
HIV-$1_{L33S/N43D/S138A}$	700 ± 150 (**880**)	45 ± 9.9 (**90**)	2.3 ± 0.4(2.3)	0.5 ± 0.2(0.7)
HIV-$1_{N43D/E137K/S138A}$	12 ± 3.6 (**15**)	2.4 ± 0.8 (4.8)	0.2 ± 0.1(0.2)	0.4 ± 0.1(0.6)
HIV-$1_{L33S/N43D/E137K/S138A}$	480 ± 47 (**600**)	36 ± 3.1 (**72**)	3.8 ± 1.3(3.8)	1.0 ± 0.4(1.4)
HIV-$1_{L33S/N43D/I69L/E137K/S138A}$	1808 ± 852(2260)	157 ± 83(314)	4 ± 2 (4)	1.0 ± 0.4(1.4)
HIV-$1_{I37N/L44M/N126K}$	200 ± 24 (**250**)	30 ± 8.7 (**60**)	17 ± 3.8 (**17**)	2.2 ± 0.3(3.1)

Anti-HIV activity was determined using the MAGI assay. Fifty percent effective concentration (EC_{50}) values and SD were obtained from the results of at least three independent experiments. Shown in parentheses are the fold-increases in resistance (increase in EC_{50} value) calculated by comparison to a wild-type virus (HIV-1_{WT}). Increases of over 10-fold are indicated in bold.

[a] To improve the replication kinetics, substitution of D36G, observed in majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (wild-type virus; HIV-1_{WT}) (Izumi et al., 2009; Mink et al., 2005).

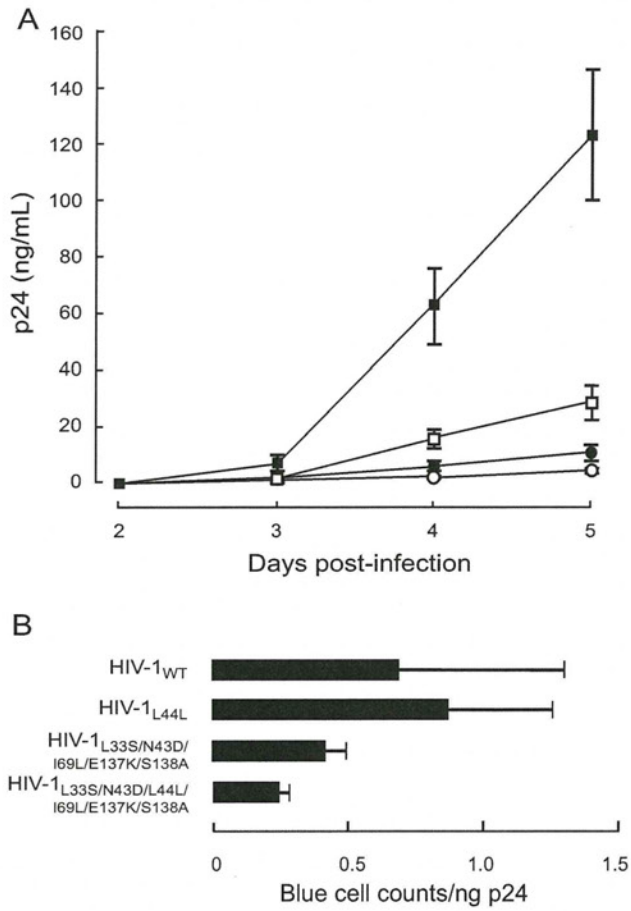


Fig. 4. Effect of secondary mutations in the N-HR on (A) replication kinetics and (B) infectivity. $L_{TTG}44L_{CTG}$ was introduced into HIV-1_{WT} and T-20_{S138A} resistant HIV-1 (HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$). Replication kinetics were determined by measuring p24 production in culture supernatants. HIV-1_{WT} (open circles), HIV-1_{L44L} (closed circles) HIV-$1_{L33S/N43D/I69L/E137K/S138A}$ (open squares), and HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$ (closed squares). $L_{TTG}44L_{CTG}$ introduction statistically enhanced both replication of HIV-1_{WT} and HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$ (Student's *t*-test, $p < 0.01$ on day 4 and 5). Relative infectivity (blue cell counts in MAGI cells divided by amount of p24) was calculated (B). Error bars indicate SD of three determinations. Decrease of infectivity between HIV-$1_{L33S/N43D/I69L/E137K/S138A}$ and HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$ were statistically significant (Student's *t*-test, $p < 0.05$).

the introduction of a secondary resistance mutation (E137K) in the backbone of a peptide fusion inhibitor is a useful change that results into more potent fusion inhibitors, even for HIV-1 strains that are resistant to peptide fusion inhibitors.

Selection of T-20_{S138A}-resistance starting with wild-type HIV-1 resulted in the emergence of I37N and L44M substitutions, which were located in the N-HR region that is thought to interact with T-20. Other substitutions at position 37 (I37T or I37K) also conferred resistance to T-20 and C34 (Nameki et al., 2005), suggesting that I37 in N-HR is critical for the attachment of C-HR-derived peptide fusion inhibitors. The L44M mutation has only been observed in subtype B HIV-1-infected patients treated with T-20 (Carmona et al., 2005), and conferred weak resistance to T-20 (Loutfy et al., 2007). In this study, L44M did not confer resistance to all peptide inhibitors; however, L44M in combination with other mutations (I37N/N126K) remarkably enhanced resistance to T-20_{S138A}, suggesting that L44M serves as a secondary mutation to enhance resistance to T-20_{S138A}. N126K also enhances resistance to some fusion inhibitors (Baldwin et al., 2004; Nameki et al., 2005; Eggink et al., 2008) by helping recover losses in intra-gp41 interactions that were caused by primary mutations, such as N43D.

When we selected T-20_{S138A}-resistant HIV-1 (HIV-$1_{N43D/S138A}$) we obtained a somehow different set of mutations that included L33S, which is located at the presumed T-20 binding site at N-HR, as well as I37N, N43D, and L44M. L33S was previously reported in HIV-1 variants resistant to T-20 (Fikkert et al., 2002), C34 (Armand-Ugon

Table 3
Antiviral activity of C-HR-derived peptides against gp160 recombinant viruses.

