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# HIV-1 acquires resistance to two classes of antiviral drugs through homologous recombination

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**Abstract**

Genetic recombination contributes to the genomic heterogeneity of human immunodeficiency virus type 1 (HIV-1). In the present study, we demonstrate that HIV-1 readily develops resistance to two classes of anti-HIV-1 drugs through in vitro genetic recombination involving large segments of the viral genome. Co-transfection of COS-7 cells with an HIV-1 plasmid (pSUM13) carrying five mutations in the reverse transcriptase (RT)-encoding region (A62V, V75I, F77L, F116Y, Q151M), conferring resistance to multiple dideoxynucleoside analogs (ddNs), and another HIV-1 plasmid (pSUM431) carrying five mutations in the protease-encoding region (V32I, L33F, K45I, I84V, L89M), conferring resistance to protease inhibitors such as KNI-272, readily produced HIV-1 carrying both sets of mutations when propagated in MT-2 cells in the presence of azidothymidine (AZT) and KNI-272. The resultant HIV-1 variant was highly resistant to both ddNs and KNI-272. Co-infection of MT-2 cells with HIV-$1_{SUM13}$ carrying the RT mutations and HIV-$1_{SUM431}$ carrying the mutations in the protease also generated HIV-1 with both sets of mutations when cultured with AZT and KNI-272. We also report here that the problematic artifactual recombination occurring during genetic analyses of heterogeneous nucleic acid sequences using polymerase chain reaction can be successfully obviated. © 1997 Elsevier Science B.V.

*Keywords:* HIV-1; Resistance; Antiviral drugs; Homologous recombination

## 1. Introduction

One of the greatest obstacles in the antiviral chemotherapy of human immunodeficiency virus (HIV-1) infection is the emergence of drug-resistant HIV-1 variants (Larder and Kemp, 1989; Richman et al., 1991; De Clercq, 1994). The accumulating data suggest that the development of such variants results in clinical deterioration in patients receiving antiviral chemotherapy (Tudor-Williams et al., 1992; Kozal et al., 1993; Mon-

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taner et al., 1993; D'Aquila et al., 1995). There has been considerable evidence that antiretroviral combination chemotherapy enables a greater and more persistent virologic and immunologic improvement compared with single drug therapy (Yarchoan et al., 1990, 1994; D'Aquila et al., 1995; Hammer et al., 1996; Katzenstein et al., 1996). It is apparent, however, that HIV-1 develops resistance to all anti-HIV-1 drugs thus far available even when used in combination (Larder et al., 1993; Shirasaka et al., 1993; Gu et al., 1994; Shafer et al., 1994; Zhang et al., 1994; Shirasaka et al., 1995). The error-prone polymerization (approximately one to ten misincorporations per genome per round of HIV-1 replication) mediated by the virally-encoded reverse transcriptase (RT) of HIV-1 is thought to contribute to the genetic diversity and hypermutability of HIV-1 (Preston et al., 1988; Roberts et al., 1988; Bebenek et al., 1989), although the studies of Mansky and Temin (1995) have suggested that HIV-1 is not different from other single-stranded RNA in terms of mutation rates. In addition to the error-prone nature of RT, homologous recombination, which occurs at a high rate in retroviral replication, also makes it possible to increase the variation in a viral population by combining various genetic segments present in multiple viral strains (Hu and Temin, 1990; Stuhlmann and Berg, 1992; Coffin, 1996). Indeed, three recent reports have suggested that homologous recombination may be involved in the development of resistance to 3′-azido-2′,3′-dideoxythymidine (AZT) (Kellam and Larder, 1995), AZT plus (-)-$\beta$-L-2′,3′-deoxy-3′-thiacytidine (3TC) (Gu et al., 1995), and AZT plus SC-52151 (Moutouh et al., 1996).

In the present work, we examined whether cross-resistance to AZT and KNI-272 could occur through homologous recombination between a multi-dideoxynucleoside resistant HIV-1 strain (Shirasaka et al., 1993, 1995; Ueno et al., 1995) and a highly KNI-272-resistant HIV-1 strain in the presence of drug selection pressure in vitro. We also report that the problematic artifactual recombination occurring during genetic analyses of heterogeneous nucleic acid sequences using polymerase chain reaction was successfully obviated.

## 2. Materials and methods

### 2.1. Reagents and cells

AZT and 2′,3′-dideoxycytidine (ddC) were purchased from Sigma (St. Louis, MO), and 2′,3′-dideoxyinosine (ddI) from Calbiochem (San Diego, CA). KNI-272 was synthesized as previously described (Mimoto et al., 1992; Kageyama et al., 1993). MT-2 cells were grown in RPMI 1640-based complete medium supplemented with 10% fetal calf serum (FCS: HyClone Laboratories, Logan, UT), 200 U/ml penicillin, 200 U/ml streptomycin, and 4 mM L-glutamine. COS-7 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM).

