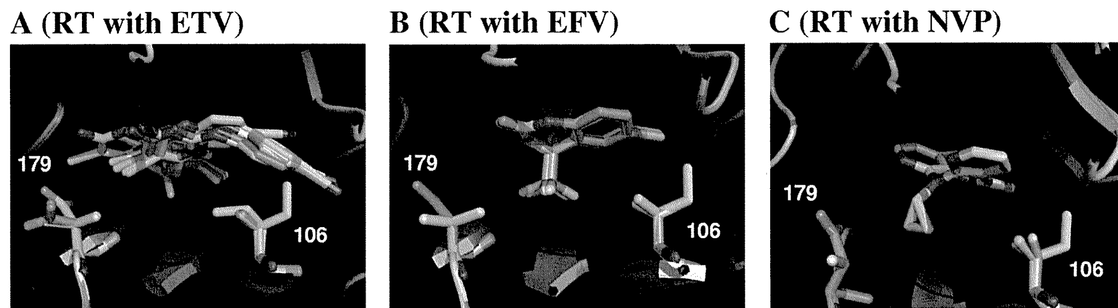

FIG. 4. Superposition of RT_{WT} and five mutant models. (A) RTs with ETV. (B) RTs with EFV. (C) RTs with NVP. NNRTIs and the 106th and 179th residues are highlighted by the stick configuration. White sticks, RT_{WT}; yellow, RT_{V106A}; green, RT_{V106I}; orange, RT_{V179D}; purple, $RT_{V106A/V179D}$; cyan, $RT_{V106I/V179D}$.

not to ETV, although each mutation alone could not alter NNRTI susceptibility (Table 2). Furthermore, one clinical HIV-1 isolate from a treatment-naïve patient who harbored V106I and V179D without any other resistance-associated mutation showed significant resistance to EFV and NVP but not to ETV (Table 3). In a previous study, Tee et al. (29) analyzed HIV-1 RT and protease sequences in 36 antiretroviral-treated patients with detectable viral loads but they could not find any known resistance-associated mutation in 8 patients. In one of their patients on EFV treatment (04MYKL1665), V106I and V179D coexisted in the HIV-1 RT according to GenBank (accession no. AY960901; accessed in October 2009). In a clinical trial of tipranavir, the HIV-1 isolate from one patient (case 48-1084), who experienced NNRTI-treatment failure, harbored V106I and V179D without any other NNRTI resistance-associated mutations (DQ880530) (2). These data strongly indicate that the combination of V106I and V179D also confers significant resistance to NNRTIs *in vivo*.

Structural modeling indicated that V106I and V179D cooperatively reduce NNRTI binding to EFV and NVP. ETV, however, exhibits structural plasticity and can avoid any disturbance caused by the combination of V106I and V179D. This specific structure probably contributes to the efficacy of ETV against many NNRTI-resistant HIV-1 strains, resulting in an excellent rate of response to ETV-containing salvage treatment (18, 21).

Both V106I and V179D are listed as minor ETV resistance-associated mutations in the current version of the IAS-USA Drug Resistance Mutation List (14), but both are not recognized as EFV and NVP resistance-associated mutations. They are often identified individually but rarely coexist in treatment-naïve individuals (Table 1). The combination of V106I and V179D, however, can be found in patients whose baseline HIV-1 held either V106I or V179D after failure of EFV- or NVP-containing treatment (2, 29). Considering that either V106I or V179D was identified in a significant portion of treatment-naïve patients (29/364; 8%) (Table 1), the above information on NNRTI resistance caused by the mutation combination should be recognized by all clinical specialists involved in the interpretation of genotype drug resistance tests and those physicians responsible for changing antiretroviral treatment regimens. In a previous study, we selected EFV-resistant HIV-1 by culture of monoclonal HIV-1 harboring another common polymorphic mutation, K103R ($HIV\text{-}1_{K103R}$), and found the additional emergence of V179D; we then confirmed that the combination of K103R and V179D conferred significant resistance to EFV and NVP (7). Considering these findings together, one assumes that the combinations of polymorphic mutations can reduce NNRTI susceptibility and that other combinations of polymorphic mutations can confer NNRTI resistance. Furthermore, mutations found to be important for one drug may actually have a greater effect on other drugs of the same class. Even polymorphic and minor resistance mutations should be considered carefully when interpreting the results of genotype testing.

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Loss of Protease Dimerization Inhibition Activity of Darunavir Is Associated with the Acquisition of Resistance to Darunavir by HIV-1[▽]

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Dimerization of HIV protease is essential for the acquisition of protease's proteolytic activity. We previously identified a group of HIV protease dimerization inhibitors, including darunavir (DRV). In the present work, we examine whether loss of DRV's protease dimerization inhibition activity is associated with HIV development of DRV resistance. Single amino acid substitutions, including I3A, L5A, R8A/Q, L24A, T26A, D29N, R87K, T96A, L97A, and F99A, disrupted protease dimerization, as examined using an intermolecular fluorescence resonance energy transfer (FRET)-based HIV expression assay. All recombinant HIV_{NL4-3}-based clones with such a protease dimerization-disrupting substitution failed to replicate. A highly DRV-resistant *in vitro*-selected HIV variant and clinical HIV strains isolated from AIDS patients failing to respond to DRV-containing antiviral regimens typically had the V32I, L33F, I54M, and I84V substitutions in common in protease. None of up to 3 of the 4 substitutions affected DRV's protease dimerization inhibition, which was significantly compromised by the four combined substitutions. Recombinant infectious clones containing up to 3 of the 4 substitutions remained sensitive to DRV, while a clonal HIV variant with all 4 substitutions proved highly resistant to DRV with a 205-fold 50% effective concentration (EC_{50}) difference compared to HIV_{NL4-3}. The present data suggest that the loss of DRV activity to inhibit protease dimerization represents a novel mechanism contributing to HIV resistance to DRV. The finding that 4 substitutions in PR are required for significant loss of DRV's protease dimerization inhibition should at least partially explain the reason DRV has a high genetic barrier against HIV's acquisition of DRV resistance.

Currently available combination therapy or highly active antiretroviral therapy (HAART) for human immunodeficiency virus type 1 (HIV) infection and AIDS has been shown to potently suppress the replication of HIV and extend the life expectancy of HIV-infected individuals (32, 34). Recent analyses have revealed that life expectancy in HIV-infected patients treated with HAART has significantly increased, that mortality rates for HIV-infected persons have recently become close to that of general population, and that the appearance of the current first-line antiretroviral therapy with boosted protease inhibitor (PI)-based regimens has made the development of HIV resistance relatively less likely (2, 7, 18, 39). However, the ability to provide effective long-term antiretroviral therapy for HIV infection remains a complex issue since many of those who initially achieved favorable viral suppression to undetectable levels still suffer treatment failure (12, 18, 29).

Dimerization of HIV protease (PR) subunits is an essential process for the acquisition of proteolytic activity of HIV PR, which plays a critical role in the maturation and replication of the virus (28, 40). Thus, inhibition of PR dimerization by chemical reagents is likely to abolish proteolytic activity and intervene in HIV replication. We have recently developed an intermolecular fluorescence resonance energy transfer (FRET)-based HIV-expression assay that employs cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-tagged HIV PR monomers to detect and quantify PR dimerization (26). Using this assay, we identified a group of nonpeptidyl small molecule inhibitors of HIV PR dimerization. These inhibitors, including darunavir (DRV) and tipranavir (TPV) as well as a series of potent experimental antiretroviral agents such as TMC126 (41), blocked PR dimerization at concentrations of as low as 0.01 μM and potently blocked HIV replication *in vitro* (26).

DRV contains a structure-based and designed privileged nonpeptidic P2 ligand, 3(*R*),3a(*S*),6a(*R*)-*bis*-tetrahydrofuranylurethane (*bis*-THF) (14, 15, 27), which potently inhibits the enzymatic activity and dimerization of HIV PR (26) and has a high-level genetic barrier against HIV development of resistance to DRV (9, 10). Nevertheless, we have witnessed that HIV acquires significant levels of resistance against DRV among HIV-infected individuals who have received long-term combi-

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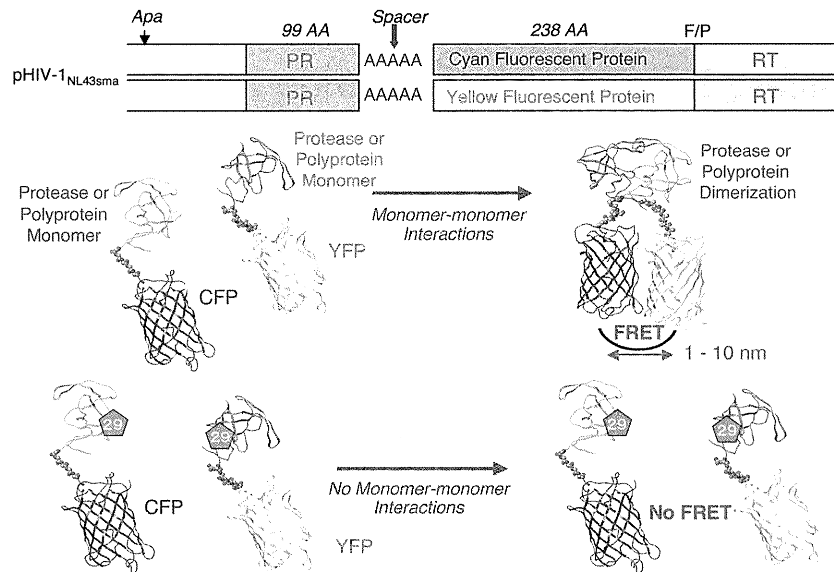

FIG. 1. FRET-based HIV expression system. Plasmids encoding full-length molecular infectious HIV (HIV_{NL4-3}) clones that produce CFP- or YFP-tagged PR were prepared using the PCR-mediated recombination method as described in Materials and Methods. A linker consisting of five alanines was inserted between PR and the fluorescent protein. A phenylalanine-proline site (F/P) that HIV PR cleaves was introduced between the fluorescent protein and RT. Shown are structural representations of PR monomers and dimer in association with the linker atoms and fluorescent proteins. FRET occurs when the two fluorescent proteins become 1 to 10 nm apart. If an agent that is capable of inhibiting the dimerization of PR monomer subunits is present when the CFP- and YFP-tagged PR monomers are produced within the cell upon cotransfection, no FRET occurs. If certain amino acid substitutions (AA) such as D29N (shown below) are introduced, PR subunits do not get dimerized and no FRET occurs.

nation chemotherapy (33, 38). Indeed, a variety of amino acid substitutions that are potentially related to HIV resistance to DRV have been reported (9, 24, 33, 38). Thus, the elucidation of the mechanism of the development of HIV drug resistance represents an urgent subject in the research area of HIV-1 infection/AIDS and therapy.

