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

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Running Title: Inhibition of SRV-4 infection/replication

Manuscript information: 29 Text pages (including this page)

1 Table, 2 Figures, and 68 References

229 words for the abstract

4,492 words for the text

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 are 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 or higher than those against HIV-1, Moloney murine leukemia virus, and feline immunodeficiency virus. In contrast, non-nucleoside 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.

INTRODUCTION

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.*, manuscript in preparation). SRV-4 is 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 similarity 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 (67-74%) 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 in 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 are 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 within 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, non-nucleoside reverse transcriptase inhibitors (NNRTIs), INSTI, and protease inhibitors (PIs), *in vitro* using single-round infection and multi-round 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, USA). 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 Institute 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 SRV-4 infectious clone into 293T cells (M. Okamoto *et al.*, detailed manuscript in preparation) were grown in Dulbecco's modified Eagle's medium (DMEM). MT-2 cells (human T lymphocytes) were grown in Roswell Park Memorial Institute (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, USA) were cultured in DMEM supplemented with 0.5 mg/mL G418. Platinum-GP cells (Plat-GP; Cell Biolabs, San Diego, CA, USA) 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.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) for quantification of the viral copy number. Viral RNA and genomic DNA were prepared from concentrated SRV-4 using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and from SRV-4-infected 293T cells using DNAzol (Invitrogen), respectively. The viral copy number was quantified using the One Step PrimeScript RT-PCR Kit (Takara, Otsu, Japan) and the StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, CA, USA) with a known copy-number control. The

primer sets and a probe used for SRV-4 amplification have been 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.*, manuscript in preparation), and a plasmid, pcDNA-VSV-G, encoding the vesicular stomatitis virus envelope glycoprotein (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 and 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-coding transfer vector, pCDF-luc-EF1-puro, a 34TF10-derived packaging vector, pFIV-34N (SBI System Biosciences, Mountain View, CA, USA), 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 NRTI, NNRTI, and 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 on 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 using the Bright-Glo Luciferase Assay System (Promega, Madison, WI, USA) and TriStar LB 941 Multimode Microplate Reader (Berthold, Bad Wildbad, Germany) 48 h postinfection. Cytotoxicity of the inhibitors was measured 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 on 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 on a six-well plate

and pretreated with inhibitors of approximately 10 × EC_{50} values 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 moi of 2.0×10^6 copies/cell.

For cell-to-cell SRV-4 infection, SRV-4-free 293T cells (2×10^5) were pretreated with inhibitors as in 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 media containing the corresponding inhibitors on days 1, 3, and 5 postinfection/coculture. SRV-4 in each collected supernatant was concentrated, and RT activity was quantified to monitor viral replication.

Statistical analysis. Dunnett's test and Bonferroni test were used to determine the statistical significance in anti-SRV-4 activity of inhibitors in single-round assays (Table 1) and in SRV-4 transmission in cell-free and cell-to-cell (Figure1), respectively.

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

University HIV Drug Resistance Database (22), are also shown.

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 using TE671 and MT-2 cells. TE671 cells, which are derived from human rhabdomyosarcoma, have frequently been used for the infection experiments of 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 routinely used for analysis of antiviral activity of inhibitors (9). Inhibitory activity against HIV-1 and FIV (lentivirus) 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} values within the micromolar range (EC_{50}: 2.7–4.4 μM), whereas d4T and FTC exhibited moderate anti-SRV-4 activities with EC_{50} values 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} values of 42 and 0.8 nM, respectively. In contrast,

almost all the NRTIs showed higher EC_{50} values using MT-2 cells as targets compared with those using TE671 cells as targets (Table 1). However, AZT and TDF exerted potent anti-SRV-4 activities with EC_{50} values 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 the 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 of the difference in drug susceptibility between the 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 anti-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 the cells (Table 1). Importantly, variation of EC_{50} values of NRTIs against HIV-1 was minimum between both the target cells (0.3–2.1-fold change in EC_{50} values 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} values within the nanomolar to subnanomolar range (0.57 – 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 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} value against