### 2.2. Virus preparation

A wild type HIV-1 construct (pSUM9) and two mutant HIV-1 constructs, pSUM13 and pSUM431, were generated as previously described (Anderson et al., 1994; Shirasaka et al., 1995). pSUM13 contained a set of five mutations in the RT-encoding region (A62V, V75I, F77L, F116Y, Q151M) conferring multi-dideoxynucleoside resistance (MDR) to 2′,3′-dideoxynucleoside analogs (ddNs) (Shirasaka et al., 1995), while pSUM431 contained five mutations in the protease-encoding region (V32I, L33F, K45I, I84V, L89M) conferring resistance to protease inhibitors such as ritonavir and KNI-272 (Anderson et al., 1994). To generate infectious HIV-1 clones, COS-7 cells were transfected with these plasmids using Lipofectamine (Life Technologies, Gaithersburg, MD). Each plasmid (2 $\mu$g) was first mixed with Lipofectamine (12 $\mu$l) in 200 $\mu$l DMEM and incubated for 15 min at room temperature. The plasmid–Lipofectamine complex was added to COS-7 cells in 0.8 ml DMEM ($2 \times 10^5$ cells/$\phi$35-mm well) which were cultured for 4 h at 37°C and 5% $CO_2$. Following the addition of 2 ml DMEM supplemented with 10% FCS, the cells were incubated for an additional 12 h, washed with phosphate buffered saline (PBS) twice, and resuspended in 2 ml DMEM with 10% FCS. After 48 h, the culture was terminated with centrifugation at $1000 \times g$

for 10 min. The 50% tissue culture infectious dose ($TCID_{50}$) values of each virus preparation was determined in MT-2 cells as previously described (Richman, 1993a). These titrated viral preparations served as a source of infectious virions.

### 2.3. Co-transfection of COS-7 cells

Co-transfection of COS-7 cells with two distinct plasmids was performed as follows. pSUM13 (1 $\mu$g) and pSUM431 (1 $\mu$g) were mixed with Lipofectamine (12 $\mu$l) in 200 $\mu$l DMEM and added to COS-7 cells in 0.8 $\mu$l DMEM ($2 \times 10^5$ cells/$\phi$35-mm well). The co-transfected COS-7 cells were cultured and the supernatant containing infectious virions was harvested as described above. The virions were further propagated in MT-2 cells in the presence or absence of 0.1 $\mu$M AZT and/or 0.3 $\mu$M KNI-272. MT-2 cell cultures were replenished with fresh complete medium containing the appropriate drug concentrations every 4–6 days.

### 2.4. Infection of MT-2 cells with infectious clones and drug exposure

MT-2 cells ($3 \times 10^5$) were exposed to each infectious HIV-1 clone at a 50 $TCID_{50}$ dose for 1 h at 37°C, washed with PBS twice, resuspended in 10 ml complete medium, and cultured in the presence or absence of 0.1 $\mu$M AZT and/or 0.3 $\mu$M KNI-272. The culture was replenished with fresh complete media containing the appropriate drug concentrations. On day 14 of culture, additional MT-2 cells ($5 \times 10^5$) were added in order to maintain the culture.

### 2.5. Preparation of HIV-1 RNA and proviral DNA and PCR amplification

The nucleotide sequence of various HIV-1 strains was determined using viral RNA or cellular proviral DNA as previously described (Shirasaka et al., 1995). Briefly, the HIV-1-containing supernatant of the COS-7 cell culture was subjected to ultracentrifugation at 30 000 × g for 1 h at 4°C in a Heraeus Sepatech high speed centrifuge (# 3753 rotor, Heraeus Instruments, Hanau, Germany) and the pelleted virus was subjected to RNA extraction as previously described (Kojima et al., 1995). Purified viral RNA was mixed with a reaction mixture (30 $\mu$l) containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 3 mM dATP, dGTP, dCTP and TTP, 40 units of RNasin (Promega, Madison, WI), 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD), and 15 pmol of the downstream primer RC10, 5′-GCC CTA TTT CTA AGT CAG ATC C-3′ (antisense, nt 3115–3137 of HXB2). The mixture was incubated at 37°C for 1 h followed by incubation at 70°C for 10 min to inactivate the reverse transcriptase. Cell lysates containing proviral DNA were prepared by lysing MT-2 cells ($10^6$) in lysis buffer [10 mM NaCl, 10 mM Tris–HCl (pH 8), 10 mM EDTA, 0.5% SDS, 100 $\mu$g/ml proteinase K] overnight at 37°C. The proviral DNA was further purified by phenol-chloroform extraction and ethanol precipitation. The cDNA product (5 $\mu$l) or the proviral DNA was amplified through the polymerase chain reaction (PCR) in a 50 $\mu$l reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dATP, dGTP, dCTP and TTP, and 2 units of *Taq* DNA polymerase (Perkin Elmer, Foster, CA) with a pair of primers RC7, 5′-TCA GGT CAC TCT TTG GCA ACG A-3′ (sense, nt 2254–2275), and RC10 (12.5 pmol each) with 35 cycles of denaturation at 94°C for 0.5 min, annealing at 58°C for 0.5 min, and polymerization at 72°C for 1 min using the GeneAmp PCR System 9600 (Perkin Elmer). RC7 and RC10 amplified a 884 bp fragment containing the protease- and reverse transcriptase-encoding regions.