MATERIALS AND METHODS

Cells, viruses, and antiviral agents. MT-4 cells were grown in RPMI 1640-based culture medium, and 293T and COS7 cells were propagated in Dulbecco's modified Eagle's medium. These media were supplemented with 10% fetal calf serum (FCS; PAA Laboratories GmbH, Linz, Austria) plus 50 U of penicillin and 50 μg of kanamycin per ml. The following HIV strains were used for the determination of 50% effective concentrations (EC_{50}s) against DRV and to construct plasmids for use in the FRET-based HIV expression assay, including HIV_{NL4-3} and HIV_{8MIX}^{P51}. Three recombinant clinical HIV isolates ($_{rCL}HIV_{F16}$, $_{rCL}HIV_{T45}$, and $_{rCL}HIV_{T48}$) used in this study were produced using recombinant HIV_{NL4-3}-based infectious molecular clones generated by ligating patient-derived amplicons encompassing approximately 200 nucleotides of 3′ Gag (beginning at the unique ApaI restriction site), the entire protease, and the first 72 nucleotides of reverse transcriptase (RT) using the expression vector pNLPFB (a generous gift from Tomozumi Imamichi of the National Institute of Infectious Diseases and Allergy). The four clinical HIV isolates examined in the present study were chosen from 32 isolates that had been obtained from multi-PI-treated patients whose protease genotype contained prototypical patterns of PI resistance. The median duration of continuous PI treatment was 7.5 years (range, 6 to 10 years). The median number of PIs received (excluding the use of ritonavir for pharmacokinetic boosting) was 5 (range, 4 to 8). According to the PhenoSense assay, two isolates ($_{rCL}HIV_{T45}$ and $_{rCL}HIV_{F16}$) had high-level resistance to all PIs, including >90-fold decreased susceptibility to DRV and >8-fold decreased susceptibility to TPV, and one isolate ($_{rCL}HIV_{T48}$) had intermediate resistance to DRV and TPV and high-level resistance to the remaining PIs (see Table 2).

DRV was synthesized by A. K. Ghosh as described previously (16, 27). GRL-0216 (37), GRL-98065 (1), and TMC-126 (41) were synthesized in a convergent manner by coupling an optically active P2 ligand and an (*R*)-hydroxyethylamino sulfonamide isostere (17). TPV was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health.

Generation of FRET-based HIV expression system. Cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-tagged HIV PR constructs were generated using BD Creator DNA cloning kits (BD Biosciences, San Jose, CA). The basic concepts of the intermolecular FRET-based HIV expression assay (FRET-HIV assay) are illustrated in Fig. 1. In brief, XhoI and HindIII fragments from the pCR-XL-TOPO vector containing the HIV PR-encoding gene excised from $pHIV_{NL4-3}$ were inserted into pDNR-1r, the donor vector, which had been digested with XhoI and HindIII. In the transfer of the PR gene from the donor vector into pLP-CFP/YFP-C1 (acceptor vector), the Cre-loxP site-specific recombination method was used according to the manufacturer's instructions. Using Cre-recombinase with the loxP site, the PR gene from pDNR-1r was inserted into pLP-CFP-C1 or pLP-YFP-C1 through Cre-mediated recombination (19), generating a plasmid expressing CFP-tagged wild-type PR (PR_{WT}) and one expressing YFP-tagged PR_{WT}, with which HIV PR was successfully expressed as a fusion protein with CFP and YFP tagged at the C terminus, respectively. Western blot assay using anti-green fluorescent protein-specific rabbit polyclonal antibodies revealed that PR was correctly tagged with CFP or YFP (26).

For the generation of full-length molecular infectious clones containing CFP- or YFP-tagged PR, the PCR-mediated recombination (PMR) method was used (11). To this end, we amplified an upstream proviral DNA fragment containing an ApaI site and HIV PR (excised from $pHIV_{NL4-3}$) with primer pair Apa-PRO-F (5′-TTG CAG GGC CCC TAG GAA AAA GG-3′) plus PR-5Ala-R (5′-GGC TGC TGC GGC AGC AAA ATT TAA AGT GCA GCC AAT CT-3′), a middle proviral DNA fragment containing CFP (excised from pCFP-C1) or YFP (excised from pYFP-C1) (Clontech, Mountain View, CA) with primer pair CFPYFP-5Ala-F (5′-GCT GCC GCA GCA GCC GTG AGC AAG GGC GAG GAG CTG-3′) plus CFPYFP-FP-R (5′-ACT AAT GGG AAA CTT GTA CAG CTC GTC CAT GCC G-3′), and a downstream proviral DNA fragment containing the 5′-DNA fragment of RT and an SmaI site from $pHIV_{NLSma}$ (13, 25), which had been created to have an SmaI site by changing two nucleotides (2590 and 2593) of $pHIV_{NL4-3}$, with primer pair FRT-F (5′-TTT CCC ATT AGT CCT ATT GAG ACT GTA-3′) plus NL4-3-RT263-R (5′-CCA GAA ATC TTG AGT TCT CTT ATT-3′). A linker consisting of five alanines was inserted between the PR and fluorescent protein. The phenylalanine-proline site that HIV PR cleaves

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was also introduced between the fluorescent protein and RT. Thus, the three DNA fragments obtained were subsequently joined by using the PMR reaction performed under the standard condition for ExTaq polymerase (Takara Bio, Inc., Otsu, Japan) with 10 pmol of Apa-PRO-F (5′-TTG CAG GGC CCC TAG GAA AAA GG-3′) and NL4-3-RT263-R (5′-CCA GAA ATC TTG AGT TCT CTT ATT-3′) and the three DNA fragments (100 ng each) in a 20-μl reaction solution. Thermal cycling was carried out at 94°C for 3 min, followed by 35 cycles of 94°C for 50 s, 53°C for 50 s, and 72°C for 2 min, and finally 72°C for 15 min. The amplified PCR products were cloned into pCR-XL-TOPO vector according to the manufacturer's instructions (Gateway cloning system; Invitrogen). PCR products were generated with pCR-XL-TOPO vector as templates, followed by digestion by both ApaI and SmaI, and the ApaI-SmaI fragment was introduced into $pHIV_{NLSma}$ (13), generating pHIV-PR_{WT}^{CFP} and pHIV-PR_{WT}^{YFP}, respectively.

Analysis of inter- and intramolecular interactions of PR subunits. Analysis of inter- and intramolecular interactions of PR subunits was conducted by examining the crystal structure of DRV with HIV PR (Protein Data Bank identification no. [PDB ID no.] 2IEN). Hydrogens were added and minimized using the OPLS2005 force field with constraints on heavy atom positions. The calculation was performed using MacroModel 9.1 from Schrödinger, LLC. Hydrogen bonds were assigned when the following distance and angle cutoff was satisfied: 3.0 Å for H-A distance, with a D-H-A angle of >90° and an H-A-B angle of >60°, where H is the hydrogen, A is the acceptor, D is the donor, and B is a neighbor atom bonded to the acceptor. The representative distance between the termini of two monomers was determined by analyzing the PR-DRV crystal structure (PDB ID no. 2IEN). The distance between the α carbons at the N termini and C termini is around 0.5 nm, whereas the distance between the α carbons of the N termini ends of two monomers is around 1.8 nm.

FRET procedure. COS7 cells plated on an EZ view cover glass bottom culture plate (Iwaki, Tokyo) were transfected with the indicated plasmid constructs, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions in the presence of various concentrations of each compound, cultured for 72 h, and analyzed under a Fluoview FV500 confocal laser scanning microscope (Olympus Optical Corp., Tokyo) at room temperature. When the effect of each compound was analyzed by FRET, test compounds were added to the culture medium simultaneously with plasmid transfection. The results of FRET were determined by quenching of CFP (donor) fluorescence and an increase in YFP (acceptor) fluorescence (sensitized emission), because part of the energy of CFP is transferred to YFP instead of being emitted. This phenomenon can be measured by bleaching YFP, which should result in an increase in CFP fluorescence. This technique, also known as acceptor photobleaching, is a well-established method of determining the occurrence of FRET (5, 6, 35, 36). Dequenching of the donor CFP by selective photobleaching of the acceptor YFP was performed by first obtaining YFP and CFP images at the same focal plane, followed by illuminating the same image for 3 min at a wavelength of 488 nm with a laser power set at the maximum intensity to bleach YFP and then recapturing the same CFP and YFP images. The changes in the CFP and YFP fluorescence intensity in the images of selected regions were examined and quantified using the Olympus FV500 Image software system (Olympus Optical Corp.). Background values were obtained from the regions where no cells were present and were subtracted from the values for the cells examined in all calculations. For each chimeric protein, the data were obtained from at least three independent experiments. Digitized image data obtained from the experiment were prepared for presentation using Photoshop 6.0 (Adobe Systems, Mountain View, CA). Ratios of intensities of CFP fluorescence after photobleaching to CFP fluorescence prior to photobleaching ($CFP^{A/B}$ ratios) were determined. It is well established that $CFP^{A/B}$ ratios of >1.0 indicate that association of CFP- and YFP-tagged proteins occurred and were interpreted to indicate that the dimerization of PR subunits occurred. $CFP^{A/B}$ ratios of <1 indicated that the association of the two subunits did not occur and were interpreted to indicate that PR dimerization was inhibited. The difference in the $CFP^{A/B}$ ratios determined in the presence or absence of test drugs was evaluated using the nonparametric Mann-Whitney U statistic test.

Replication kinetics of various NL-PR_{mutant}^{YFP} strains. MT-4 cells (10^5) were exposed to each infectious HIV-PR^{YFP} clone (100 ng of p24 Gag protein/ml) for 6 h, washed twice with phosphate-buffered saline (PBS), and cultured in 7 ml of complete medium with some modification as described previously (3, 13). Culture supernatants (50 μl) were harvested every other day, and virus replication was monitored by the amounts of p24 Gag produced in the culture supernatants.

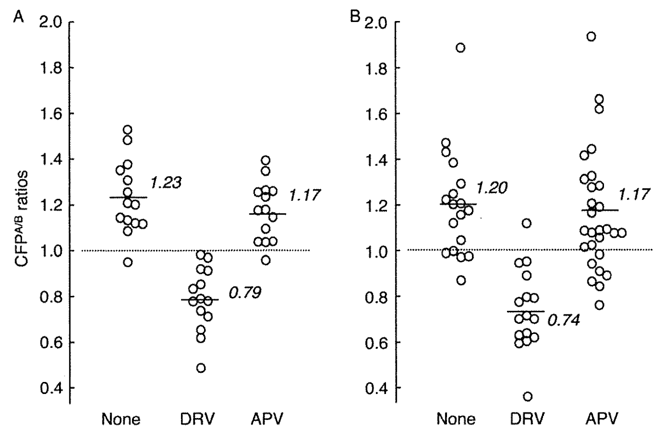

FIG. 2. DRV blocks the dimerization of both pHIV-PR_{WT}-encoded PR and pPR_{WT}-encoded PR. (A) COS7 cells were cotransfected with pHIV-PR_{WT}^{CFP} plus pHIV-PR_{WT}^{YFP} in the absence or presence of 1 μM DRV or APV. On day 3 after transfection, $CFP^{A/B}$ ratios were determined using an FV500 confocal laser microscope. When the average value of $CFP^{A/B}$ ratios was greater than 1.0, it was judged that the dimerization of PR occurred, whereas when it was less than 1.0, it was judged that the dimerization did not occur. (B) COS7 cells were cotransfected with a pair of wild-type PR-expressing plasmids (pPR_{WT}^{CFP} plus pPR_{WT}^{YFP}) in the absence or presence of 1 μM DRV or APV, and $CFP^{A/B}$ ratios were determined as described above. Note that DRV inhibited the dimerization of PR when it was expressed as HIV virions and virion-free PR. The results of statistical evaluation of the changes in the $CFP^{A/B}$ ratios, determined in the presence or absence of DRV or APV, using the nonparametric Mann-Whitney U test, are as follows. (A) For the $CFP^{A/B}$ ratios in the absence of drug ($CFP^{A/B\text{No Drug}}$) versus the $CFP^{A/B}$ ratios in the presence of 1.0 μM DRV ($CFP^{A/B1.0\text{ DRV}}$), $P = 0.00001$, and for $CFP^{A/B\text{No Drug}}$ versus $CFP^{A/B1.0\text{ APV}}$, $P = 0.42$. (B) For $CFP^{A/B\text{No Drug}}$ versus $CFP^{A/B1.0\text{ DRV}}$, $P = 0.000003$, and for $CFP^{A/B\text{No Drug}}$ versus $CFP^{A/B1.0\text{ APV}}$, $P = 0.60$.