FIV was at a 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 INSTI is a preferential class of inhibitor 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 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 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 (Figure 1A). Similarly, viral expansion through *de novo* SRV-4 transmission was observed in the cell-to-cell model (Figure 1B). However, SRV-4 expanded more efficiently through the cell-to-cell mechanism than through the cell-free mechanism, as judged by the 2–3-fold higher RT activity observed in the cell-to-cell model 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} values of AZT, TDF, and RAL, previously measured in single-round infection assays, completely inhibited cell-free SRV-4 infection up to 3 days (Figure 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 (Figure 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%–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) (M. Okamoto *et al.*, manuscript in preparation). 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 of 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 (more than 10-fold) varied between TE671 and MT-2 cells (Table 1). A similar variation was previously reported with 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 condition 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 63–234 of HIV-1) and integrase catalytic core domain (IN-CCD; residues 50–212) were compared with those of HIV-1, MoMLV, and FIV (Figure 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 (Figure 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 than HIV-1 infection. In addition, although mutations at Q151 in the LPQG motif and M184 in the YMDD motif are involved in higher 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 RAL resistance by mutations such as Q148H/R/K and N155H (52, 58). Although SRV-4 IN contains H166, which corresponds to N155 in HIV-1 (Figure 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 with 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 retro/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 (Figure 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 is speculated that that 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 exclusively transmits by a cell-to-cell pathway (62-66). In the present study, we compared the inhibitory activity 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 only showed marginal effects on cell-to-cell SRV-4 transmission (Figure 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 suppresses 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 in 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.

ACKNOWLEDGMENTS

We thank H. Miyoshi and Y. Sakurai for providing lentiviral vectors and the pDON-AI-2-luc vector, respectively. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Emtricitabine (FTC), tenofovir disoproxil fumarate (TDF), darunavir (DRV; from Tibotec, Inc.), and raltegravir (RAL; from Merck & Company, Inc.). This work was supported in part by JSPS KAKENHI [Grant-in-Aid for Young Scientists (B)] to K.S. (No. 24791021) and [Grant-in-Aid for Scientific Research (B)] to M.O. (No. 24300153)].

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FIGURE LEGENDS

Figure 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; open column), TDF (10 nM; hatched column), RAL (150 nM; dotted column), or vehicle (DMSO; solid column). 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 is shown as means and standard deviations obtained from three independent experiments. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, -: $P\geq0.05$ (Bonferroni test).

Figure 2. Protein sequence alignments of RT and IN. Reference amino acid sequences of SRV-4 (GenBank accession number: NC_014474.1), HIV-1 (NC_001802.1), MoMLV (NC_001501.1), and FIV (NC_001482.1) were aligned using the program Clustal W, and the regions corresponding to the RT polymerase domain (residues 63–234) (*A*) and IN catalytic core domain (50–212) (*B*) of HIV-1 are shown.

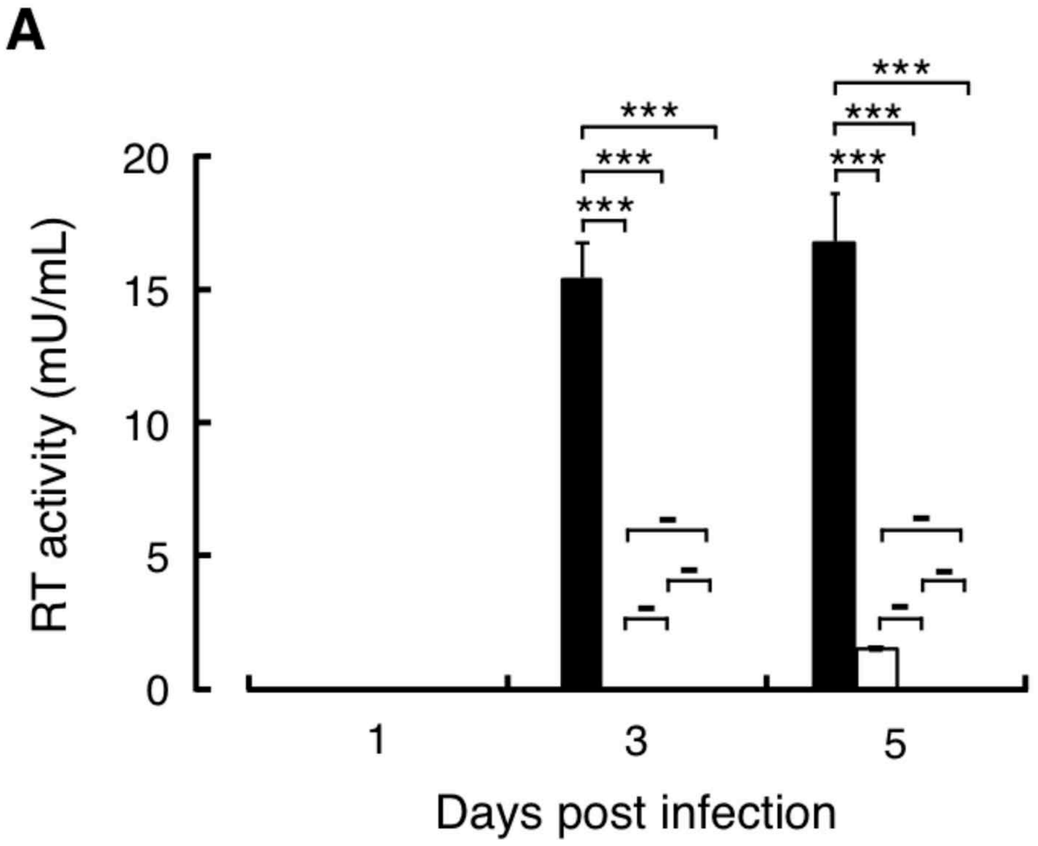
The amino acid number of SRV-4 IN is based on that of SRV-3 (68). Absolutely conserved residues and conserved substitutions are shown in black and gray boxes, respectively. Symbols above the sequences: closed circle, residue associated with drug resistance; asterisk, catalytic residue.