Compound	EC_{50} (nM)	
ddC	771 ± 272	
T-20 derivatives		
T20	>10,000	(NA)
T20EK	2729 ± 1113	(NA)
$T20_{S138A}$	3126 ± 453	(NA)
$T20_{E137K}$	2761 ± 1477	(NA)
$T20_{E137K/S138A}$	203 ± 54	(0.6)
C34 derivatives		
C34	171.0 ± 106	(3.4)
$C34_{N126K}$	25.9 ± 4.6	(NA)
SC34EK	1.0 ± 0.8	(1)
$C34_{E137K}$	7.0 ± 4.4	(0.4)
$C34_{E137K/S138A}$	0.3 ± 0.1	(0.3)

Anti-HIV activity was determined using the MAGI assay. Fifty percent effective concentration (EC_{50}) values and SD were obtained from the results of at least three independent experiments. Shown in parentheses are the fold-increases in resistance (increase in EC_{50} value) calculated by comparison to the resistant clone with mutations only in gp41 (HIV-$1_{L33S/N43D/I69L/E137K/S138A}$). To improve the replication kinetics, substitution of D36G, observed in majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (Izumi et al., 2009; Mink et al., 2005). NA, not available; ddC, dideoxycytidine.

et al., 2003), and a membrane-anchored C-HR-derived peptide, M87 (Lohrengel et al., 2005). Although our work clearly demonstrates that L33S is involved in resistance to T-20 derivatives, it was not possible to discern whether L33S affected binding affinity to C-HR in the CD analyses because L33 was not in the sequence of the N36 N-HR peptide that we had to use in this study. As shown in Fig. 2, L33S did not significantly affect replication kinetics compared with HIV-1_{WT}, suggesting that L33S might sustain binding affinity with C-HR to form a stable six-helix bundle. The L33S mutation is located in the loop of stem IIc of the RRE (Ueno et al., 2009). Hence, nucleotide changes for L33S do not require compensatory mutations to maintain secondary structure of the RRE. Therefore, it is likely that L33S has little effect on replication kinetics. In this study, L33S conferred little resistance to C34 in this study, while it was previously reported to confer up to 10-fold resistance (Armand-Ugon et al., 2003), suggesting that some other viral background might affect the resistance, since Armand-Ugon et al. (2003) examined bulk virus samples obtained from the selection.

A prevalent polymorphism, E137K, which was associated with N43D *in vivo* (Svicher et al., 2008), has been proven to restore infectivity that has been impaired by N43D (Tolstrup et al., 2007). E137K did not affect susceptibility to all peptide fusion inhibitors by itself, but in combination with primary mutations, it remarkably enhanced resistance to T-20_{S138A}. Moreover, introduction of the E137K change into N43D/S138A enhanced the viral replication kinetics as shown in Fig. 2. A possible hydrogen bond between K137 and D43 may partially restore the reported loss in six-helix bundle stability conferred by the N43D mutation (Bai et al., 2008), suggesting that E137K can compensate for losses in the interactions between N-HR_{N43D} and C-HR. This hypothesis is consistent with our CD results presented in Fig. 3.

Because E137K restored binding affinity with N-HR similar to the S138A mutation, we expected that introduction of E137K into T-20 would effectively suppresses replication of T-20-resistant HIV-1. We examined the antiviral activity of E137K- and E137K/S138A-containing T-20 and C34 to T-20_{S138A}-resistant HIV-1. We found that T-20_{E137K} had similar antiviral activity with other T-20 derivatives such as T-20_{S138A} and T-$20_{E137K/S138A}$. Hence, we believe that the combination of few substitution secondary mutations can enhance the antiviral activity of peptide fusion inhibitors. Therefore, it is possible to design peptides that include the secondary mutations in the C-HR and use them by themselves and/or in combinations to block fusion inhibitor resistant viruses. Importantly, we have successfully applied this strategy to suppress HIV-1 resistance to next generation fusion inhibitor SC34EK (Shimura et al., 2010).

In this study, we identified two distinct pathways to escape pressure of T-20_{S138A}. Emergence of drug resistance mutants under drug pressure involves a stochastic selection. Nonetheless, the makeup of the final population depends on both the ability of specific populations to evade the drug, as well as their fitness that determines their representation in the escape population. There are several examples in the literature where HIV becomes resistant to the same drug by different mechanisms. For example, in the case of the most commonly used drugs that target HIV reverse transcriptase (RT), the virus can develop multidrug resistance by either the Q151M complex pathway (Kavlick et al., 1998; Shirasaka et al., 1995) or by accumulation of thymidine associated mutations (TAMs) (Hachiya et al., 2008; Kosalaraksa et al., 1999). We recently report some of background polymorphisms can also influence resistance pathways, such 172R/K in the RT region (Hachiya et al., 2012). In the case of the T-20S138A inhibitor, the N43D/S138A may also act as such polymorphisms despite the presence of primary mutations (Izumi et al., 2009) and preferentially affect the emergence of specific mutations.

5. Conclusion

As previously discussed (Izumi et al., 2009), although other developed peptide-based fusion inhibitors need many amino acid additions and/or substitutions for the enhancement of their antiviral activity (Chinnadurai et al., 2007; Eggink et al., 2008; Dwyer et al., 2007; Otaka et al., 2002), application of secondary mutations similar to T-20_{S138A} and T-$20_{E137K/S138A}$ is straightforward. It is based on information from viral evolution studies under drug pressure that help design improved inhibitors.

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Comprehensive *In Vitro* Analysis of Simian Retrovirus Type 4 Susceptibility to Antiretroviral Agents

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Simian retrovirus type 4 (SRV-4), a simian type D retrovirus, naturally infects cynomolgus monkeys, usually without apparent symptoms. However, some infected monkeys presented with an immunosuppressive syndrome resembling that induced by simian immunodeficiency virus infection. Antiretrovirals with inhibitory activity against SRV-4 are considered to be promising agents to combat SRV-4 infection. However, although some antiretrovirals have been reported to have inhibitory activity against SRV-1 and SRV-2, inhibitors with anti-SRV-4 activity have not yet been studied. In this study, we identified antiretroviral agents with anti-SRV-4 activity from a panel of anti-human immunodeficiency virus (HIV) drugs using a robust *in vitro* luciferase reporter assay. Among these, two HIV reverse transcriptase inhibitors, zidovudine (AZT) and tenofovir disoproxil fumarate (TDF), potently inhibited SRV-4 infection within a submicromolar to nanomolar range, which was similar to or higher than the activities against HIV-1, Moloney murine leukemia virus, and feline immunodeficiency virus. In contrast, nonnucleoside reverse transcriptase inhibitors and protease inhibitors did not exhibit any activities against SRV-4. Although both AZT and TDF effectively inhibited cell-free SRV-4 transmission, they exhibited only partial inhibitory activities against cell-to-cell transmission. Importantly, one HIV integrase strand transfer inhibitor, raltegravir (RAL), potently inhibited single-round infection as well as cell-free and cell-to-cell SRV-4 transmission. These findings indicate that viral expansion routes impact the inhibitory activity of antiretrovirals against SRV-4, while only RAL is effective in suppressing both the initial SRV-4 infection and subsequent SRV-4 replication.