In the nested PCR conducted, the first PCR was performed with 20 cycles, followed by seven cycles of reaction after replenishment with polymerase and dNTPs. A portion of the first PCR product (5 $\mu$l) was subjected to nested PCR in a new reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dATP, dGTP, dCTP and TTP, 2 units of *Taq* DNA polymerase, and a pair of nested primers, 12.5 pmol RC8, 5′-ATT GAT AGA TAA CTA TGT CTG G-3′ (antisense, nt 3074-3095) and 12.5 pmol RC9, 5′-GCA ACG

ACC CCT CGT CAC AAT A-3′ (sense, nt 2269–2290). The nested PCR product was then subjected to DNA sequencing as described below.

### 2.6. Determination of nucleotide sequence of HIV-1

The PCR-amplified DNA was purified with spin columns (PCR Select III, 5 Prime 3 Prime, Boulder, CO), cloned into pGEM-T (Promega, Madison, WI) as previously described (Shirasaka et al., 1995), and was subjected to sequencing with an automated DNA sequencer, ABI 373 stretch (Applied Biosystems, Foster City, CA) using dye-labeled M13 forward and reverse primers as previously described (Shirasaka et al., 1995).

### 2.7. Drug sensitivity assay

Sensitivity of each HIV-1 preparation to selected anti-HIV-1 agents was determined as previously described (Richman et al., 1993b). Briefly, MT-2 cells ($1.2 \times 10^5$) were exposed to HIV-1 at a 100 $TCID_{50}$ dose for 1 h at 37°C, washed with PBS twice, dispensed in 96-well microtiter culture plates (2000 cells/well), and cultured in the presence or absence of drugs. On day 7, the amount of p24 Gag protein in the culture supernatant was determined using a commercially available radioimmunoassay (RIA) kit (Du Pont, Boston, MA). All assays were performed in triplicate. Drug sensitivities are shown as the concentration of the drug that inhibited viral replication by 50% ($IC_{50}$).

### 2.8. Construction of an infectious clone carrying the SUM13 and SUM431 mutations

A molecular infectious clone carrying both sets of mutations was generated using a cartridge mutagenesis system as previously described (Shirasaka et al., 1995; Ueno et al., 1995). To this end, pTZNX151-8, a plasmid containing a *Xma*I-*Nhe*I fragment (carrying the five mutations in the RT-encoding region (Ueno et al., 1995) and pHAS431, a plasmid containing a *Apa*I-*Sal*I fragment (carrying the five mutations in the protease-encoding region of pSUM431 (Anderson et al., 1994) were employed. pTZNX151-8 was digested with *Xma*I and *Nhe*I, and the excised 759-bp segment was cloned into *Xma*I-*Nhe*I-digested pHAS431, producing pHAS13/431. Subsequently, the *Apa*I-*Sal*I fragment (3.8 kb) of pHAS13/431 was inserted into *Apa*I-*Sal*I-digested pSUM3 (Shirasaka et al., 1995), generating pSUM13/431. HIV-$1_{SUM13/431}$ was generated through transfection of COS-7 cells with pSUM13/431, followed by propagation of virions in MT-2 cells in the absence of drugs. The presence of introduced mutations and the absence of unintended mutations were confirmed by sequencing both the RT- and protease-encoding regions of the proviral DNA isolated from the cell lysates of the HIV-$1_{SUM13/431}$-infected MT-2 cells.

## 3. Results

### 3.1. Co-transfection with pSUM13 and pSUM431 produces HIV-1 which replicates in the presence of AZT + KNI-272

When we transfected COS-7 cells with pSUM9 which contained wild type HIV-1 (HIV-$1_{HXB2D}$) and propagated the resultant infectious virions in MT-2 cells in the presence of 0.1 $\mu$M AZT and/or 0.3 $\mu$M KNI-272, viral replication was virtually completely suppressed, while there was a significant level of viral replication in the absence of drugs by day 10 of culture as assessed by the amount of p24 Gag protein released into the culture medium (Fig. 1). When we transfected COS-7 cells with pSUM13, the resultant HIV-1 (HIV-$1_{SUM13}$) replicated and produced a significant amount of p24 in the presence of AZT, but failed to replicate in the presence of KNI-272 or AZT + KNI-272 (Fig. 1). When pSUM431 was employed, the resultant HIV-1 (HIV-$1_{SUM431}$) continued to replicate and produced a significant amount of p24 in the presence of KNI-272, but not in the presence of AZT or AZT + KNI-272. By contrast, when COS-7 cells were co-transfected with a 50:50 mixture of pSUM13 and pSUM431 and the resultant infectious virions were propagated in MT-2 cells in the presence or absence of AZT and/or KNI-272, the virions started to repli-