RESULTS

DRV inhibits the dimerization of HIV PR expressed as a single protein. The basic concepts of the intermolecular FRET-based HIV-expression assay (FRET-HIV expression assay) to assess PR dimerization are illustrated in Fig. 1 (26). Using the FRET-based HIV expression assay, we previously identified a group of PR dimerization inhibitors (PDIs), including DRV and TPV, although other conventional PR inhibitors (PIs), such as amprenavir (APV), failed to block dimerization (Fig. 2A) (26). In the FRET-based HIV expression assay, YFP- or CFP-tagged PR should be primarily expressed as a part of $Pr160^{gag\text{-}pol}$ polyprotein, and it was assumed that DRV blocks the dimerization of the PR subunit within the polyprotein. Thus, it remained to be determined whether DRV also blocks the dimerization of PR in the form of a single PR molecule. We, therefore, generated a pair of plasmids encoding wild-type $HIV_{NL4\text{-}3}$ PR tagged with YFP and CFP in the 3′ terminus (pPR_{WT}^{YFP} and pPR_{WT}^{CFP}, respectively), transfected COS7 cells with the pair, and determined whether DRV blocked the dimerization of PR_{WT}^{YFP} and PR_{WT}^{CFP}. As shown in Fig. 2B, the average value of $CFP^{A/B}$ ratios obtained in the absence of drug was 1.20 ± 0.24, which indicated that the dimerization between PR_{WT}^{YFP} and PR_{WT}^{CFP} occurred. The average value of the ratios determined in the presence of 1 μM DRV was 0.74 ± 0.18 ($P = 0.000003$), signifying that DRV clearly blocked the

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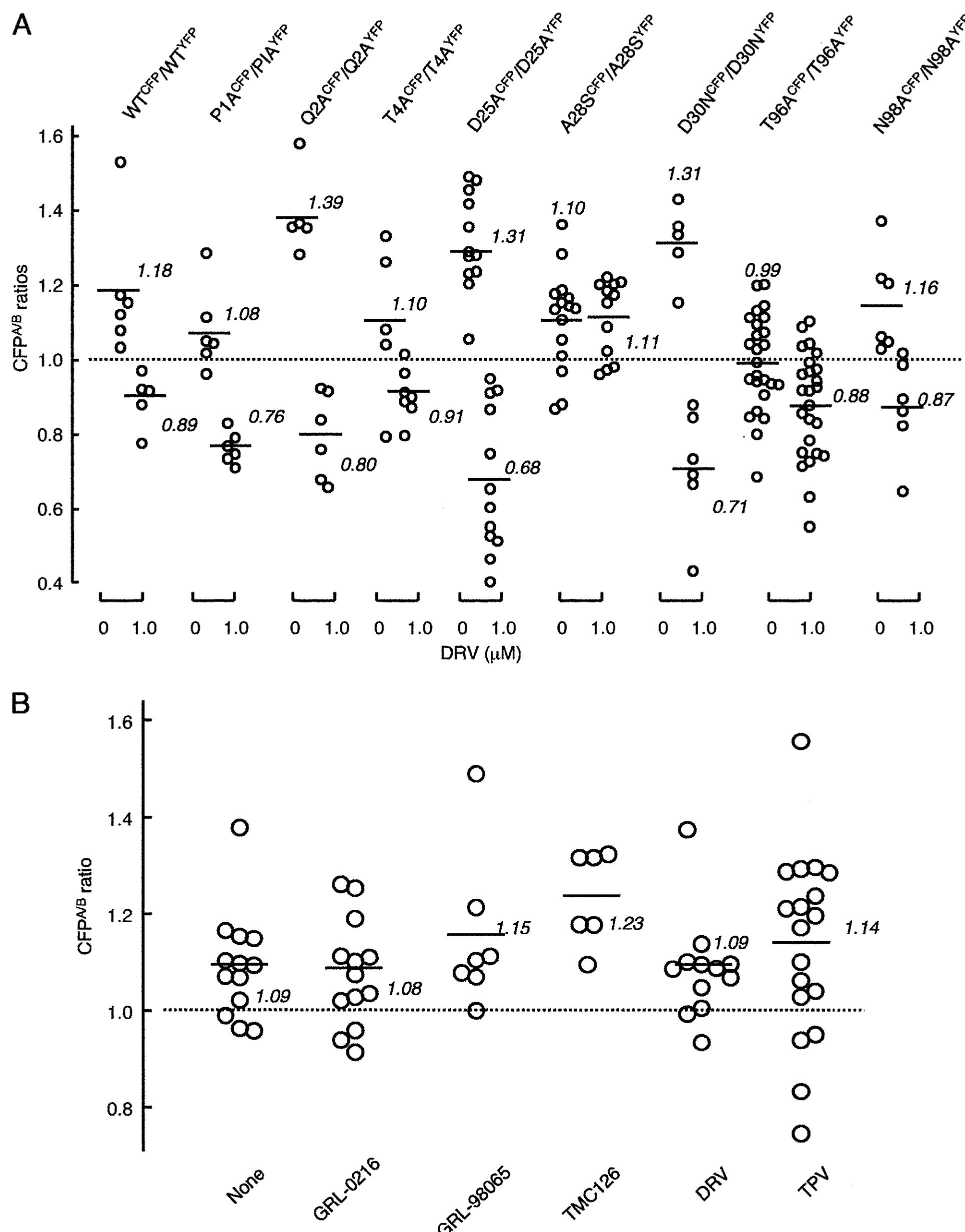

FIG. 3. Dimerization profiles of single PR mutants in the presence of DRV. (A) COS-7 cells were cotransfected with pHIV-PR_{WT}^{CFP} plus pHIV-PR_{WT}^{YFP} (shown as WT^{CFP}/WT^{YFP}) or mutated pairs such as pHIV-PR_{P1A}^{CFP} plus pHIV-PR_{P1A}^{YFP} (shown as $P1A^{CFP}/P1A^{YFP}$) in the absence or presence of 1 μM DRV. On day 3 after transfection, $CFP^{A/B}$ ratios were determined. (B) COS7 cells were cotransfected with plasmid pair pHIV-PR_{A28S}^{CFP} and pHIV-PR_{A28S}^{YFP} in the absence or presence of an agent (1 μM GRL-0216, DRV, GRL-98065, TPV, or TMC126), and $CFP^{A/B}$ ratios were determined as described above. (A) The statistical evaluation of all the changes in the $CFP^{A/B}$ ratios determined in the presence or absence of DRV using the nonparametric Mann-Whitney U test, gave P values ranging 0.000037 to 0.044, except for the P value for the pair $A28S^{CFP}$ and $A28S^{YFP}$, which was 0.57. (B) The differences between the $CFP^{A/B}$ ratios in the absence of drug ($CFP^{A/B_{No\ Drug}}$) and the $CFP^{A/B}$ ratios in the presence of 1.0 μM DRV ($CFP^{A/B_{1.0\ DRV}}$) were statistically insignificant, indicating that all of the agents examined failed to block the dimerization of $A28S^{CFP}/A28S^{YFP}$.

dimerization of PR_{WT}^{YFP} and PR_{WT}^{CFP}, while the value with APV was 1.17 ± 0.27 (P = 0.60), indicating that APV failed to block the dimerization, in line with our previous data (26). These results strongly suggest that DRV blocks dimerization of the PR monomer subunit in the form of $Pr160^{gag-pol}$ polyprotein as well as in the form of a single molecule.

Dimerization profiles of single PR mutants in the presence of DRV. Certain amino acids in the termini and active site interfaces, both of which are critical for the dimerization of PR monomer subunits (28, 40), do not significantly affect the dimerization process of PR. Such amino acids include Pro-1, Gln-2, Thr-4, Asp-25, Ala-28, Asp-30, Thr-96, and Asn-98 (26). It is assumed that DRV blocks PR dimerization by binding to a certain structural domain or domains within or in the proximity of either or both of the two interfaces (4, 22, 23). We, therefore, examined whether amino acid substitutions at posi-

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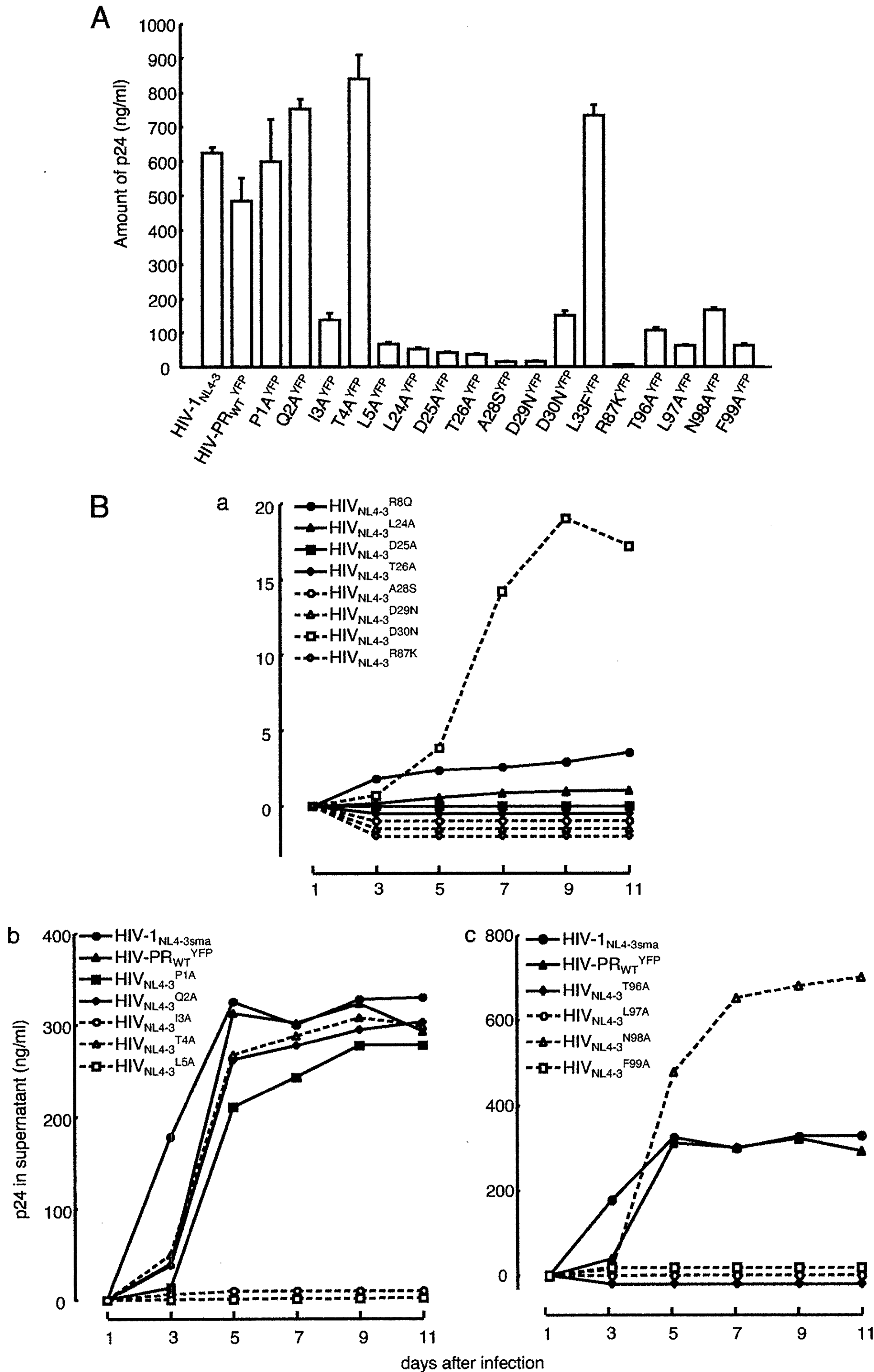