Simian type D retroviruses (SRV/Ds) are prevalent among wild and colony-born macaque monkeys, including *Macaca fascicularis* (cynomolgus) and *M. mulatta* (rhesus) (1–3). Although SRV/D infection is asymptomatic in most of these monkeys, mild immunosuppression accompanied by anemia, diarrhea, and splenomegaly has been observed in infected cynomolgus monkeys (3, 4). Recently, Japanese macaques (*M. fuscata*) housed in the Primate Research Institute (PRI) of Kyoto University, Japan, died of a hemorrhagic syndrome with symptoms such as anorexia, pallor, and nasal hemorrhage (5). Extensive investigations revealed that this illness was caused by an infection with an SRV/D known as simian retrovirus type 4 (SRV-4) (5; M. Okamoto et al., unpublished data). SRV-4 has been reported to be distantly related to other SRV/Ds, including SRV-1, -2, -3, -5, -6, and -7; e.g., the previously isolated SRV-4 showed genome sequence similarities of 78, 76, and 74% to SRV-1, -2, and -3, respectively (6). Although there is more than 80% amino acid sequence identity between Gag, Prt, and Pol of SRV-4 and SRV-1, -2, or -3, the Env sequence of SRV-4 is relatively diverse (67 to 74%) compared to other SRV/Ds (6). Although SRV-4 asymptomatically infects cynomolgus monkeys (7), SRV-4 infection of Japanese macaques has not been reported to date. Because the cause of the high mortality observed only for SRV-4-infected Japanese monkeys at PRI remains unclear, it is important to study SRV-4 pathogenesis in Japanese monkeys and to develop a prevention/treatment strategy for controlling SRV-4 infection.

Human immunodeficiency virus (HIV) infection remains a significant threat to humans. Over 20 antiviral drugs have been approved for the treatment of HIV-1-infected individuals. Antiretroviral therapy (ART) can efficiently suppress viral load and enable the recovery of immune function in HIV-1-infected individuals. Some of these drugs suppress infections caused by other retroviruses, including murine leukemia virus (MLV) (8, 9), xenotropic murine leukemia-related retrovirus (XMRV) (10, 11), feline immunodeficiency virus (FIV) (12, 13), and human T-cell leukemia virus type 1 (HTLV-1) (14, 15), indicating that some anti-HIV drugs are widely active against several other retroviruses. There have been some reports on the anti-SRV/D activity of anti-HIV drugs. Tsai et al. reported that three nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), zidovudine (AZT), zalcitabine (ddC), and 2′,3′-deoxyadenosine (ddA), exhibited inhibitory activity against SRV-2 infection *in vitro* (16). Moreover, although ddC treatment induced no major change in viral titers in pigtailed monkeys (*M. nemestrina*) naturally infected with SRV-2, the prophylactic use of ddC blocked *de novo* SRV-2 infection in this species (17). Rosenblum et al. reported that anti-SRV-1 and anti-SRV-2 activities of several NRTIs were relatively comparable with anti-HIV-1 activity (18). Furthermore, elvitegravir (EVG) and raltegravir (RAL), which are HIV-1 integrase strand transfer inhibitors (INSTIs), efficiently block SRV-3 (also known as Mason-Pfizer monkey virus) infection at nanomolar concentrations (19). Thus, some NRTIs and INSTIs exhibit anti-SRV/D activity; however, whether these drugs are active against SRV-4 infection remains unclear.

In this study, we extensively evaluated the anti-SRV-4 activity of a series of anti-HIV inhibitors, including NRTIs, nonnucleo-

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side reverse transcriptase inhibitors (NNRTIs), an INSTI, and protease inhibitors (PIs), *in vitro* using single-round infection and multiround viral spread by cell-free and cell-to-cell transmission. Among the NRTIs tested, AZT and tenofovir disoproxil fumarate (TDF) efficiently blocked single-round infection and cell-free transmission of SRV-4, although they were less effective against cell-to-cell transmission. RAL, an INSTI, blocked single-round infection and cell-free transmission of SRV-4 within the nanomolar range, and notably, it was also effective against cell-to-cell SRV-4 transmission. These results indicate that AZT, TDF, and RAL are effective in blocking the initial SRV-4 infection, and particularly, RAL is the most promising drug for the control of SRV-4 replication.

MATERIALS AND METHODS

Antiviral agents. Didanosine (ddI) (NRTI), lamivudine (3TC) (NRTI), stavudine (d4T) (NRTI), ddC (NRTI), AZT (NRTI), and nelfinavir (NFV) (PI) were purchased from Sigma (St. Louis, MO). Efavirenz (EFV) (NNRTI), nevirapine (NVP) (NNRTI), and saquinavir (SQV) (PI) were purchased from Toronto Research Chemicals (Ontario, Canada). Emtricitabine (FTC) (NRTI), TDF (NRTI), darunavir (DRV) (PI), and RAL (INSTI) were obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH).

Cells and viruses. TE671 (human rhabdomyosarcoma), 293T (human embryonic kidney), and 293T/SRV-4 (a persistently SRV-4-infected 293T cell line) cells, which have been established by the transfection of an SRV-4 infectious clone into 293T cells (Okamoto et al., unpublished), were grown in Dulbecco's modified Eagle's medium (DMEM). MT-2 cells (human T lymphocytes) were grown in RPMI 1640 medium. These media were supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin. 293FT cells (Invitrogen, Carlsbad, CA) were cultured in DMEM supplemented with 0.5 mg/ml G418. Platinum-GP cells (Plat-GP; Cell Biolabs, San Diego, CA) were maintained in DMEM supplemented with 10 μg/ml blasticidin.