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

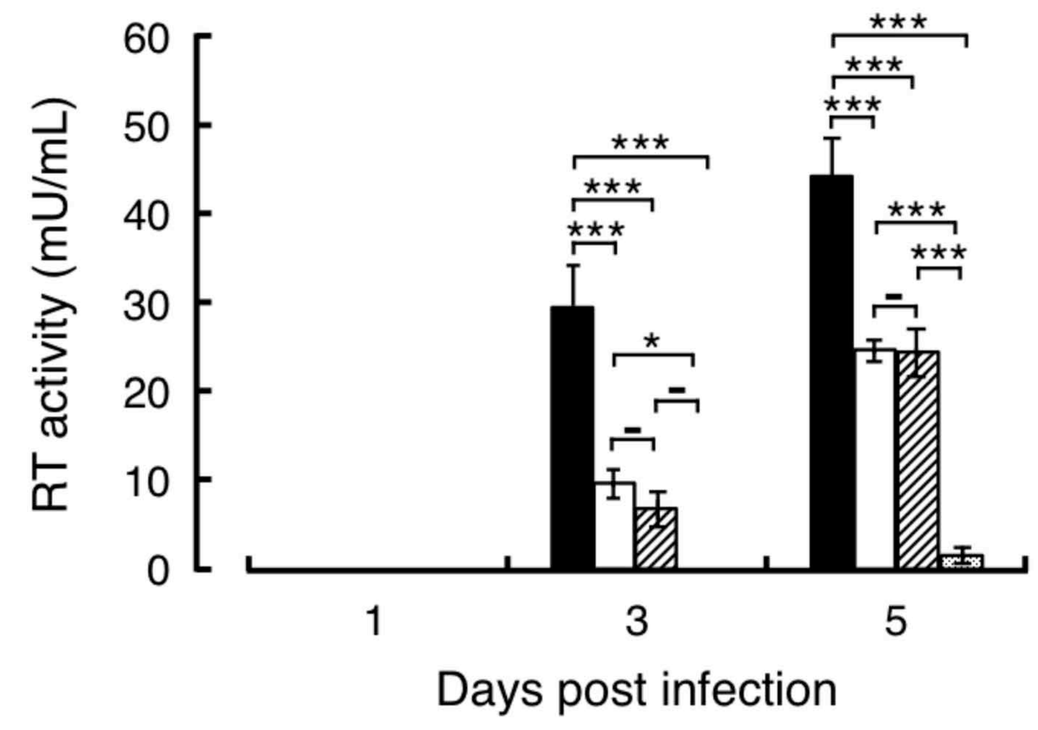
Target cells	Inhibitors	EC_{50} (μM)			
		HIV-1	SRV-4	MoMLV	FIV
TE671	*NRTI*				
	Thymidine analog				
	AZT	0.018 ± 0.0074	0.042 ± 0.012*	0.019 ± 0.0043	0.029 ± 0.0035
	d4T	0.40 ± 0.10	0.17 ± 0.039	3.7 ± 0.82**	0.52 ± 0.0055
	Inosine analog				
	ddI	10 ± 1.7	4.4 ± 1.3**	>10 [0%]	19 ± 1.7**
	Cytidine analog				
	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	*NRTI*				
	Thymidine analog				
	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 analog				
	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 INSTI against VSV-G-pseudotyped SRV-4, HIV-1, MoMLV, and FIV were determined using luciferase assay.
Data is shown as means and standard deviations obtained from three or more independent experiments, and statistical analysis were performed (*: $P<0.05$, **: $P<0.01$, not indicated: $P\geq0.05$; Dunnett's test against control HIV-1). EC_{50} values shown as >10 indicate that more than 10 μM of drugs is required to block viral infection by 50%. In this case, percentage inhibition of viral infection at 10 μM is shown in brackets, and considered as 10 μM for statistical analysis.