FIG. 4. Replication kinetics of HIV-PR^{YFP} with wild-type or mutated PR. (A) 293T cells were transfected with pHIV-PR$_{WT}$^{YFP} or mutated pHIV-PR^{YFP} (if pHIV-PR$_{P1A}$^{YFP} was used, it is shown as P1A^{YFP}), and the amounts of p24 Gag in the culture supernatants were determined 48 h after transfection. (B) MT-4 cells (10^5) were exposed to the harvested supernatant of each infectious HIV-PR^{YFP} clone shown in panel A (100 ng of p24 Gag protein/ml) for 6 h, washed twice with phosphate-buffered saline (PBS), and further cultured in 7 ml of complete medium. Culture supernatants (50 μl) were harvested every other day, and virus replication was monitored by the amounts of p24 Gag produced in the culture supernatants. Replication kinetics of various HIV-PR^{YFP} mutants are shown over 11 days. In subpanels a, b, and c, the replication kinetics of infectious clones carrying mutations in the active site, N ternimus, and C terminus, respectively, are shown. Note that recombinant HIV clones, whose replication rates were relatively poor, are illustrated in subpanel a. The experiments that generated data in subpanels a and b were performed on the same occasion. Thus, two controls (HIV-1$_{NL4-3sma}$ and HIV-PR$_{WT}$^{YFP}) in subpanel b serve as controls in subpanel a as well.

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tions 1, 3, 5, 25, 28, 30, 96, and 98, which allow PR to dimerize, affected the PR dimerization disruption by DRV. We reasoned that if any of the amino acid substitutions at these positions would affect PR dimerization inhibition by DRV, such amino acids could possibly be associated with the binding of DRV to the PR subunit. However, 1 μM DRV effectively blocked the dimerization of all of the mutated PR species, except that of the species with the A28S substitution (Fig. 3A). These data suggest that all amino acid residues examined except A28S were not associated with the binding of DRV to the PR monomer subunit.

We have previously shown that, in addition to DRV and TPV, the three compounds GRL-0216 (37), GRL-98065 (1), and TMC126 (41) effectively blocked PR dimerization in the FRET-based HIV expression assay (26). Since the structures of these five compounds differ from each other, it was thought that the binding profiles of each compound also differed. We, therefore, examined if the four compounds other than DRV disrupted the dimerization of the A28S-carrying PR subunit. As shown in Fig. 3B, all four compounds failed to block protease dimerization, suggesting that Ala-28 is likely involved directly or indirectly in the binding of all four compounds to the PR monomer subunit.

Replication kinetics of HIV variants with failed PR dimerization. The failure of PR dimerization should completely block or significantly compromise the replication of HIV. In order to confirm that the observed dimerization failure elicited by a single amino acid substitution (26) causes replication failure of HIV, we generated a panel of HIV variants carrying YFP-tagged PR with a single amino acid substitution and examined the replicative capability of each variant. When the amount of p24 antigen produced into culture medium following transfection of COS7 cells with each plasmid was quantified, an HIV variant containing I3A, L5A, L24A, D25A, T26A, A28S, D29N, R87K, T96A, L97A, or F99A produced no or a significantly small amount of p24 (Fig. 4A). Among these variants, we have previously shown that I3A, L5A, T26A, D29N, R87K, T96A, L97A, and F99A disrupted PR dimerization (26). Figure 5 shows that R8A (P = 0.000099), R8Q (P = 0.000084), and L24A (P = 0.0000014) also disrupted PR dimerization, as examined in the FRET-based HIV expression assay (Fig. 5). When fresh MT-4 cells were exposed to each cell-free culture supernatant of the transfected COS7 cells, as described above, no further replication was seen over the 11-day period of the culture (Fig. 4B). The HIV variant carrying A28S also failed to replicate; however, this failure was explained by an observation that protease with A28S has almost no enzymatic activity, as reported by Hong et al. (20, 21). In contrast, recombinant HIV clones containing either of P1A, Q2A, T4A, D30N, or N98A continued to replicate (Fig. 4B).

DRV resistance profiles of PR species carrying DRV-resistance-associated amino acid substitutions. Using the standardized protocol for selection of drug-resistant HIV variants, we along with others have experienced difficulty in selecting DRV-resistant HIV variants *in vitro* (8, 24). The emergence of DRV-resistant HIV variants was substantially slower than that of variants resistant to other FDA-approved PIs when a single HIV strain was employed as a starting viral strain (8, 24). In this respect, we have recently succeeded in selecting a highly DRV-resistant HIV variant by using a mixture of 8 highly multi-PI-resistant, DRV-susceptible clinical HIV strains (HIV_{8MIX} strains HIV_A, HIV_B, HIV_C, HIV_G, HIV_{TM}, HIV_{MM}, HIV_{JSL}, and HIV_{SS}), which were originally isolated from patients with AIDS, who had failed then-existing anti-HIV regimens after receiving 9 to 11 anti-HIV drugs over the previous 32 to 83 months in the late 1990s and contained 9 to 14 amino acid substitutions in the PR-encoding region (42). By passage 39 in the selection with DRV, HIV_{8MIX} ($HIV_{8MIX}{}^{P39}$) became highly resistant to DRV, with an EC_{50} ~333-fold greater than that against HIV_{NL4-3}. HIV_{8MIX} at passage 39 ($HIV_{8MIX}{}^{P39}$) was capable of replicating in the presence of 1 μM DRV with a replication fitness comparable to that of HIV_{NL4-3} (24). HIV_{8MIX} at passage 51 ($HIV_{8MIX}{}^{P51}$), which was also capable of replicating in the presence of 5 μM DRV, was found to contain the following 14 mutations: L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54M, L63P, K70Q, V82I, I84V, and L89M (24). As illustrated in Table 1, when $HIV_{8MIX}{}^{P51}$ was propagated in the presence of 0.1 and 1.0 μM DRV in $CD4^+$ MT-4 cells, the virus replicated comparably to HIV_{NL4-3} during the 9-day period of culture, while HIV_{NL4-3} completely failed to replicate in the presence of 0.1 or 1.0 μM DRV, as examined according to the amounts of Gag protein produced in the culture supernatant, indicating that $HIV_{8MIX}{}^{P51}$ had acquired a high-level resistance against DRV, while it maintained its robust replication fitness.

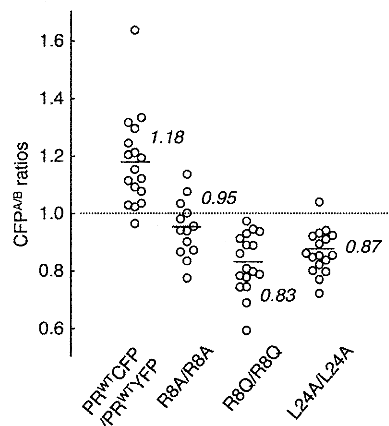

FIG. 5. Dimerization inhibition profiles of selected HIV-1 PR mutants. COS7 cells were cotransfected with a pair of HIV-PR^{CFP} and HIV-PR^{YFP} strains either wild type or carrying single amino acid (AA) substitutions, such as the R8A, R8Q, or L24A, in the absence of drug. The $CFP^{A/B}$ ratios were determined at the conclusion of the 3-day period of culture. The differences between the $CFP^{A/B}$ ratios of the WT and the $CFP^{A/B}$ ratios of the mutant had P values of 0.000099 for R8A, 0.000084 for R8Q, and 0.0000014 for L24A.

We, therefore, asked if the dimerization of the PR of $HIV_{8MIX}{}^{P51}$ was blocked by DRV, exploiting the FRET-based HIV expression system by using a pair of newly generated plasmids encoding a full-length molecular infectious HIV clone containing CFP- or YFP-tagged PR with all 14 amino acid substitutions. As shown in Fig. 6, DRV significantly blocked the dimerization of the wild-type PR of HIV_{NL4-3} at concentrations of 0.1 and 1 μM. However, DRV failed to block the dimerization of the PR of $HIV_{8MIX}{}^{P51}$ at 0.1 μM (P = 0.42). These data suggested that all amino acid substitutions present in the PR of $HIV_{8MIX}{}^{P51}$ or subsets of them were associated with the $HIV_{8MIX}{}^{P51}$ strain's acquisition of DRV resistance.

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TABLE 1. HIV DRV-resistant strain HIV_{8MIX}^{P51} is capable of replicating in the presence of DRV[a]

Virus	DRV (μM)	Replication (ng/ml) at day postexposure:				
		1	3	5	7	9
HIV_{NL4-3} (WT)	0	0	33 ± 9.9	955 ± 9.9	993 ± 57	1152 ± 127
	0.1	0	0	0	0	0
	1.0	0	0	0	0	0
HIV_{8MIX}^{P51}	0	0	701 ± 45	734 ± 68	771 ± 19	877 ± 88
	0.1	1.5 ± 2.1	590 ± 103	682 ± 199	729 ± 3	909 ± 178
	1.0	0.5 ± 0.7	270 ± 10	886 ± 117	936 ± 18	1,201 ± 170

[a] $CD4^+$ MT-4 cells were exposed to HIV_{NL4-3} or HIV_{8MIX}^{P51} (a highly DRV-resistant HIV variant derived from the mixture of 8 highly-PI-resistant clinical HIV isolates exposed to increasing concentrations of DRV up to 1 μM) (24), cultured in the absence or presence of 0.1 or 1.0 μM DRV. Viral replication was monitored by the amounts of p24 Gag protein (ng/ml) produced in the culture supernatant.

Effects of V32I, L33F, I54M/L, and/or I84V substitutions on HIV susceptibility to DRV and PR dimerization inhibition by DRV. When we examined the sequence of the PR-encoding gene in HIV_{8MIX}^{P51} and three clinical HIV variants isolated from individuals with AIDS who did not respond to DRV-containing antiviral regimens (Table 2) (33), four amino acid substitutions (V32I, L33F, I54M, and I84V) were found to be mostly in common and thought to be relatively unique in such DRV-resistant HIV variants. The locations of the four amino acid substitutions are illustrated in Fig. 7A. This notion was further confirmed when we examined reports by others (9, 24, 33, 38) regarding the sequence of the PR-encoding region of DRV-resistant variants, as illustrated in Table 2. We consequently examined whether the notion described above was plausible by incorporating one of the four amino acid substitutions or subsets of them.