Concentrated SRV-4 was prepared as follows: 293T/SRV-4 cells (10^6 cells) were cultured in a T-75 flask. After 3 days, culture supernatants were recovered and filtered through a 0.45-μm membrane, followed by the addition of a 30% polyethylene glycol (PEG) solution and 1.2 M sodium chloride. The mixture was then incubated overnight at 4°C, followed by centrifugation at 3,000 rpm for 45 min at 4°C. The resultant pellet was resuspended in DMEM and used for assays immediately after titration.

Quantification of the proviral copy number. Viral RNA and genomic DNA were prepared from concentrated SRV-4 by using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany) and from SRV-4-infected 293T cells by using DNAzol (Invitrogen), respectively. The viral copy number was quantified by using the One Step PrimeScript reverse transcriptase PCR (RT-PCR) kit (TaKaRa, Otsu, Japan) and the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) with a known copy number control. The primer sets and a probe used for SRV-4 amplification were described previously (20). PCR conditions were 5 min at 42°C, 10 s at 95°C, and 55 cycles of 5 s at 95°C and 34 s at 62°C.

VSV-G-pseudotyped luciferase expression vectors. An envelope-deleted SRV-4-based firefly luciferase expression vector, Δenv-SRV-4-luc (R. Yoshikawa et al., unpublished data), and a plasmid, pcDNA-VSV-G, encoding the vesicular stomatitis virus envelope glycoprotein (VSV-G) (provided by H. Miyoshi, Riken Bioresource Center, Tsukuba, Japan) were used to generate VSV-G-pseudotyped luciferase-expressing SRV-4. These plasmids were cotransfected into 293FT cells. After 48 h of transfection, culture supernatants were recovered, filtered through a 0.45-μm membrane, and stored at −80°C until use.

The VSV-G-pseudotyped luciferase-expressing HIV-1-based lentiviral vector was generated as reported previously (9). The Moloney MLV (MoMLV)-based retroviral vector was produced by cotransfection of the pDON-AI-2-luc plasmid, a firefly luciferase gene-containing pDON-AI-2 retroviral vector (TaKaRa) (provided by Y. Sakurai, Institute for Virus Research, Kyoto University, Kyoto, Japan), and pcDNA-VSV-G into a MoMLV-based packaging cell line, Plat-GP. The FIV-based lentiviral vector was prepared by cotransfection of a luciferase-encoding transfer vector, pCDF-luc-EF1-puro; a 34TF10-derived packaging vector, pFIV-34N (SBI System Biosciences, Mountain View, CA); and pcDNA-VSV-G into 293FT cells. All the recombinant viruses were collected and stored as mentioned above.

Evaluation of the anti-SRV-4 activities of NRTIs, NNRTIs, and an INSTI in single-round infection. To evaluate the inhibitory activities of anti-HIV drugs against VSV-G-pseudotyped luciferase-expressing SRV-4, HIV-1, MoMLV, and FIV, TE671 cells (10^4 cells/well) were plated onto white 96-well flat plates. After 24 h of incubation, the cells were infected with each virus in the presence of various concentrations of inhibitors. Similarly, 3×10^5 MT-2 cells were infected separately. Luciferase activity was determined by using the Bright-Glo luciferase assay system (Promega, Madison, WI) and a TriStar LB 941 multimode microplate reader (Berthold, Bad Wildbad, Germany) at 48 h postinfection. Cytotoxicity of the inhibitors was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, as described previously (9). Antiviral activity and cytotoxicity of the inhibitors are presented as the concentration that blocks viral infection by 50% (50% effective concentration [EC_{50}]) and the concentration that inhibits cell viability by 50% (50% cytotoxic concentration [CC_{50}]), respectively.

Evaluation of the inhibitory activity of PI against SRV-4 production. 293T/SRV-4 cells were washed three times with phosphate-buffered saline (PBS) and plated at a density of 2×10^5 cells/well onto a six-well culture plate in the presence of various concentrations of PIs. After 72 h of incubation, culture supernatants were collected and concentrated as described above. The resultant pellet was solubilized with lysis buffer supplied in the Reverse Transcriptase Assay, colorimetric (Roche, Mannheim, Germany), and RT activity was quantified to evaluate viral production.

Effects of AZT, TDF, and RAL on SRV-4 replication. To test cell-free SRV-4 infection, 293T cells were plated at a density of 2×10^5 cells/well onto a six-well plate and pretreated with inhibitors of approximately 10× EC_{50}s determined by the single-round luciferase assay (AZT [400 nM], TDF [10 nM], and RAL [150 nM]) or dimethyl sulfoxide (DMSO) as a control for 4 h. Following this, culture media were replaced with fresh medium containing identical concentrations of each inhibitor, and the cells were infected with concentrated (37.5-fold) replication-competent SRV-4 at a multiplicity of infection (MOI) of 2.0×10^6 copies/cell.

For cell-to-cell SRV-4 infection, SRV-4-free 293T cells (2×10^5 cells) were pretreated with inhibitors as described above for the cell-free infection assay. Following this, 293T/SRV-4 cells (4×10^3 cells; proviral copy number, $5 \times 10^{1.3}$ copies/cell) were cocultured in the presence of identical concentrations of inhibitors.

In both the experimental approaches, culture supernatants were collected and replenished with an equal volume of fresh medium containing the corresponding inhibitors on days 1, 3, and 5 postinfection/postcoculture. SRV-4 in each collected supernatant was concentrated, and RT activity was quantified to monitor viral replication.

Statistical analysis. Dunnett's test and the Bonferroni test were used to determine the statistical significance of anti-SRV-4 activity of inhibitors in single-round assays (Table 1) and of SRV-4 transmission in cell-free and cell-to-cell infection assays (Fig. 1), respectively.

Protein sequence alignment. Standard amino acid sequences of SRV-4 (GenBank accession number NC_014474.1), HIV-1 (accession number NC_001802.1), MoMLV (accession number NC_001501.1), and FIV (accession number NC_001482.1) were aligned by using the program Clustal W (21), as described previously (9). Residues associated with drug resistance in HIV-1, reported in the Stanford University HIV Drug Resistance Database (22), are also shown.