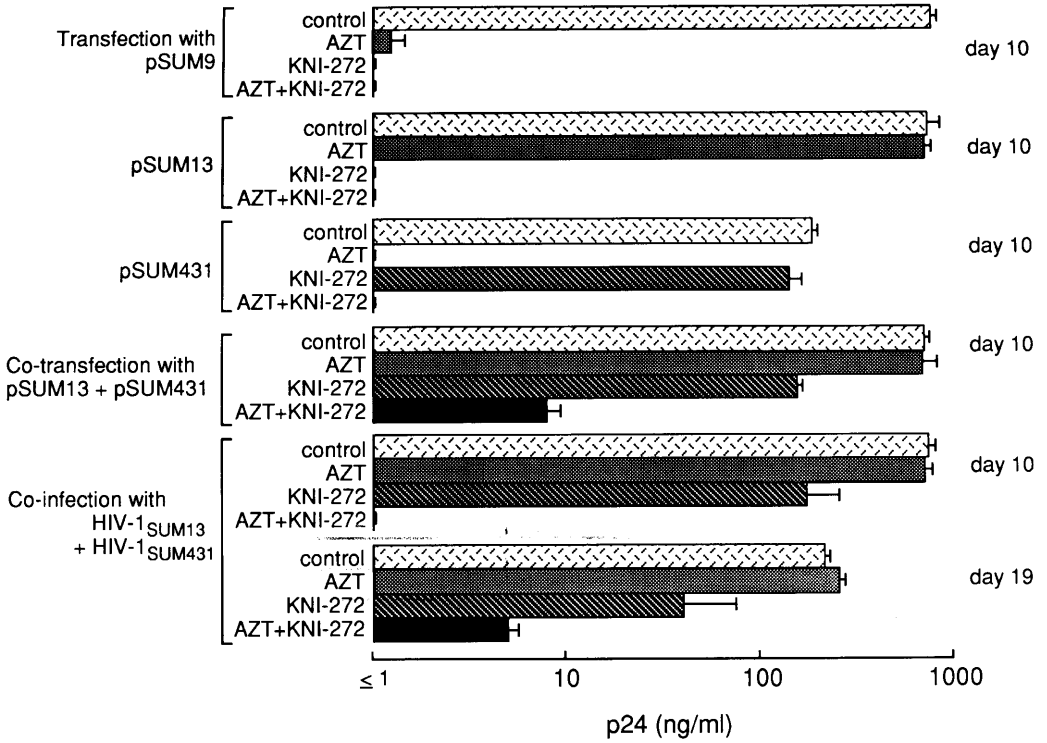


Fig. 1. Emergence of HIV-1 replicating in the presence of AZT and KNI-272 following co-transfection and co-infection. COS-7 cells were transfected with various plasmids, followed by viral propagation in MT-2 cells in the presence or absence of 0.1 $\mu$M AZT and/or 0.3 $\mu$M KNI-272. Co-infection of MT-2 cells with HIV-$1_{SUM13}$ and HIV-$1_{SUM431}$ at an equal infectious viral dose (25 $TCID_{50}$ each) was also conducted, followed by propagation in the presence or absence of AZT and/or KNI-272. In both regimens, HIV-1 replicating in the presence of AZT + KNI-272 (solid columns) emerged. The amounts of p24 Gag protein produced in the culture supernatant were determined by RIA. All experiments were performed in triplicate.

cate in all four cultures by day 10, although the amount of p24 antigen produced in the presence of AZT + KNI-272 was less than those in the other three cultures as assessed on day 10 (Fig. 1). It was possible, however, that the HIV-1 resistant to both drugs was simply derived from recombination between these two transfecting plasmid DNAs when they were transcribed. We, therefore, asked whether HIV-1 resistant to both AZT and KNI-272 emerged when MT-2 cells were co-infected with two infectious clones, HIV-$1_{SUM13}$ and HIV-$1_{SUM431}$.