When we introduced each of the four substitutions into the wild-type strain, HIV_{NL4-3}, there was no increase observed in the EC_{50}s of DRV against such infectious recombinant clones,

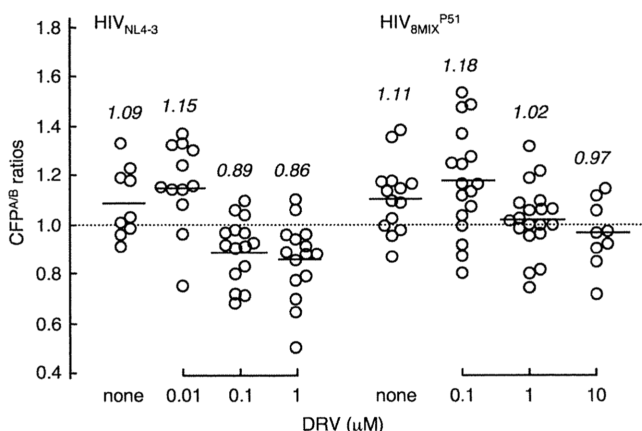

FIG. 6. DRV fails to inhibit the dimerization of the protease of a highly DRV-resistant HIV_{8MIX}^{P51} variant. COS7 cells were transfected with a pair of plasmids encoding a full-length molecular infectious HIV-1 clone (HIV_{8MIX}^{P51}) containing CFP- or YFP-tagged PR with 14 amino acid substitutions (L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54M, L63P, K70Q, V82I, I84V, and L89M) in the presence or absence of 0.1, 1, or 10 μM DRV. On day 3 after transfection, $CFP^{A/B}$ ratios were determined as described in the legend to Fig. 2. HIV_{NL4-3} served as a reference. Note that 0.1 and 1 μM DRV failed to block the dimerization of the protease of HIV_{8MIX}^{P51}, while the same concentration of DRV blocked protease dimerization in HIV_{NL4-3}. The differences between the $CFP^{A/B}$ ratios in the absence of drug ($CFP^{A/B\text{No Drug}}$) and the $CFP^{A/B}$ ratios in the presence of 0.01 μM DRV ($CFP^{A/B0.01\text{ DRV}}$), between the $CFP^{A/B}$ ratios in the presence of 0.01 μM DRV ($CFP^{A/B0.01\text{ DRV}}$) and 0.1 μM DRV ($CFP^{A/B0.1\text{ DRV}}$), and between the $CFP^{A/B}$ ratios in the presence of 0.1 μM DRV ($CFP^{A/B0.1\text{ DRV}}$) and 1.0 μM DRV ($CFP^{A/B1.0\text{ DRV}}$) had P values of 0.32, 0.0025, and 0.34 for HIV_{NL4-3}, respectively. The differences between the $CFP^{A/B\text{No Drug}}$ and the $CFP^{A/B0.1\text{ DRV}}$, between the $CFP^{A/B0.1\text{ DRV}}$ and $CFP^{A/B1.0\text{ DRV}}$, and between the $CFP^{A/B1.0\text{ DRV}}$ and the $CFP^{A/B10.0\text{ DRV}}$ had P values of 0.42, 0.022, and 0.26, respectively, for HIV_{8MIX}^{P51}.

TABLE 2. Amino acid substitutions associated with DRV resistance

DRV resistance-associated amino acid substitutions identified[a]	Variant[b]	EC_{50}, μM (fold change)[c]
L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54 M, L63P, K70Q, V82I, I84V, L89 M	HIV_{8MIX}^{P51}	>1 (>333)
L10F, V11I, I13V, L19Q, K20 M, V32I, L33F, E35A, M36I, M46I, I47V, I54 M, R57K, I62V, L63P, I64V, G73T, T74A, I84V, L89V, L90 M	$_{rCL}HIV_{F16}$	0.30 (97)
L10F, V11I, T12P, I13V, I15V, L19P, K20T, V32I, L33F, E35G, M36I, I54V, I62V, L63P, K70T, A71I, G73S, P79A, I84V, L89V, L90 M	$_{rCL}HIV_{T45}$	0.33 (105)
L10I, I13V, I15V, L19V, L24I, V32I, L33F, K43E, M46L, I54L, D60E, L63P, A71V, I72V, V82A, I84V	$_{rCL}HIV_{T48}$	0.17 (54)
V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, L89V		
V32I, I50V, I54L, I54 M, L76V, V82F		

[a] Amino acid substitutions identified in the protease-encoding region of HIV are shown. The amino acid substitutions shown in the second row from the bottom were reported by De Meyer et al. (9) and Mitsuya et al. (33). Those in the bottom row were reported by Van Marck et al. (38) and were reported to have the greatest impact on HIV-1 resistance to DRV. Those for HIV_{8MIX}^{P51} were reported by Koh et al. (24).

[b] Three infectious clones ($_{rCL}HIV_{F16}$, $_{rCL}HIV_{T45}$, and $_{rCL}HIV_{T48}$) were derived from clinical strains isolated from patients who failed to respond to DRV-containing regimens.

[c] Shown are EC_{50}s of DRV for each infectious clone. Values in parentheses represent fold changes of EC_{50}s of DRV compared to EC_{50}s against a wild-type clinical strain, HIV-$1_{ERS104pre}$. All assays were conducted in triplicate, and the mean values are shown. The EC_{50}s of ritonavir and lopinavir against HIV_{8MIX}^{P51}, $_{rCL}HIV_{F16}$, $_{rCL}HIV_{T45}$, and $_{rCL}HIV_{T48}$ were all >1 μM.

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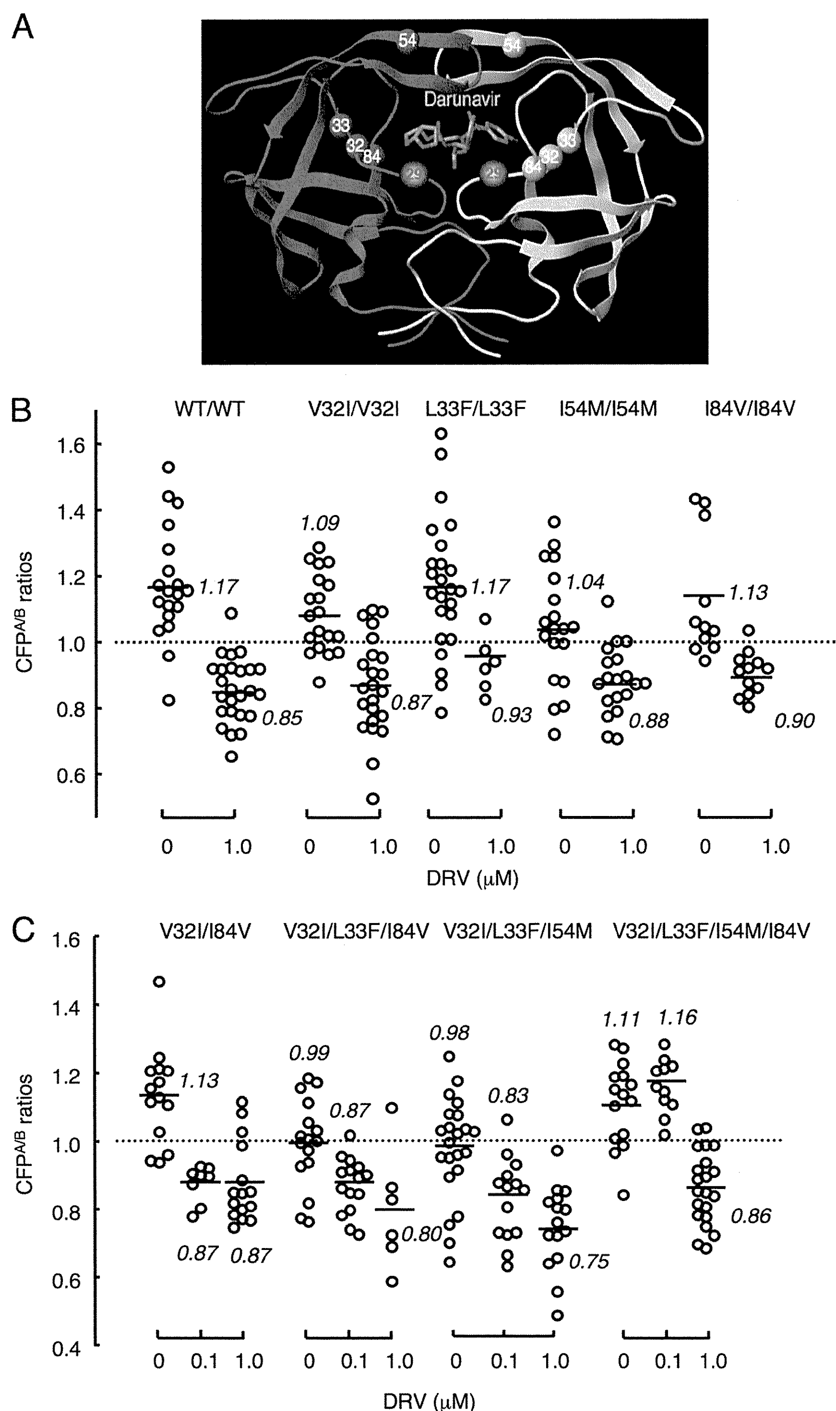

FIG. 7. Amino acid changes conferring DRV resistance on HIV. (A) Locations of amino acid substitutions V32I, L33A/F, I54M, and I84V associated with HIV's DRV resistance. The location of Asp29 (D29), which is known to be an essential amino acid for dimerization, is also shown. (B) Profiles of DRV's dimerization inhibition of PR carrying a single amino acid substitution. COS7 cells were cotransfected with a pair of HIV-PR^{CFP} and HIV-PR^{YFP} variants carrying wild-type PR or a single amino acid substitution such as V32I, L33F, I54M, or I84V, each of which was found to be associated with the development of HIV resistance to DRV, in the presence of 1 μM DRV, further cultured, and the CFP$^{A/B}$ ratios were determined. Note that none of the amino acid substitutions introduced blocked the dimerization of PR. The statistical evaluation of all the changes in the CFP$^{A/B}$ ratios determined in the presence or absence of DRV, conducted using the nonparametric Mann-Whitney U test, showed *P* values ranging from 0.00000034 (3.4E−07) to 0.0026. (C) Profiles of DRV's dimerization inhibition of PR carrying combined amino acid substitutions. COS7 cells were cotransfected with a pair of HIV-PR^{CFP} and HIV-PR^{YFP} variants carrying combined amino acid substitutions such as V32I and I84V, V32I, L33F, and I84V, V32I, L33F, and I54M, or V32I, L33F, I54M, and I84V. The COS7 cells were further cultured in the continuous presence of 0, 0.1, and 1 μM DRV, and the CFP$^{A/B}$ ratios were determined at the conclusion of the 3-day period of culture. The differences between the CFP$^{A/B}$ ratios in the absence of drug (CFP$^{A/B_{No\ Drug}}$) and the CFP$^{A/B}$ ratios in the presence of 0.1 μM DRV (CFP$^{A/B_{0.1\ DRV}}$) and between the CFP$^{A/B}$ ratios in the presence of 0.1 μM DRV (CFP$^{A/B_{0.1\ DRV}}$) and 1.0 μM DRV (CFP$^{A/B_{1.0\ DRV}}$) had *P* values of 0.0015 and 0.42 for V32I and I84V, 0.0047 and 0.15 for V32I, L33F, and I84V, 0.033 and 0.07 for V32I, L33F, and I54M, and 0.3 and 0.0000073 for V32I, L33F, I54M, and I84V, respectively.