TABLE 1 Susceptibility of VSV-G-pseudotyped luciferase-expressing SRV-4 and related retroviruses/lentiviruses to NRTIs and an INSTI in single-round infection[a]

Target cell line and inhibitor	Mean EC_{50} ± SD (μM) (mean % inhibition ± SD)			
	HIV-1	SRV-4	MoMLV	FIV
TE671				
NRTIs				
Thymidine analogs				
AZT	0.018 ± 0.0074	0.042 ± 0.012*	0.019 ± 0.0043	0.029 ± 0.0035
d4T	0.40 ± 0.10	0.17 ± 0.039	3.7 ± 0.82**	0.52 ± 0.0055
Inosine analog				
ddI	10 ± 1.7	4.4 ± 1.3**	>10 (0)	19 ± 1.7**
Cytidine analogs				
ddC	5.6 ± 1.1	2.7 ± 0.13**	>10 (0)**	3.2 ± 0.50**
3TC	4.4 ± 0.75	3.9 ± 1.2	>10 (0)**	2.2 ± 0.50*
FTC	0.48 ± 0.15	0.50 ± 0.082	>10 (0)**	0.35 ± 0.030
Adenosine analog				
TDF	0.0043 ± 0.00058	0.00080 ± 0.00037**	0.0035 ± 0.0012	0.0015 ± 0.00076**
INSTI				
RAL	0.0031 ± 0.0015	0.015 ± 0.0065	0.0017 ± 0.00036	0.049 ± 0.00090*
MT-2				
NRTIs				
Thymidine analogs				
AZT	0.037 ± 0.014	0.11 ± 0.037	0.71 ± 0.36*	1.4 ± 0.40**
d4T	0.50 ± 0.13	2.3 ± 0.44	3.5 ± 0.61	21 ± 6.6**
Inosine analog				
ddI	3.4 ± 0.70	>10 (42 ± 4.4)*	>10 (0)*	16 ± 4.7**
Cytidine analogs				
ddC	7.2 ± 2.4	0.59 ± 0.45**	>10 (0)	3.5 ± 1.1*
3TC	2.8 ± 1.0	>10 (17 ± 3.3)**	>10 (0)**	1.1 ± 0.10**
FTC	0.52 ± 0.12	3.0 ± 0.40**	>10 (0)**	0.22 ± 0.12
Adenosine analog				
TDF	0.0071 ± 0.0018	0.0016 ± 0.00025**	0.0071 ± 0.00056	0.0039 ± 0.0021
INSTI				
RAL	0.0033 ± 0.0010	0.0024 ± 0.00068	0.00064 ± 0.00057	0.062 ± 0.032**

[a] Antiviral activities of NRTIs and an INSTI against VSV-G-pseudotyped SRV-4, HIV-1, MoMLV, and FIV were determined by using a luciferase assay. Data are shown as means and standard deviations obtained from three or more independent experiments, and statistical analyses were performed (*, $P < 0.05$; **, $P < 0.01$; not indicated, $P \geq 0.05$ [determined by Dunnett's test against control HIV-1]). EC_{50}s shown as >10 indicate that more than 10 μM drugs is required to block viral infection by 50%. In this case, percent inhibition of viral infection at 10 μM is shown in parentheses and is considered 10 μM for statistical analysis.

RESULTS

Anti-SRV-4 activity of HIV NRTIs in single-round infection. To date, there is no convenient assay system for evaluating the anti-SRV-4 activity of compounds; therefore, we first established a simple and quantitative assay system by employing VSV-G-pseudotyped luciferase-expressing SRV-4 as a model virus. The anti-SRV-4 activity of the test compounds was evaluated by using TE671 and MT-2 cells. TE671 cells, which are derived from human rhabdomyosarcoma, have frequently been used for infection experiments with several retroviruses, including SRVs (23). MT-2 cells, which are derived from human T lymphocytes, are also susceptible to some viruses, including HIV (24) and hepatitis C virus (25), and are routinely used for analysis of antiviral activity of inhibitors (9). Inhibitory activity against HIV-1 and FIV (lentiviruses) and MoMLV (gammaretrovirus) was also evaluated.

Some HIV NRTIs reportedly possess anti-SRV-1 and anti-SRV-2 activities (16, 18); therefore, we first evaluated the anti-SRV-4 activity of seven NRTIs which have been approved for the treatment of HIV-1-infected patients. When TE671 cells were used as targets, ddI, ddC, and 3TC exhibited weak anti-SRV-4 activities, with EC_{50}s within the micromolar range (EC_{50}, 2.7 to 4.4 μM), whereas d4T and FTC exhibited moderate anti-SRV-4 activities, with EC_{50}s within the submicromolar range (EC_{50}, 0.2 and 0.5 μM, respectively) (Table 1). Remarkably, AZT and TDF exhibited potent anti-SRV-4 activities, with EC_{50}s of 42 and 0.8 nM, respectively. In contrast, almost all the NRTIs showed higher EC_{50}s using MT-2 cells as targets than those using TE671 cells as targets (Table 1). However, AZT and TDF exerted potent anti-SRV-4 activities, with EC_{50}s of 110 and 1.6 nM, respectively, even in the less sensitive MT-2 cells. Notably, all the NRTIs tested in this study exhibited no cytotoxicity against both cell types up to 100 μM, indicating that the observed anti-SRV-4 activity was not because of cell damage (data not shown).

One possible explanation for the difference in drug susceptibility between TE671 and MT-2 cells would be the different phosphorylation efficacies of NRTIs, which require sequential phosphorylations by cellular kinases to reach the active form (26, 27). To confirm this, we next evaluated anti-HIV-1, anti-FIV, and an-