### *3.2. Co-infection of MT-2 cells with HIV-$1_{SUM13}$ and HIV-$1_{SUM431}$ produces HIV-1 which replicates in the presence of AZT + KNI-272*

HIV-$1_{SUM13}$ and HIV-$1_{SUM431}$ were independently prepared and titrated, and MT-2 cells were exposed to a mixture of these two viral preparations at an equal infectious viral dose (25 $TCID_{50}$). HIV-1 was then propagated in the presence or absence of AZT and/or KNI-272. By day 10 of propagation there was no significant amount of p24 in the supernatants in the presence of AZT + KNI-272 (Fig. 1), however, the forma-

Table 1
Artifactural recombination during PCR amplification of heterogeneous nucleic acid sequences

| Cycles | Number of clones | Codons of RT[a] | | | | | Codons of protease[a] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 62 | 75 | 77 | 116 | 151 | 32 | 33 | 45 | 84 | 89 |
| | | A | V | F | F | Q | V | L | K | I | L |
| 35 cycles[b] | | | | | | | | | | | |
| | 5 | V | I | I | Y | M | — | — | — | — | — |
| | 3 | — | — | — | — | — | I | F | I | V | M |
| | 2 | V | I | I | Y | M | — | — | — | — | M |
| | 1 | V | I | I | Y | M | — | — | — | Y | M |
| | 1 | V | — | — | Y | M | — | — | — | — | — |
| | 1 | V | I | I | — | — | — | — | — | Y | — |
| | 1 | V | I | — | Y | M | — | — | — | — | — |
| 20 cycles followed by seven cycles with nested primers[c] | | | | | | | | | | | |
| | 6 | — | — | — | — | — | I | F | I | V | M |
| | 8 | V | I | I | Y | M | — | — | — | — | — |

[a] Wild type codons of RT and protease are shown.
[b] A reaction mixture (50 $\mu$l) containing pSUM13 (0.2 pg), pSUM431 (0.2 pg) was subjected to 35 cycles of PCR amplification in a 50-$\mu$l reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 2 units of polymerase and RC7 and RC10 (12.5 pmole each).
[c] The same condition as the one used for the 35 cycles of PCR amplification was employed for the first 20 cycles of amplification. A portion (5 $\mu$l) of the first PCR products was further subjected to seven cycles of PCR amplification with the nested primers, RC8 and RC9 and fresh polymerase and dNTP replenished.

tion of HIV-1-induced syncytia was observed by day 17 of culture. On day 19 of propagation, there was a substantial amount of p24 in the HIV-1 culture containing both AZT + KNI-272, in agreement with a recent report by Moutouh et al. (1996).

### *3.3. Artifactual recombination occurs during PCR amplification of mixed HIV-1 population*

We then attempted to determine the nucleotide sequence of the various HIV-1 replicating in the presence or absence of drugs, however, we noted that certain molecular clones contained an incomplete set(s) of the SUM13 and/or SUM431 mutations. Since artifactual recombination occurs during PCR amplification of heterogeneous nucleic acid sequences (Meyerhans et al., 1990; Brown and Simmonds, 1995), we evaluated the rate of artifactual recombination which could have occurred during our analyses of nucleotide sequences of HIV-1. To this end, we employed a 50:50 mixture of pSUM13 (0.2 pg) and pSUM431 (0.2 pg) as a template and performed 35 cycles of denaturation, primer annealing, and polymerization. Eight of 14 molecular clones generated contained a complete set of the SUM13 or SUM431 mutations (Table 1). However, six of 14 clones contained an incomplete set or mixed sets of the mutations. To obviate this problem, we chose to subject a 50:50 mixture of pSUM13 and pSUM431 to 20 cycles of amplification followed by seven cycles of amplification with polymerase and a pair of nested primers replenished. The sequence data revealed that six of 14 molecular clones contained the SUM13 mutations alone and the remainder contained the SUM431 mutations alone. No clones contained incomplete sets of the mutations (Table 1).

Table 2
Emergence of HIV-1 variants carrying both the SUM13 and SUM431 mutations[a]

| | Days in culture | Clones examined | SUM13 | SUM431 | SUM13/SUM431 |
|---|---|---|---|---|---|
| Co-transfection | 0 | 15 | 7 | 8 | 0 |
| | 8 | 16 | 4 | 12 | 0 |
| | 13 | 20 | 0 | 0 | 20 |
| Co-infection | 0 | 12 | 7 | 5 | 0 |
| | 13 | 20 | 0 | 19 | 1 |
| | 21 | 20 | 0 | 0 | 20 |

[a] Viral RNA isolated from the virus preparation used for infection of MT-2 cells was reverse-transcribed and the resultant cDNA was sequenced (day 0). On days 8, 13 and 21, proviral DNA isolated from infected MT-2 cells was analyzed. See Section 2 for detail.