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TABLE 3. The four amino acid substitutions V32I, L33F, I54M, and I84V confer on HIV_{NL4-3} variants high-level resistance to DRV[a]

Wild-type strain or recombinant HIV variant	Amino acid substitution(s) in PR	Mean EC_{50} ± SD (μM)	Fold resistance
HIV-1_{NL4-3}	None (wild type)	0.0031 ± 0.0002	1
HIV-1_{NL4-3}^{V32I}	V32I	0.00022 ± 0.00006	0.07
HIV-1_{NL4-3}^{L33F}	L33F	0.0028 ± 0.0008	0.9
HIV-1_{NL4-3}^{I54M}	I54M	0.0026 ± 0.0001	0.8
HIV-1_{NL4-3}^{I84V}	I84V	0.0035 ± 0.0001	1
HIV-$1_{NL4-3}^{V32I/I54V}$	V32I, I54M	0.0017 ± 0.0002	0.5
HIV-$1_{NL4-3}^{V32I/I84V}$	V32I, I84V	0.00028 ± 0.00008	0.09
HIV-$1_{NL4-3}^{V32I/L33F/I54V}$	V32I, L33F, I54M	0.0019 ± 0.0006	0.6
HIV-$1_{NL4-3}^{V32I/L33F/I84V}$	V32I, L33F, I84V	0.0030 ± 0.0004	0.9
HIV-$1_{NL4-3}^{V32I/L33F/I54MV/82A}$[b]	V32I, L33F, I54M, V82A		
HIV-$1_{NL4-3}^{V32I/L33F/I54 M/V82I}$	V32I, L33F, I54M, V82I	0.034 ± 0.018	11
HIV-$1_{NL4-3}^{V32I/L33F/I54V/I84V}$	V32I, L33F, I54M, I84V	0.64 ± 0.02	205

[a] The data shown represent mean values derived from the results of three independent experiments conducted in triplicate. The EC_{50}s were determined by employing MT-4 cells exposed to each infectious recombinant HIV-1 clone (50 $TCID_{50}$) and cultured in the presence of various concentrations of DRV, using the inhibition of p24 Gag protein production by DRV by 50% as an endpoint.

[b] Note that there are no EC_{50} or fold resistance values for HIV-$1_{NL4-3}^{V32I/L33F/I54MV/82A}$ because this variant was replication incompetent.

with resistance ranging from 0.07- to 1.0-fold, as shown in Table 3. Introduction of the combinations of two or three amino acid substitutions did not increase the EC_{50}s of DRV against such clones either, with resistance ranging from 0.09- to 0.9-fold. However, when we introduced all four amino acid substitutions (V32I, L33F, I54M, and I84V), into HIV_{NL4-3}, generating $HIV_{NL4-3}^{V32I/L33F/I54M/I84V}$, the EC_{50} of DRV against $HIV_{NL4-3}^{V32I/L33F/I54M/I84V}$ was as high as 0.64 ± 0.02 μM with resistance of 205-fold (Table 3). These data suggested that the four amino acid substitutions are associated with the high-level resistance to DRV seen in HIV_{8MIX}^{P51}.

We next examined the effects of one of the four amino acid substitutions or subsets of them on PR dimerization inhibition by DRV using the FRET-based HIV expression assay. Figure 7B shows that each single mutation of the four mutations allowed PR to undergo dimerization, and 1.0 μM DRV effectively blocked the dimerization (P values ranging from 3.4E−07 to 0.0026). We next determined the effects of various combinations of the four amino acid mutations on dimerization inhibition by DRV (Fig. 7C). Two mutations such as V32I and I84V still allowed PR to undergo dimerization, and DRV effectively blocked the dimerization at 0.1 and 1 μM. When combinations of three amino acid substitutions such as V32I, L33F, and I84V and V32I, L33F, and I54M were introduced, the mean $CFP^{A/B}$ ratios were close to 1.0, the threshold for indication of the occurrence of dimerization, with 0.99 and 0.98, respectively, in the absence of DRV, suggesting that possibly such groups of three substitutions somewhat compromised PR dimerization, although DRV still significantly blocked the dimerization at 0.1 and 1 μM, giving mean ratios ranging from 0.75 to 0.87. PR dimerization still occurred with all four substitutions, giving a mean $CFP^{A/B}$ ratio of 1.11; however, 0.1 μM DRV clearly failed the dimerization, giving a ratio of 1.16 (P = 0.3), while 1.0 μM DRV blocked dimerization (P = 0.00000073) (Fig. 7C).

V82I, not V82A, contributes to the loss of DRV's activity to inhibit PR dimerization. Since V82A and V82I were seen in $_{rCL}HIV_{T48}$ and HIV_{8MIX}^{P51}, respectively (Table 2), we examined whether V82A and V82I had effects on the loss of DRV's protease dimerization inhibition activity. Figure 8 shows that with V82A alone, PR dimerization occurred and 1 μM DRV effectively blocked the dimerization. When V82A was combined with the three amino acid substitutions V32I, L33F, and I54M, PR dimerization still occurred, which DRV effectively blocked at both 0.1 and 1 μM, suggesting that V82A substitution plays no significant role in conferring on HIV reduced sensitivity to the PR dimerization inhibition by DRV. We also examined the effects of the V82I substitution, which was identified in HIV_{8MIX}^{P51}, with or without the three amino acid substitutions, on inhibition of PR dimerization by DRV. Interestingly, the four combined amino acid substitutions V32I, L33F, I54M, and V82I abrogated DRV's PR dimerization inhibition activity at

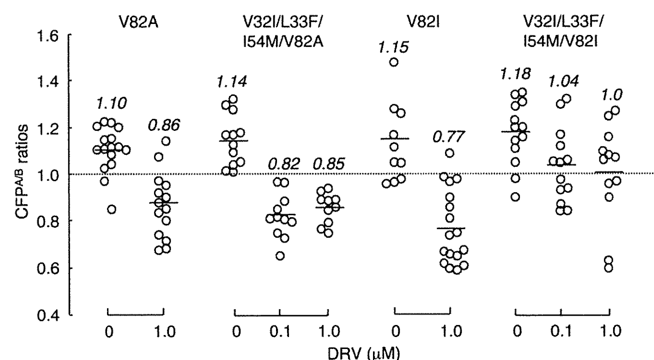

FIG. 8. Effects of V82A and V82I substitutions on DRV's activity to inhibit PR dimerization. COS7 cells were cotransfected with the pHIV-PR^{CFP} and pHIV-PR^{YFP} pair of plasmids carrying a single V82A or V82I substitution or four combined mutations (V32I, L33F, and I54M plus V82A or V82I). The COS7 cells were further cultured in the continuous presence of 0.1 and 1 μM DRV, and the $CFP^{A/B}$ ratios were determined at the conclusion of the 3-day period of culture. Note that combined with other three substitutions V32I, L33F, and I54M, V82A did not have a significant effect on DRV's dimerization inhibition activity, while V82I compromised the dimerization inhibition of DRV at 0.1 and 1.0 μM. The differences between the $CFP^{A/B}$ ratios in the absence of drug ($CFP^{A/B\text{No Drug}}$) and the $CFP^{A/B}$ ratios in the presence of 1.0 μM DRV ($CFP^{A/B1.0\text{ DRV}}$) had P values of 0.00017 for V82A and 0.0027 for V82I. The differences between the $CFP^{A/B}$ ratios in the absence of drug ($CFP^{A/B\text{No Drug}}$) and the $CFP^{A/B}$ ratios in the presence of 0.1 μM DRV ($CFP^{A/B0.1\text{ DRV}}$) and between the $CFP^{A/B}$ ratios in the presence of 0.1 μM DRV ($CFP^{A/B0.1\text{ DRV}}$) and 1.0 μM DRV ($CFP^{A/B1.0\text{ DRV}}$) were 0.000055 and 0.38 for V32I, L33F, I54M, and V82A and 0.026 and 0.91 for V32I, L33F, I54M, and V82I, respectively.

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both 0.1 and 1.0 μM (Fig. 8), strongly suggesting that V82I with other three amino acid substitutions contributes to the loss of DRV's activity to inhibit PR dimerization.

DISCUSSION

In the FRET-based HIV expression assay we previously reported, YFP- or CFP-containing PR should be initially expressed as a part of $Pr160^{gag-pol}$ polyprotein, and it was assumed that PDIs block the dimerization of PR monomer subunit within the polyprotein. In the present study, we examined whether one of the PDIs, DRV, blocked the dimerization of HIV PR expressed as a single protease molecule. As expected, DRV effectively blocked the dimerization (Fig. 2B), indicating that PDIs can block the dimerization of the PR monomers in the form of polyprotein as well as after autolysis (30, 31). The dimerization of two identical PR monomers is required for the acquisition of PR catalytic activity (28, 40), and the failure of PR dimerization should result in the loss of viral replication or compromised viral replication.

In our previous study (26) and in the present study, we demonstrated that a single amino acid substitution, such as I3A, L5A, R8Q, L24A, T26A, D29N, R87K, T96A, L97A, or F99A, effectively disrupts PR dimerization, as examined in the FRET-based HIV expression assay. In the present work, we constructed plasmids containing HIV_{NL4-3}-based recombinant clones with one of those amino acid substitutions and examined if such clones replicated in MT-4 cells. To this end, we attempted to propagate such recombinant clones in MT-4 cells using the supernatants obtained through transfection of 293T cells; however, all clones failed to replicate (Fig. 4B). In contrast, similarly generated recombinant clones containing a single amino acid substitution such as P1A, Q2A, T4A, D30N, and N98A (26), none of which disrupted PR dimerization, as examined with the FRET-based HIV expression assay (26), continued to replicate in MT-4 cells (Fig. 4B). These data further confirmed the validity of the results obtained in the FRET-based HIV expression assay.

It is of note that HIV-1 protease containing A28S has virtually no enzymatic activity, as previously published by Hong et al. (20), and as expected, recombinant $HIV-1_{NL4-3}$ with A28S ($HIV_{NL4-3}{}^{A28S}$) was totally replication incompetent, as shown in Fig. 4B. The D25A substitution is also known to render HIV-1 protease virtually enzymatically inactive; however, that substitution does not disrupt the dimerization of D25A protease as we previously published (26). Yet, 1 μM DRV clearly blocks the dimerization of D25A protease (Fig. 3A), suggesting that D25 is not significantly associated with the putative DRV binding site in monomer subunit. These observations have led us to conclude that A28 is likely involved directly or indirectly in binding of DRV to the protease monomer subunit.

In recent clinical studies, a set of mutations, including V32I, L33F, I47V, I54L, and L89V has been identified in HIV strains isolated from patients failing DRV-containing regimens and has been associated with a diminished virological response to a regimen containing DRV boosted with ritonavir (DRV/r) (9, 33). More recently, Van Marck et al. reported that a set of amino acid substitutions (V32I, I50V, I54L/M, L76V, and V82F) in the PR-encoding region of HIV might be associated with the failure of HIV-1 infected individuals receiving DRV-containing antiviral regimens (38). We have recently selected a highly DRV-resistant HIV variant ($HIV_{8MIX}{}^{P51}$) by using a mixture of eight highly multi-PI-resistant (but DRV-sensitive) clinical HIV strains, isolated from those who had received various PIs and failed to respond to PI (not DRV)-containing regimens, by propagating the mixture in the presence of increasing concentrations of DRV in phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBMCs) followed by MT-4 cells. $HIV_{8MIX}{}^{P51}$ proved to have an EC_{50} of DRV ~333-fold greater than that against a wild-type HIV strain. $HIV_{8MIX}{}^{P51}$ was highly resistant to amprenavir, indinavir, nelfinavir, ritonavir, lopinavir, and atazanavir (all EC_{50}s of >1 μM) and moderately resistant to saquinavir and TPV (EC_{50}s 33- and 18-fold greater, respectively) and replicated as rapidly as the wild-type HIV_{NL4-3} strain in the presence of 1 μM DRV (Table 1). The amino acid substitutions identified in $HIV_{8MIX}{}^{P51}$ were L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54M, L63P, K70Q, V82I, I84V, and L89M (24). When we determined the amino acid sequences of three highly DRV-resistant clinical HIV strains, ${}_{rCL}HIV_{F16}$, ${}_{rCL}HIV_{T45}$, and ${}_{rCL}HIV_{T48}$, which had EC_{50}s of DRV of 0.30 μM (97-fold), 0.33 μM (105-fold), and 0.33 μM (105-fold), respectively, we recognized that all three clinical isolates contained V32I, L33F, I54V, and I84V in common.