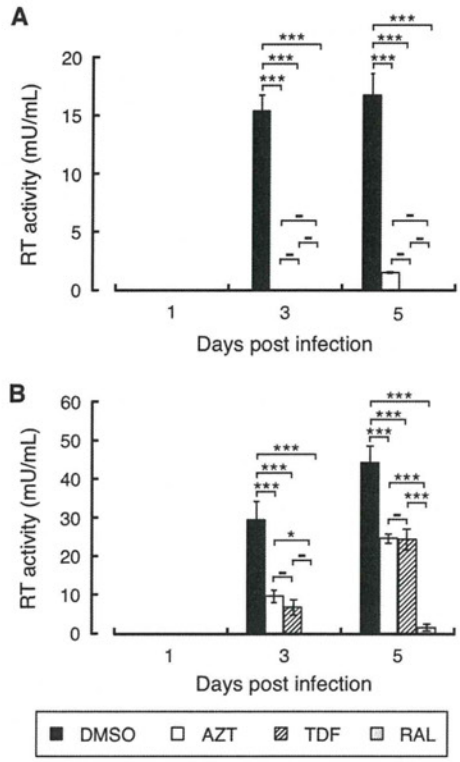

FIG 1 Effects of AZT, TDF, and RAL on SRV-4 replication. Anti-SRV-4 activities of AZT, TDF, and RAL were evaluated in cell-free transmission (A) and cell-to-cell transmission (B) models. SRV-4-free 293T cells were treated with AZT (400 nM), TDF (10 nM), RAL (150 nM), or vehicle (DMSO). After 4 h, culture media were replaced with fresh medium containing identical concentrations of each drug with replication-competent SRV-4 (A) or with SRV-4-infected 293T cells at a ratio of uninfected to infected cells of 50:1 (B). Culture supernatants were periodically collected, and SRV-4 was concentrated. The viral pellet was lysed, and reverse transcriptase activity derived from SRV-4 was quantified with a standard of known activity to monitor viral production. Data are shown as means and standard deviations obtained from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; −, $P \geq 0.05$ (determined by the Bonferroni test).

ti-MoMLV activities using the same assay system, in which TE671 or MT-2 cells were infected with VSV-G-pseudotyped luciferase-expressing HIV-1- or FIV-based lentiviral vectors or MoMLV-based retroviral vectors in the presence of various concentrations of inhibitors. HIV-1 infection was blocked by all the tested NRTIs to various extents (Table 1). Among these, AZT and TDF exhibited potent activities against SRV-4 and MoMLV infections, while d4T was less active than AZT (Table 1). In FIV infection, AZT and d4T were active within the submicromolar range only in TE671 cells; however, TDF exhibited potent anti-FIV activity in both cell types (Table 1). Importantly, variation of EC_{50}s of NRTIs against HIV-1 was minimum between both target cell types (0.3- to 2.1-fold change in EC_{50}s measured with TE671 and MT-2 cells), suggesting that cell-derived factors are not a major cause of target cell-based differences in anti-SRV-4 activity.

Taken together, these findings indicate that HIV NRTIs have inhibitory activity against SRV-4 infection to various extents. Among these, AZT showed preferential anti-SRV-4 activity, a trend different from that previously observed against SRV-1 and SRV-2 (18). In addition, TDF exhibited the most potent anti-SRV-4 activity in the single-round infection assay.

Inhibitory effect of HIV-1 NNRTIs on SRV-4 infection. HIV-1 NNRTIs, including NVP and EFV, efficiently suppress HIV-1 infection by inhibiting HIV-1 RT activity by binding to a hydrophobic pocket near the RT polymerase active site (28, 29). In the present study, EFV showed slight cytotoxicity, with CC_{50} values of 57 and 48 μM in TE671 and MT-2 cells, respectively. However, both NVP and EFV potently inhibited HIV-1 infection, with EC_{50}s within the nanomolar to subnanomolar range (0.57 to 82 nM) in both cell types. In contrast, NVP and EFV were completely inactive against SRV-4 infection as well as against MoMLV and FIV infections, even at 10 μM. These results correlate well with the impressive narrow spectrum of NNRTI activity; i.e., NNRTIs are active against HIV-1 but not against HIV-2 and other retroviruses (11, 30–32).

Inhibitory activity of an HIV INSTI against SRV-4 infection. We next evaluated the inhibitory effect of RAL, the first INSTI approved for clinical use, on SRV-4 infection. RAL has potent anti-HIV-1 activity in addition to a broad antiviral spectrum including simian immunodeficiency virus (SIV) (33), MLV (34), XMRV (11), and SRV-3 (19). We also observed that RAL inhibited HIV-1 and MoMLV infections (Table 1). FIV was less susceptible to RAL than HIV-1 and MoMLV, although the RAL EC_{50} against FIV was at the nanomolar level. Most importantly, SRV-4 infection was potently inhibited by RAL within a nanomolar concentration (Table 1). We previously observed that EVG, a new INSTI contained within a recently approved anti-HIV drug, was active against not only HIV but also MoMLV and SIV (9), indicating that INSTIs are a preferential class of inhibitors for a wide range of retroviral infections. We report for the first time the potential blockage of SRV-4 infection by RAL without cytotoxicity.

Effect of HIV PIs on SRV-4 production. We then evaluated the inhibitory activity of PIs against SRV-4 replication. It is impossible to evaluate the anti-SRV-4 activity of PIs with the replication-deficient SRV-4 used to evaluate the inhibitory activities of NRTIs, NNRTIs, and an INSTI. To overcome this limitation, we evaluated persistently SRV-4-infected cells, in which the production of progeny infectious virions from SRV-4-infected 293T cells was monitored in the presence of various concentrations of PIs. Viruses released into culture supernatants were quantified by virion-derived RT activity.

First, we measured the cytotoxicity of three PIs (NFV, SQV, and DRV) against 293T cells. Although DRV showed no cytotoxicity up to 100 μM, NFV and SQV decreased cell viability, with CC_{50} values of 22 and 28 μM, respectively. To exclude a cell toxicity-based reduction in viral production, we used 0.1 and 1 μM concentrations of PIs in this study, which are sufficiently high to exert anti-HIV-1 activity (11, 35, 36). However, none of the PIs inhibited late-phase SRV-4 replication steps even at 1 μM (data not shown), indicating that SRV-4 is intrinsically less susceptible to PIs.

Effects of AZT, TDF, and RAL on SRV-4 replication. As observed in the early part of this study, two NRTIs (AZT and TDF) and one INSTI (RAL) efficiently inhibited replication-deficient SRV-4 infection in a single-cycle luciferase assay. To further elucidate the anti-SRV-4 property of these inhibitors, we assessed their effect on SRV-4 replication.

To precisely evaluate the inhibitory activity against SRV-4 replication, we distinguished the SRV-4 replication pattern into two viral expansion pathways: cell-free and cell-to-cell transmission. In the cell-free model, SRV-4-free 293T cells were infected with

cell-free SRV-4 in the presence of inhibitors, and further viral expansion was monitored by virus-derived RT activity. In contrast, SRV-4-infected 293T cells were used as the source of infection for cell-to-cell transmission.