### *3.4. HIV-1 acquires both SUM13 and SUM431 mutations through recombination*

Using the nested PCR as described above, we determined the nucleotide sequence of 15 molecular clones generated from HIV-1, which was isolated 48 h after co-transfection of COS-7 cells with pSUM13 and pSUM431 (Table 2, day 0). Seven of 15 clones had the SUM13 mutations only, eight clones had the SUM431 mutations, and none had both sets of mutations (Table 2). When we sequenced 16 molecular clones generated from HIV-1 propagated for 8 days in the presence of AZT + KNI-272, four of 16 clones had the SUM13 mutations alone, the remainder had the SUM431 mutations alone. However, when assessed on day 13 of viral propagation, 20 of 20 molecular clones examined contained both SUM13 and SUM431 mutations (Table 2). This data indicates that the resultant virions replicating in the presence of AZT + KNI-272 had acquired both the MDR-associated and KNI-272-resistance-associated mutations. These virions were designated HIV-$1_{SUM13/SUM431}$.

We also examined whether co-infection of MT-2 cells with two infectious clones, HIV-$1_{SUM13}$ and HIV-$1_{SUM431}$, followed by propagation in the presence of AZT + KNI-272 generated recombinant HIV-1 carrying both sets of mutations (Table 2). MT-2 cells were exposed to HIV-$1_{SUM13}$ and HIV-$1_{SUM431}$ and propagated in the presence of AZT and KNI-272. On day 13 of viral propagation, when no apparent syncytia formation had been identified, only one of 20 molecular clones examined contained both SUM13 and SUM431 mutations while the rest contained the SUM 431 mutations alone. However, on day 21 of culture, when significant syncytia formation and cytopathic effect of HIV-1 were observed, all 20 molecular clones examined contained both sets of mutations (Table 2).

### *3.5. HIV-$1_{SUM13/SUM431}$ is resistant to ddNs and KNI-272 and fully capable of replicating in the presence of AZT and KNI-272*

We then determined the drug sensitivity of various HIV-1 strains which emerged in culture as well as the infectious clones resistant to three ddNs (AZT, ddC, and ddI) or KNI-272 in vitro (Table 3). HIV-$1_{SUM13}$ was less sensitive to ddNs than wild type HIV-$1_{HXB2D}$ but was comparably sensitive to KNI-272. HIV-$1_{SUM431}$ was less sensitive to KNI-272 than HIV-$1_{HXB2D}$, but comparably sensitive to ddNs. In contrast, HIV-$1_{SUM13/SUM431}$ was less sensitive to both ddNs and KNI-272 than HIV-$1_{HXB2D}$. Since the progeny HIV-$1_{SUM13/SUM431}$ was likely to be a mixed viral population, it was possible that minor populations (HIV-$1_{SUM13}$ and HIV-$1_{SUM431}$) could affect the $IC_{50}$ values of drugs against HIV-$1_{SUM13/SUM431}$, a recombinant infectious clone, HIV-$1_{SUM13/431}$, was generated, which also proved to be less sensitive to both ddNs and KNI-272 (Table 3).

We also examined whether HIV-$1_{SUM13/SUM431}$ and HIV-$1_{SUM13/431}$ were as replication-competent as wild type HIV-1 in vitro. We independently prepared and titrated HIV-$1_{HXB2D}$, HIV-$1_{SUM13}$, HIV-$1_{SUM431}$, and HIV-$1_{SUM13/SUM431}$ and HIV-$1_{SUM13/431}$, exposed MT-2 cells to each HIV-1 preparation at the same infectious dose of the virus

Table 3
Sensitivity of HIV-1 variants carrying the SUM13 and SUM431 mutations to 2′,3′-dideoxynucleosides and KNI-272

| Virus | $IC_{50}^{a}$ ($\mu$M) | | | |
|---|---|---|---|---|
| | AZT | ddC | ddI | KNI-272 |
| HIV-$1_{HXB2-D}$ | <0.03 | $0.032 \pm 0.11$ | $3.5 \pm 2.4$ | $0.029 \pm 0.014$ |
| HIV-$1_{SUM13}$ | $2.1 \pm 0.1$ | $0.30 \pm 0.04$ | $15 \pm 3$ | $0.023 \pm 0.010$ |
| HIV-$1_{SUM431}$ | <0.03 | $0.034 \pm 0.012$ | $3.0 \pm 0.8$ | $1.0 \pm 0.5$ |
| HIV-$1^{b}_{SUM13/SUM431}$ | $2.6 \pm 0.1$ | $0.50 \pm 0.07$ | $17 \pm 4$ | $1.2 \pm 0.3$ |
| HIV-$1^{c}_{SUM13/431}$ | $1.4 \pm 0.2$ | $0.23 \pm 0.02$ | $14 \pm 0.3$ | $1.3 \pm 0.3$ |

[a] MT-2 cells were exposed to 100 $TCID_{50}$ HIV-1, and cultured in the presence or absence of drugs. On day 7, the amount of p24 Gag protein in the culture supernatant was determined. Drug sensitivities are shown as the concentration of drug that inhibited viral replication by 50% ($IC_{50}$). All assays were done in triplicate and mean values ± S.D. are shown.
[b] The virions produced by COS-7 cells transfected with pSUM13 and pSUM431 were propagated in the presence of AZT and KNI-272 for 7 days. The HIV-$1_{SUM13/SUM431}$ is HIV-1 replicating with AZT and KNI-272 (Fig. 1).
[c] HIV-$1_{SUM13/431}$ is the virus prepared from an infectious clone pSUM13/431 carrying the two sets of mutations, A62V/V75I/F77L/F116Y/Q151M in the RT-encoding gene and V32I/L33F/K45I/I84V/L89M in the protease-encoding gene.