Thus, we hypothesized that PR dimerization inhibition by DRV might contribute to the antiviral activity of DRV and that the loss of PR dimerization inhibition by DRV might be associated with the decreased antiviral activity of DRV. When we introduced single, double, or triple amino acid substitutions of the four substitutions into pHIV-$PR_{WT}{}^{CFP}$ and pHIV-$PR_{WT}{}^{YFP}$ and cotransfected COS7 cells with such two plasmids, the dimerization of HIV-$PR_{WT}{}^{CFP}$ and HIV-$PR_{WT}{}^{YFP}$ clearly occurred in the absence of DRV, giving $CFP^{A/B}$ ratios ranging from 1.04 to 1.17 (Fig. 7B). DRV at 1 μM effectively blocked the dimerization of the PR containing a single one of the four amino acid substitutions (Fig. 7B), giving average $CFP^{A/B}$ ratios ranging from 0.88 to 0.93. DRV at 0.1 and 1.0 μM also blocked dimerization when two amino acid substitutions (V32I and I84V) and three substitutions (V32I, L33F, and I84V or V32I, L33F, and I54M) were introduced. However, when all four combined substitutions were introduced, DRV at 0.1 μM failed to block the dimerization (Fig. 7C), suggesting that the combination of the four substitutions compromised the activity of DRV to block the dimerization, presumably through altering the conformation of the monomer's putative binding site for DRV.

Considering that (i) the four amino acid substitutions in PR V32I, L33F, I54M, and I84V, were identified in the highly DRV-resistant HIV variant ($HIV_{8MIX}{}^{P51}$) and various DRV-resistant clinical HIV strains, such as ${}_{rCL}HIV_{F16}$, ${}_{rCL}HIV_{T45}$, and ${}_{rCL}HIV_{T48}$, isolated from individuals failing DRV-containing regimens (9, 33); that (ii) multi-PI-resistant, but DRV-sensitive HIV variants (24) do not contain the combination of the four substitutions; and (iii) that with the four substitutions combined, the activity of DRV to block PR dimerization is compromised, it is strongly suggested that the loss of PR dimerization activity is associated with the acquisition of resistance of HIV against DRV. More critically, the fact that the emergence of the four combined mutations is often seen in individuals failing DRV-containing regimens strongly suggests that

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the protein dimerization inhibition activity of DRV is in operation for the drug to exert its anti-HIV activity in a clinical setting. Our finding that four combined amino acid substitutions are required for the loss of PR dimerization inhibition activity of DRV should explain at least in part the reason why DRV has a high level of genetic barrier to HIV acquisition of DRV resistance. It is noteworthy that the crystallographic data of HIV PR monomer complexed with DRV are as yet unavailable; however, if the results of such structural analysis are obtained, it should help our understanding of the dynamics of the dimerization process of HIV PR, the mechanisms of the activity of DRV and other PDIs to block the dimerization, and the mechanisms of the emergence of HIV resistance against PDIs.

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In Vitro Selection of Highly Darunavir-Resistant and Replication-Competent HIV-1 Variants by Using a Mixture of Clinical HIV-1 Isolates Resistant to Multiple Conventional Protease Inhibitors$^{\nabla}$†

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We attempted to select HIV-1 variants resistant to darunavir (DRV), which potently inhibits the enzymatic activity and dimerization of protease and has a high genetic barrier to HIV-1 development of resistance to DRV. We conducted selection using a mixture of 8 highly multi-protease inhibitor (PI)-resistant, DRV-susceptible clinical HIV-1 variants (HIV-1_{MIX}) containing 9 to 14 PI resistance-associated amino acid substitutions in protease. HIV-1_{MIX} became highly resistant to DRV, with a 50% effective concentration (EC_{50}) ~333-fold greater than that against HIV-1_{NL4-3}. HIV-1_{MIX} at passage 51 (HIV-$1_{MIX}{}^{P51}$) replicated well in the presence of 5 μM DRV and contained 14 mutations. HIV-$1_{MIX}{}^{P51}$ was highly resistant to amprenavir, indinavir, nelfinavir, ritonavir, lopinavir, and atazanavir and moderately resistant to saquinavir and tipranavir. HIV-$1_{MIX}{}^{P51}$ had a resemblance with HIV-1_C of the HIV-1_{MIX} population, and selection using HIV-1_C was also performed; however, its DRV resistance acquisition was substantially delayed. The H219Q and I223V substitutions in Gag, lacking in HIV-$1_C{}^{P51}$, likely contributed to conferring a replication advantage on HIV-$1_{MIX}{}^{P51}$ by reducing intravirion cyclophilin A content. HIV-$1_{MIX}{}^{P51}$ apparently acquired the substitutions from another HIV-1 strain(s) of HIV-1_{MIX} through possible homologous recombination. The present data suggest that the use of multiple drug-resistant HIV-1 isolates is of utility in selecting drug-resistant variants and that DRV would not easily permit HIV-1 to develop significant resistance; however, HIV-1 can develop high levels of DRV resistance when a variety of PI-resistant HIV-1 strains are generated, as seen in patients experiencing sequential PI failure, and ensuing homologous recombination takes place. HIV-$1_{MIX}{}^{P51}$ should be useful in elucidating the mechanisms of HIV-1 resistance to DRV and related agents.

Successful antiviral drugs, in theory, exert their virus-specific effects by interacting with viral receptors, virally encoded enzymes, viral structural components, viral genes, or their transcripts without disturbing cellular metabolism or function. We have designed and synthesized a series of nonpeptidyl protease inhibitors (PIs) that are potent against HIV-1 variants resistant to a number of PIs. One such anti-HIV-1 agent, darunavir (DRV), containing a structure-based designed privileged nonpeptidic P2 ligand, 3(*R*),3a(*S*),6a(*R*)-*bis*-tetrahydrofuranyl-urethane (*bis*-THF) (9, 10, 16), has been used worldwide as a first-line drug for the treatment of drug-naive patients with HIV-1 infection and those who harbor multidrug-resistant HIV-1 (HIV-1_{MDR}) variants and do not respond to previously existing highly active antiretroviral therapy (HAART) regimens. It has been reported that DRV has a high genetic barrier to development of HIV-1 resistance (4, 5) and that most patients with HIV-1 infection treated with other PIs respond favorably to DRV-based salvage therapy (19), while a variety of amino acid substitutions potentially related to HIV-1 resistance to DRV have been reported (4, 19). For elucidation of the mechanism of development of resistance to DRV by HIV-1, it is critical to acquire highly DRV-resistant HIV-1 variants, which should be of high utility in further designing more efficacious and resistance-deferring anti-HIV-1 drugs.

In the present work, we attempted to select DRV-resistant variants by propagating wild-type HIV-1_{NL4-3}, a mixture (HIV-1_{MIX}) of 8 highly PI-resistant HIV-1 clinical isolates, and each of the isolates separately. *In vitro* selection of HIV-1 variants highly resistant to DRV was unsuccessful when HIV-1_{NL4-3} was used, in agreement with previous findings of De Meyer et al. (3). In contrast, when HIV-1_{MIX} was propagated, a highly DRV-resistant viral population was selected at relatively early passages. The population that replicated in the presence of 5 μM DRV at passage 51 (HIV-$1_{MIX}{}^{P51}$) contained as many as 14-amino-acid substitutions in the protease-encoding region. HIV-$1_{MIX}{}^{P51}$ should be useful in elucidating the mechanisms of HIV-1 resistance to DRV and related agents.

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MATERIALS AND METHODS

Cells and viruses. MT-4 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (FCS) (PAA Laboratories GmbH, Linz, Austria) plus 50 U of penicillin and 100 μg of kanamycin per ml. COS7 and 293T cells were grown in Dulbecco's modified Eagle medium (DMEM)-based culture medium supplemented with 10% FCS plus 50 U penicillin and 100 μg kanamycin per ml. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat of HIV-1-seronegative individuals using Ficoll-Hypaque density gradient centrifugation and cultured in RPMI 1640-based culture medium containing 10% FCS and antibiotics with 10 μg of phytohemagglutinin (PHA-PBMC) for 3 days prior to drug susceptibility assays. The following HIV strains were used for the drug susceptibility assay and selection experiments: HIV-1_{NL4-3}, clinical HIV-1 strains from drug-naive patients with AIDS (HIV-$1_{ERS104pre}$) (22), and 8 HIV-1 clinical isolates, HIV-1_A, HIV-1_B, HIV-1_C, HIV-1_G, HIV-1_{TM}, HIV-1_{MM}, HIV-1_{JSL}, and HIV-1_{SS}, which were originally isolated from patients with AIDS who had failed existing anti-HIV regimens after receiving 9 to 11 anti-HIV-1 drugs over the previous 32 to 83 months in the late 1990s and which contained 9 to 14 amino acid substitutions corresponding to the protease-encoding region which have reportedly been associated with HIV-1 resistance to various PIs; these were genotypically and phenotypically characterized as multi-PI-resistant HIV-1 variants (25). All of the variants employed were susceptible to DRV, with 50% effective concentrations (EC_{50}s) less than 0.029 μM (10-fold increase in EC_{50}) (16; also unpublished data).

Antiviral agents. Darunavir (DRV) (previously designated TMC114), a novel nonpeptidic PI containing *bis*-THF, was designed and synthesized by A. K. Ghosh as described previously (9, 11). TMC126 is the prototype of DRV. Both TMC126 and DRV contain the *bis*-THF moiety (24), while TMC126 and DRV have 4-methoxybenzenesulfonamide and sulfonamide isostere, respectively (see Fig. S1 in the supplemental material). GRL-02031 and GRL-03021, both of which are structurally related to DRV and highly potent against multi-PI resistant HIV-1 *in vitro* (14), were newly designed and were synthesized by A. K. Ghosh and S. Leshchenko-Yashchuk (see Fig. S1 in the supplemental material). These two compounds were used as control drugs in the *in vitro* drug selection experiments. Detailed synthetic methods for GRL-02031 and GRL-03021 will be described elsewhere. Saquinavir (SQV) and ritonavir (RTV) were kindly provided by Roche Products Ltd. (Welwyn Garden City, United Kingdom) and Abbott Laboratories (Abbott Park, IL), respectively. Amprenavir (APV) was a kind gift from GlaxoSmithKline (Research Triangle Park, NC). Nelfinavir (NFV), indinavir (IDV), and lopinavir (LPV) were kindly provided by Japan Energy Inc., Tokyo, Japan. Atazanavir (ATV) was a kind gift from Bristol Myers Squibb (New York, NY). Tipranavir (TPV) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health.

Generation of PI-resistant HIV-1 using HIV-1_{NL4-3} *in vitro*. MT-4 cells (10^5/ml) were exposed to HIV-1_{NL4-3} (500 50% tissue culture infected doses [$TCID_{50}$s]) and cultured in the presence of various PIs at an initial EC_{50}. Viral replication was monitored by determining the amount of p24 Gag produced by MT-4 cells. The culture supernatants were harvested on day 7 and used to infect fresh MT-4 cells for the next round of culture in the presence of increasing concentrations of each drug. When the virus began to propagate in the presence of the drug, the drug concentration was generally increased 2- to 3-fold. Proviral DNA samples obtained from the lysates of infected cells were subjected to nucleotide sequencing.