We observed that in the cell-free model, SRV-4 efficiently infected 293T cells and reached the maximum level at 3 days postinfection (Fig. 1A). Similarly, viral expansion through *de novo* SRV-4 transmission was observed in the cell-to-cell model (Fig. 1B). However, SRV-4 expanded more efficiently through the cell-to-cell mechanism than through the cell-free mechanism, as judged by the 2- to 3-fold-higher RT activity observed in the cell-to-cell model at 5 days postinfection, indicating that cell-derived SRV-4 is a favorable source of SRV-4. Under these conditions, the 10-fold-higher EC_{50}s of AZT, TDF, and RAL, previously measured in single-round infection assays, completely inhibited cell-free SRV-4 infection up to 3 days (Fig. 1A). However, on day 5, only 7% of the viral production was observed in the presence of AZT, whereas TDF and RAL still almost completely blocked SRV-4 expansion. This tendency was well correlated with the antiviral activity measured during the single-round SRV-4 infection (Table 1). In contrast, when cell-associated SRV-4 was used as the infectious source, inhibitory activities of AZT and TDF were only partial; therefore, *de novo* SRV-4 transmission was ongoing at 3 and 5 days postinfection (Fig. 1B). Notably, we sequenced the RT regions of the proviral DNA at the end of this study, and no changes from the original SRV-4 were observed (data not shown). Thus, drug resistance was not associated with insufficient activity. However, only 3% to 5% of viral replication was observed in the presence of RAL on day 5 ($P < 0.001$, compared to AZT and TDF), indicating that RAL potently inhibited SRV-4 replication; therefore, it should be highly effective in controlling SRV-4 infection and replication.

DISCUSSION

To date, several SRV serotypes have been identified, and their distributions in monkeys have been revealed (1–3, 37–41). For example, SRV-4 and SRV-5 infect cynomolgus and rhesus monkeys, respectively, while the Japanese monkey is not a natural host of these SRVs (7, 42). However, the recent outbreak of SRV-4 at PRI revealed that Japanese monkeys are susceptible to SRV-4 (5), since fatal disease could be induced in some of them (43) (Okamoto et al., unpublished). These epidemics reflect the necessity for effective drugs against SRV-4 infection. In addition, human SRV infection has been reported, although no associated diseases have been identified (44). This finding also suggests that the identification of anti-SRV drugs is important to prevent the entry of these viruses into the human population.

Among the identified SRVs, the inhibitory activity of anti-HIV drugs against SRV-1 and SRV-2 has been relatively well analyzed. In these studies, the evaluation of anti-SRV activity was performed by time-consuming, cost-intensive, and hazardous procedures, e.g., using wild-type SRVs and infected monkeys (17, 18). In the present study, we used a VSV-G-pseudotyped luciferase reporter SRV-4 to screen inhibitors with anti-SRV-4 activity from a panel of clinically approved anti-HIV drugs. In this system, the luciferase reporter gene enabled sensitive and rapid evaluation. Moreover, replacement of the intrinsic envelope with VSV-G avoids the restriction of target cell tropism, thereby enabling the direct comparison of antiviral activity with other viruses in the same cells. Using this assay system, we reported for the first time that two anti-HIV NRTIs (AZT and TDF) and one INSTI (RAL) efficiently inhibited SRV-4 infection. The tendency for drug susceptibility of SRV-4 is different from that of SRV-1 and SRV-2, as reported in a previous study in which SRV-1 and SRV-2 infections were more potently inhibited by ddC than by AZT, 3TC, and d4T (18). Reportedly, SRV-4 is genetically distinct from SRV-1 and SRV-2 (3), suggesting that this intrinsic diversity reflects drug susceptibility.

Among the NRTIs tested, AZT and TDF exhibited potent anti-SRV-4 activities in single-round infection and cell-free viral transmission and also inhibited HIV-1, MoMLV, and FIV infections to various extents. However, the inhibitory activities of some NRTIs, particularly the thymidine analogs AZT and d4T, against SRV-4, MoMLV, and FIV infections were markedly (>10-fold) varied between TE671 and MT-2 cells (Table 1). A similar variation was previously reported for several viruses (18, 45). Major factors accounting for the different sensitivities of viruses to NRTIs in different target cells include the endogenous levels of some kinases as well as the levels of the intracellular pool of nucleotides (45–47). Moreover, although HIV-1 preferentially infects lymphoid cells, SRV infects a wide variety of cells, including not only $CD4^+$, $CD8^+$, and B cells *in vivo* but also lung fibroblast and kidney cells of monkeys *in vitro* (48). It is likely that the nature of the virus and assay conditions affects the susceptibility of SRV-4 to NRTIs in different cells, although further analyses are required to completely elucidate this phenomenon. TDF preferentially inhibited all the tested retroviruses. All the nucleoside-type RT inhibitors required three sequential phosphorylations, whereas TDF requires only a two-step phosphorylation to be active (49, 50), suggesting that this kinetic advantage reflects potent antiviral properties.

To gain deeper insights into the drug susceptibility of SRV-4, amino acid sequences of regions corresponding to the RT-polymerase domain (residues 63 to 234 of HIV-1) and the integrase catalytic core domain (IN-CCD) (residues 50 to 212) were compared with those of HIV-1, MoMLV, and FIV (Fig. 2). Because genotypic studies to elucidate drug susceptibility based on amino acid changes have been extensively performed for HIV-1 (22, 51, 52), we applied those observations to genotypic analysis of SRV-4. Overall, we confirmed that some amino acid residues in SRV-4 are identical to reported mutations affecting drug susceptibility in HIV-1. For example, HIV-1 RT mutations at positions 41, 67, 70, 210, 215, and 219, known as thymidine analog mutations (TAMs), are frequently observed in AZT and D4T resistance (52–54). In SRV-4 RT, some residues corresponding to TAMs differ from those of wild-type HIV-1 (Fig. 2A), although they must not be involved in drug susceptibility of SRV-4, since AZT and d4T inhibited SRV-4 infection at a similar or superior level compared to HIV-1 infection. In addition, although mutations at Q151 in the LPQG motif and M184 in the YMDD motif are involved in higher-level resistance to some NRTIs (55–57), these motifs are completely conserved in SRV-4 RT. In contrast, MoMLV showed complete insensitivity to certain NRTIs, including 3TC, at 10 μM (Table 1), in agreement with previous reports (8, 18). Taken together, as apparent from genotypic analysis of SRV-4 RT, AZT and TDF are thought to be potent therapeutic agents for the inhibition and control of SRV-4.