(50 $TCID_{50}$), and propagated them in the presence and absence of 0.1 $\mu$M AZT and/or 0.3 $\mu$M KNI-272. The replication rate of HIV-$1_{SUM13}$ in the presence or absence of AZT was comparable to that of HIV-$1_{HXB2D}$ in the absence of drugs as determined with the amount of p24 antigen produced in the culture medium (Fig. 2). The replication rate of HIV-$1_{SUM431}$ in the presence of KNI-272 was comparable to that of HIV-$1_{SUM431}$ in the absence of KNI-272. It was noted that the replication rate of HIV-$1_{SUM13/SUM431}$ was not affected by the presence of AZT and/or KNI-272. The replication rate of HIV-$1_{SUM13/431}$ was not affected by the presence of drugs, either (Fig. 2).

## 4. Discussion

Homologous recombination occurs at a high rate in the replication of HIV-1 and increases viral variation (Hu and Temin, 1990; Stuhlmann and Berg, 1992; Coffin, 1996), which, coupled to the error-prone nature of HIV-1 RT (Roberts et al., 1988; Bebenek et al., 1989; Mansky and Temin, 1995), likely contributes to the development of viral drug resistance (Hu and Temin, 1990; Coffin, 1996). In the present study, we examined whether recombinant HIV-1 resistant to multiple classes of HIV-1 inhibitors emerged through homologous recombination under the selection pressure of anti-HIV-1 drugs in vitro. Since retroviral recombination requires the formation of heterozygous progeny virions (Coffin, 1996), we first co-transfected COS-7 cells with two distinctly different plasmids: one carrying mutations conferring a high level of resistance to RT inhibitors and the other carrying mutations conferring a high level of resistance to protease inhibitors such as KNI-272 and ritonavir (Anderson et al., 1994). We expected that co-transfection should readily produce heterozygous virions in COS-7 cells. Indeed, propagation of HIV-1 in the presence of AZT and KNI-272 following transfection rapidly produced progeny HIV-1 resistant to both AZT and KNI-272. It was possible, however, that the HIV-1 resistant to both drugs was simply derived from recombination between these two transfecting plasmid DNAs when they were transcribed. We, therefore, co-infected MT-2 cells with two infectious clones, HIV-$1_{SUM13}$ and HIV-$1_{SUM431}$, and propagated them in the presence of AZT and KNI-272. This co-infection resulted in the generation of HIV-1 resistant to both drugs, confirming recent data (Moutouh et al., 1996).

During the genetic analysis of HIV-1, we noted that artifactual recombination occurred during PCR amplification of heterogeneous nucleic acid sequences (Meyerhans et al., 1990; Brown and Simmonds, 1995). When 35 cycles of PCR amplification of a 50:50 mixture of pSUM13 and