A


RT activity (mU/mL)
20
15
10
5
0
***
***
***
***
***
***
1
3
5
Days post infection

B


RT activity (mU/mL)
60
50
40
30
20
10
0
***
***
***
*
***
***
***
***
1
3
5
Days post infection
DMSO
AZT
TDF
RAL

A

```
HIV-1    63   IKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAG--LKKKKSVTVLD
SRV-4    73   IKKK-SGKWRLLQDLRAVNATMILMGALQPGLPSPVA--IPQNYLKIIID
MoMLV   100   VKKPGTNDYRPVQDLREVNKRVEDIHPTVPNPYNLLSGLPPSHQWYTVLD
FIV      91   IKKK-SGKWRMLIDFRELNKLTEKGAEVQLGLPHPAG--LQIKKQVTVLD

HIV-1   111   VGDAYFSVPLDEDFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIF
SRV-4   120   LKDCFFTIPLHPNDQKRFAFSLPSTNFKEPMKRYQWKVLPQGMANSPTLC
MoMLV   150   LKDAFFCLRLHPTSQPLFAFEWRDPEMGISG-QLTWTRLPQGFKNSPTLF
FIV     138   IGDAYFTIPLDPDYAPYTAFTLPRKNNAGPGRRFVWCSLPQGWILSPLIY

HIV-1   161   QSSMTKILEPFRKQNPDIVIYQYMDDLYVGSDLEIGQHRTKIEELRQHLL
SRV-4   170   QKFVAMAIQTVRDTWKQIYIIHYMDDILLAG-ADGQQVLQCFAQLKEKLV
MoMLV   199   DEALHRDLADFRIQHPDLILLQYVDDLLLAATSELDCQQGTRALLQTLGN
FIV     188   QSTLDNIIQPFIRQNPQLDIYQYMDDIYIGSNLSKKEHKEKVEELRKLLL

HIV-1   211   RWGLTTPDKKHQKEPPFLWMGYEL   234
SRV-4   219   IAGLHIAPEKLQLHDPYTYLGFQL   242
MoMLV   249   LGYRASAKKAQICQKQVKYLGYLL   272
FIV     238   WWGFETPEDKLQEEPPYTWMGYEL   262
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HIV-1    50   MHGQVDCSPGIWQLDCTHLEG-----KVILVAVHVASGYIEAEVIPAETG
SRV-4    56   VNPRGLLPNMLWQMDITHCSEFNN-LKYIHVSIDTFSGFILATLQTGEAT
MoMLV   106   TRVRGHRPGTHWEIDFTEIKPGLYGYKYLLVFIDTFSGWIEAFPTKKETA
FIV      52   VGGQLKIGPGIWQMDCTHFDG-----KIILVGIHVESGYIWAQIISQETA

HIV-1    95   QETAYFLLKLAGRW--PVKTIHTDNGSNFTGATVRAACWWAGIKQEFGIP
SRV-4   105   KHVIAHLLHCFSILG-PPRQLKTDNGPGYTSRNFHDFCSKLNIKHTTGIP
MoMLV   156   KVVTKKLLEEIFPRFGMPQVLGTDNGPAFVSKVSQTVADLLGIDWKLHCA
FIV      97   DCTVKAVLQLLSAHN--VTELQTDNGPNFKNQKMEGVLNYMGVKHKFGIP

HIV-1   143   YNPQSQGVVESMNKELKKIIGQVRDQAEHLKTAVQMAVFIHNFKRKGGIG
SRV-4   154   YNPQGQGIVERAHLSLKTTIDKIKKGEWYPTKGTPRNILNHALFILNFLN
MoMLV   206   YRPQSSGQVERMNRTIKETLTKLTLATGSRDWVLLLPLALYRARNTPGPH
FIV     145   GNPQSQALVENVNHTLKVWIRKFLPETTSLDNALSLAVHSLNFKRRGRIG

HIV-1   193   GYSAGERIVDIIATDIQTKE   212
SRV-4   204   LDDQGKSAADRFWHSDPKKQ   223
MoMLV   256   GLTPYEILYGAPPPLVNFPD   275
FIV     195   GMAPYELLAQQESLRIQDYF   214
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