RAL, an HIV INSTI, showed potent inhibitory activity against SRV-4 infection as well as against HIV-1, MoMLV, and FIV infections. HIV-1 acquires high-level RAL resistance by mutations such as Q148H/R/K and N155H (52, 58). Although SRV-4 IN

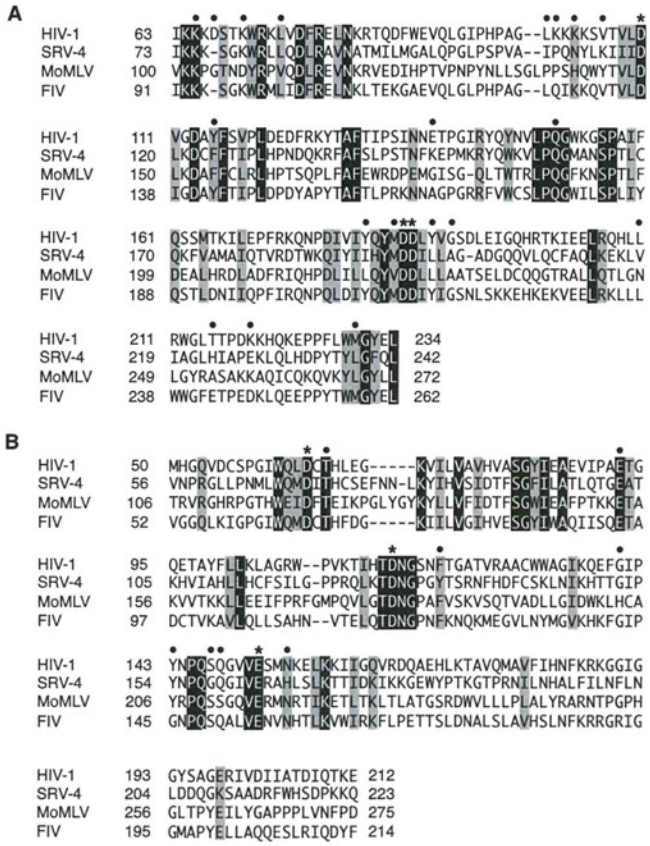

FIG 2 Protein sequence alignments of RT and IN. Reference amino acid sequences of SRV-4 (GenBank accession number NC_014474.1), HIV-1 (accession number NC_001802.1), MoMLV (accession number NC_001501.1), and FIV (accession number NC_001482.1) were aligned by using the program Clustal W, and the regions corresponding to the RT-polymerase domain (residues 63 to 234) (A) and the IN catalytic core domain (residues 50 to 212) (B) of HIV-1 are shown. The amino acid numbering of SRV-4 IN is based on that of SRV-3 (68). Absolutely conserved residues and conserved substitutions are shown in black and gray boxes, respectively. For symbols above the sequences, closed circles indicate residues associated with drug resistance for HIV-1, and asterisks indicate catalytic residues.

contains H166, which corresponds to N155 in HIV-1 (Fig. 2B), SRV-4 retained susceptibility within levels similar to those of wild-type HIV-1 (Table 1). Reportedly, SRV-3 also contains amino acids corresponding to N155H and F121Y, which are other INSTI resistance mutations; however, SRV-3 shows complete susceptibility to RAL (19). In contrast, bovine immunodeficiency virus (BIV) reportedly showed 23-fold resistance to RAL compared to wild-type HIV-1, although BIV contains a histidine (H) residue at the position corresponding to N155 (19), as seen in SRV-4, indicating that N155H is not a determinant of RAL susceptibility in retroviruses other than HIV. Although in the present study, FIV showed less susceptibility to RAL than the other retroviruses/lentiviruses tested (Table 1), FIV does not contain major INSTI resistance mutations. However, one distinct difference was observed: FIV IN carries G145, whereas it corresponds to Y143 in HIV-1 (Fig. 2B). The Y143G mutation has rarely been observed in RAL-treated patients (59); therefore, the precise effect of this mutation on RAL resistance remains unclear. However, it was speculated that that the Y143G mutation lacks the interaction with RAL (19), and interestingly, Y143G reportedly affects proviral formation (60), although this is apparent in nondividing cells (61), likely suggesting that FIV IN G145 affects susceptibility of FIV not only to INSTIs but also to NRTIs.

To expand viral infection *in vitro* and *in vivo*, viruses utilize two main pathways: cell-free and cell-to-cell transmission. However, the transmission pathway depends on the nature of the viruses. For example, cell-free HIV-1 efficiently infects $CD4^+$ T cells and also spreads in a cell-to-cell manner, whereas HTLV-1 transmits exclusively by a cell-to-cell pathway (62–66). In the present study, we compared the inhibitory activities of some inhibitors against SRV-4 replication in both cell-free and cell-to-cell transmission. We observed that although AZT and TDF could almost completely block cell-free SRV-4 transmission, they showed only marginal effects on cell-to-cell SRV-4 transmission (Fig. 1). In contrast, RAL completely suppressed SRV-4 replication in both cell-free and cell-to-cell transmission. These results indicate that a favorable pathway is intrinsically present in anti-HIV-1 drugs; AZT and TDF preferentially block cell-free infection, whereas RAL is active in both the pathways. A similar observation was reported for HIV-1, in which tenofovir preferentially suppressed cell-free transmission compared with cell-to-cell transmission (67). These observations may highlight the importance of the kinetics of viral replication and drug activation because AZT and TDF require tri- and diphosphorylation, respectively, to become active metabolites, whereas RAL does not require any modification to exert its antiviral activity. In addition, it is likely that the kinetics of SRV-4 replication steps, including reverse transcription and integration, vary between cell-free and cell-to-cell transmission, as seen for HIV-1; this may be another determinant of viral transmission pathway-dependent anti-SRV-4 activities.

Taken together, the present study demonstrated that AZT, TDF, and RAL potently inhibited SRV-4 infection. These inhibitors suppressed single-round infection and cell-free virus transmission of SRV-4; however, cell-to-cell transmission was blocked only by RAL. To effectively control SRV-4 infection and maintain a minimum risk of the emergence of drug resistance, a combination therapy of drugs such as ART in HIV-1 infection is important.

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研究成果の刊行に関する一覧表（総合）

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雑誌

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