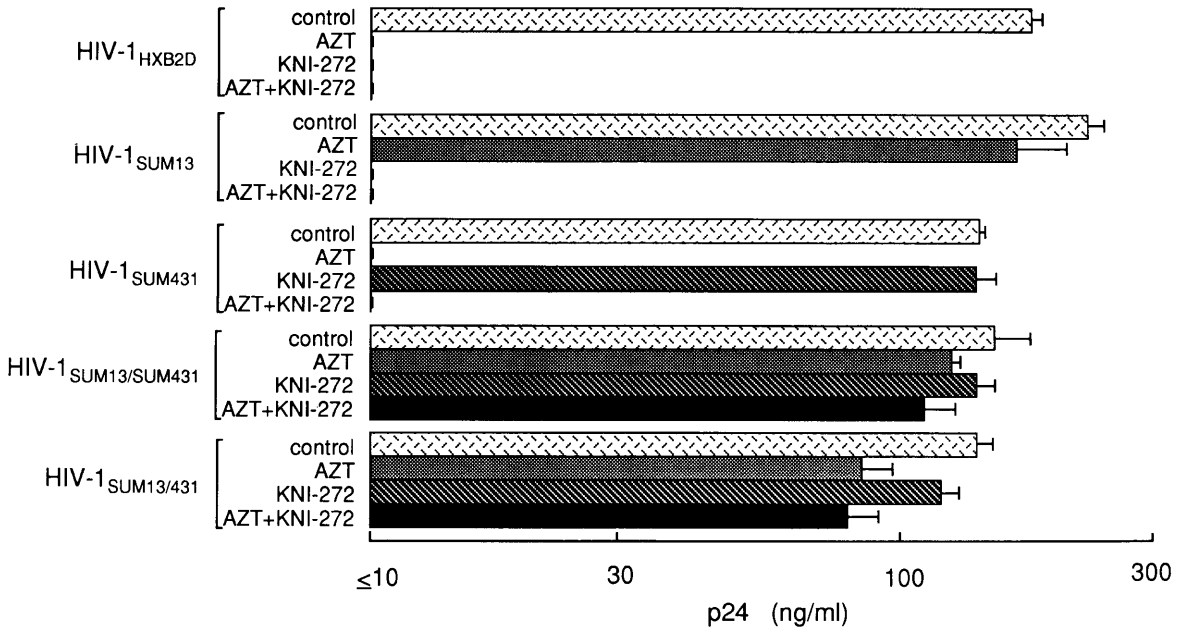


Fig. 2. HIV-$1_{SUM13/SUM431}$ replicates without regard to the presence of drugs. MT-2 cells were exposed to each viral preparation at a 50 $TCID_{50}$ dose and cultured in the presence of 0.1 $\mu$M AZT and/or 0.3 $\mu$M KNI-272. On day 7 of culture, the level of p24 Gag protein produced in the culture supernatant was determined by radioimmunoassay. All experiments were performed in triplicate.

pSUM431 was performed to produce a 884 bp fragment, six of 14 clones examined had an incomplete set or mixed sets of the mutations (Table 1). This artifact, termed artifactual recombination (Brown and Simmonds, 1995) or polymerase halt-mediated linkage of primers (PHMLP) (Frohman and Martin, 1990), is known to occur during the latter cycles of the PCR process, where there is insufficient active DNA polymerase to complete the synthesis of all primed DNA strands. It has been estimated that one in five sequences is artifactual recombinants after amplification of a 300 bp fragment of DNA over 25 thermal cycles (Meyerhans et al., 1990). The frequency of recombinants increases dramatically with longer primer spacings, producing PCR products which may be hybrid copies of two, three, or more of the original viral sequences (Brown and Simmonds, 1995). In order to obviate this problem, we subjected template DNA to 20 cycles of PCR amplification, followed by seven cycle nested PCR with polymerase and dNTPs replenished. The amount of the nested PCR product was relatively small, but was sufficient to determine the sequences of the RT- and protease-encoding region of the virus. This nested PCR product showed no evidence of artificial recombination (Table 1). Thus, it should be stressed that caution should be exercised in amplifying a relatively long DNA fragment over many thermal cycles, particularly when PCR is performed with quasi-species HIV-1 or a mixed population of HIV-1 containing various arrays of mutations, particularly in examining clinical HIV-1 strains isolated from patients.

The $IC_{50}$ values of AZT and KNI-272 against HIV-$1_{SUM13}$ and HIV-$1_{SUM431}$ were apparently comparable to those against HIV-$1_{SUM13/SUM431}$, respectively. These results imply that the mutations in the polymerase-encoding region and those in the protease-encoding region studied in this work do not significantly affect the conformation or interaction with substrates of those enzymes, although it is possible that certain mutations in the protease-encoding region may affect the polymerase function through altered cleavage of the polyprotein by the inflicted protease.

We also examined whether the progeny HIV-$1_{SUM13/SUM431}$ was replication-competent as compared with other HIV-1 preparations. The replication rate of HIV-$1_{SUM13}$ in the absence of drugs was apparently comparable to that of wild type HIV-$1_{HXB2D}$ as assessed by the amount of p24 Gag protein produced (Fig. 1), in agreement with our previous data (Maeda and Mitsuya, 1996), and the replication of HIV-$1_{SUM431}$ was not significantly affected by the presence of KNI-272. Importantly, HIV-$1_{SUM13/SUM431}$ showed a replication rate unaffected by the presence of AZT and/or KNI-272 (Fig. 2), suggesting that once multi-class-resistant progeny virus which has an appropriate fitness emerges, such a virus population may continue to propagate and persist in the presence of two drugs and even in the presence of just one drug, i.e. AZT or KNI-272. The background HIV-1 clone, HIV-$1_{HXB2D}$, does not replicate well in PBMC or monocytes/macrophages and the issue as to whether the replication of HIV-$1_{SUM13/SUM431}$ in such cells was compromised was not addressed in the present work.

Taken together, the observation in this study appears to underline that monitoring drug resistance-associated mutations in HIV-1 in patients receiving long-term combination chemotherapy represents a rational approach to tailoring an individual's treatment strategy.

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