Generation of highly DRV-resistant HIV-1 using HIV-1_{MDR} *in vitro*. Eight highly multi-PI-resistant primary HIV-1 strains (HIV-1_A, HIV-1_B, HIV-1_C, HIV-1_G, HIV-1_{TM}, HIV-1_{MM}, HIV-1_{JSL}, and HIV-1_{SS}) were isolated from patients with AIDS who had failed existing anti-HIV regimens after receiving 9 to 11 anti-HIV-1 drugs over the previous 32 to 83 months. These strains, which contained 9 to 14 amino acid substitutions corresponding to the protease-encoding region that have reportedly been associated with HIV-1 resistance to various PIs, were mixed and propagated in both MT-4 cells and PHA-PBMCs as previously described (25). The mixture on day 7 of culture was propagated in fresh MT-4 cells. The culture supernatant was harvested and used to infect fresh MT-4 cells for the selection experiment. To determine the existence of 8 clinical isolates, viral RNA was purified from each indicated supernatant using the QIAamp viral RNA minikit (Qiagen Inc., Valencia, CA), and reverse transcription-PCR (RT-PCR) was carried out using the Superscript First-Strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each primary strain was also harvested, and the culture supernatant was used for the selection experiment. The culture supernatants were harvested on day 7 and used to infect fresh MT-4 cells for the next round of culture in the presence of increasing concentrations of DRV. When the virus began to propagate in the presence of the drug, the drug concentration was generally increased 2- to 3-fold. Proviral DNA samples obtained from the lysates of infected cells were subjected to nucleotide sequencing. This DRV selection procedure was carried out until the DRV concentration reached 1 or 5 μM.

Replication kinetics of DRV-resistant HIV-1 variant and wild-type HIV-1_{NL4-3}. MT-4 cells (2.4×10^5) were exposed to the DRV-selected HIV-1 variant at passage 39 (HIV-$1_{MIX^{P39}}$) or a wild-type HIV-1_{NL4-3} preparation containing 30 ng p24 in 6-well culture plates for 3 h, and these MT-4 cells were divided into three fractions, each cultured with or without DRV (final concentration of MT-4 cells, 10^4/ml; drug concentrations, 0, 0.1, and 1.0 μM). The amounts of p24 were measured every 2 days for up to 9 days in culture.

Drug susceptibility assay. To determine the sensitivity of HIV-$1_{ERS104pre}$ and clinical multidrug-resistant HIV-1 isolates, PHA-PBMC (10^6/ml) were exposed to 50 $TCID_{50}$s of each HIV-1 isolate and cultured in the presence or absence of various concentrations of drugs in 10-fold serial dilutions in 96-well microtiter culture plates. PHA-PBMCs were derived from a single donor in each independent experiment. Thus, to obtain the data, three different donors were recruited. To determine the drug susceptibilities of a laboratory HIV-1 strain (HIV-1_{NL4-3}) and DRV-selected HIV-1 variants, MT-4 cells were used as target cells. MT-4 cells (10^5/ml) were exposed to 100 $TCID_{50}$s of wild-type HIV-1_{NL4-3} and DRV-selected HIV-1 variants in the presence or absence of various concentrations of drugs and were incubated at 37°C. On day 7 of culture, the supernatant was harvested and the amount of p24 Gag protein was determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo, Japan) (18). The drug concentrations that suppressed the production of the p24 Gag protein by 50% (EC_{50}) were determined by comparison with the level of p24 production in drug-free control cell cultures. All assays were performed in duplicate or triplicate.

RESULTS

***In vitro* selection of HIV-1 variants resistant to DRV using wild-type HIV-1_{NL4-3}.** We attempted to select HIV-1 variants with DRV by propagating a wild-type laboratory HIV-1 strain, HIV-1_{NL4-3}, in MT-4 cells in the presence of increasing concentrations of DRV as previously described (24). HIV-1_{NL4-3} was initially exposed to 0.003 μM DRV and underwent 90 passages in the presence of DRV at concentrations up to 0.1 μM. We simultaneously and independently selected HIV-1 variants in the presence of RTV, APV, LPV, or ATV. As shown in Fig. S2A in the supplemental material, HIV-1 variants that replicated in the presence of 1 μM RTV, APV, LPV, and ATV emerged by passages 13, 21, 30, and 39, respectively, while HIV-1 exposed to DRV continued to replicate poorly and failed to further replicate in the presence of ~0.1 μM DRV, indicating that the emergence of a DRV-resistant HIV-1 variant was substantially delayed compared to results with other PIs examined and HIV-1 failed to acquire significant resistance to DRV. We also determined the nucleic acid sequence of the protease-encoding region of the proviral DNA isolated from infected MT-4 cells at passages 1, 30, 60, and 90 in DRV selection. As shown in of Fig. S3 (Exp. 1) in the supplemental material, the virus contained R41I, L63P, and V82I substitutions at passage 30 and beyond.

Selection of DRV-resistant HIV-1 using HIV-1_{NL4-3} preselected against TMC126. Since we failed to obtain HIV-1 variants highly resistant to DRV as described above, we next used as a starting strain an HIV-1_{NL4-3} variant that was selected over 9 passages against TMC126 (HIV-$1_{TMC126^{P9}}$), a *bis*-THF-containing PI prototype of DRV, which was potent against a wide spectrum of PI-resistant HIV-1 variants as previously described (24). This HIV-$1_{TMC126^{P9}}$, at a later passage (by passage 15), developed the A28S substitution, located at the active site of the enzyme, and acquired a high level of resistance to TMC126 and DRV (24). HIV-$1_{TMC126^{P9}}$ was initially

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                              10         20         30         40         50         60         70         80         90         99   Fraction
Day 0   pNL4-3         PQITLWQRPL VTIKIGGQLK EALLDTGADD TVLEEMNLPG RWKPKMIGGI GGFIKVRQYD QILIEICGHK AIGTVLVGPT PVNIIGRNLL TQIGCTLNF  of clones
        HIV-1_A        .........I ....V..... .......... ....D.E... ....R..... ...V...... ..P....... V......... .T.......M ..L.F....  2/22
                       .........I .......... ..I..I.... .....I.... ..L.R..... .VP....... V.S....... .A.......M ..L......  2/22
        HIV-1_B        .........I ...R...... ..I..I.... .....I.... ..L.R..... .VP....... V.S....... .A.......M ..L......  1/22
        HIV-1_C        .........I ....V....R ...I...... .....I.... .....L.... ...V...... .VP......Q .......... .A......M. .........  4/22
                       .........I IE..V...I. .......... .......... K....L.... .......... ..P....... T......... .A.......M .........  1/22
        HIV-1_G        .........I IE..V...I. .......... .....I.... K....L.... .......... ..P....... T......... .A.......M .........  1/22
                       .........I IE..V...I. .......... .......... K....L.... E......... ..P....... T......... .A.......M ....R....  1/22
                       .........I ...R...... .......... .......... K....L.R.. ...V...... ..P....... V......... .A.......M ..L......  1/22
        HIV-1_TM       .........I ...R...... .......... .......... K....L.... ...V...... ..P....... V......... .A.......M ..L......  1/22
                       .........I ...R...... .........G .......... K....L.R.. ...V...... ..P....... V......... .A.......M ..L......  1/22
                       .........I .......... .......... .......... ..T..L.... ...V...... ..P....... V......... .A.......M ..K......  2/22
        HIV-1_MM       .........I .......R.. .......... .......... ..T..L.... ...V...... ..P....... V......... .A.......M ..K......  1/22
                       .........I .......... ...I...... .......... ..T..L.... ...V...... ..P....... V......... .A.......M ..K......  1/22
        HIV-1_JSL      .........I .......... ...I...... ..F.DIS... .....L..E. ...V..K... .VP....... V.S....... .A.......M .........  1/22
                       .........R .......... .......... ......D... .....I.... .......... .VP....... V.S...I... .T.......M ..L......  1/22
        HIV-1_SS       .........R .......... .......... ......D... .....T.... .......... .VP....... V.S...I... .T.......M ..L......  1/22

                       .........I ....V....R ...I...... .....I.... .....L.... ...V...... .VP......Q .......... .A......M. .........  3/6
Day 7   HIV-1_MIX      .........I IE..V...I. .......... .......... K....L.... .......... ..P....... T......... .A.......M .........  2/6
                       .........I ....V....R ...I...... .....I.... .....L.... ...V...... .VP.....YQ .......... .A......M. .........  1/6

                       .........I ....V....R ...I...... .....I.... .....L.... ...V...... .VP......Q .......... .A......M. .........  4/6
Day 14  HIV-1_MIX      .........I ....V....G ...I...... .....I.... .....L.... ...V...... .VP......Q .......... .A......M. .........  1/6
                       .........I ....V....G ...I...... .....I.... .....L.... ...V...... RVP......Q .......... .A......M. .........  1/6

Day 21  HIV-1_MIX      .........I ....V....R ...I...... .....I.... .....L.... ...V...... .VP......Q .......... .A......M. .........  4/5
                       .........I ....V....R ...I...... .....V.... .....L.... ...V...... .VP......Q .......... .A......M. .........  1/5
```

FIG. 1. Sequence analysis of the protease-encoding regions in the mixture of 8 HIV_{MDR} isolates. Viral RNA was purified from each indicated supernatant using the QIAamp viral RNA minikit (Qiagen Inc., Valencia, CA), and RT-PCR was carried out using the Superscript First-Strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The amino acid sequences of protease deduced from nucleotide sequences of the protease-encoding region of HIV-1 clones determined are shown. The fraction of clones examined is indicated on the right. The amino acid sequence of protease of a wild-type pNL4-3 clone is shown as a reference. Identity with this sequence at individual amino acid positions is indicated (dots).

exposed to 0.003 μM DRV, representing an EC_{50} of DRV for the virus, and underwent 85 passages in the presence of DRV. In these selection experiments, we also selected HIV-1 variants against two *bis*-THF-containing or *bis*-THF-related ligands, cyclopentanyl-THF-containing-PIs GRL-02031 and GRL-03021 (see Fig. S1). It took only 16 and 36 passages for the concentrations of GRL-02031 and GRL-03021 under which HIV-$1_{TMC126^{P9}}$ replicated to reach 1 μM, respectively. However, HIV-$1_{TMC126^{P9}}$ selected against DRV gradually lost its replication capability, and when DRV went beyond ~0.1 μM, the virus again failed to replicate, and the maintenance of the viral culture became highly difficult (see Fig. S2B in the supplemental material). HIV-$1_{TMC126^{P9}}$ replicating in the presence of 5 μM GRL-02031 contained M46I, I47V, V82I, I84V, and N98I encoded in the protease-encoding region of the gene at passage 23, while the virus replicating in the presence of 5 μM GRL-03021 contained L10I, G16E, V32I, M46I, I47V, I54L, V82A, and I84V at passage 53 (see Table S1 in the supplemental material).

The protease-encoding region of the proviral DNA isolated from HIV-$1_{TMC126^{P9}}$-infected MT-4 cells was cloned and sequenced at passages 1, 25, 55, and 85 upon DRV selection. The nucleic acid sequences of the protease-encoding region at various passages are depicted in of Fig. S3 (Exp. 2) in the supplemental material. By passage 25, the virus had acquired the V82I substitution. By passage 55, the virus had additionally acquired the R41S substitution, and by passage 85, the virus had acquired the K70E and V82M substitutions. The A28S substitution, which was seen when HIV-1 was selected against *bis*-THF-containing TMC126 (24) or brecanavir/GW640385 (BCV) (23), did not emerge.

It is of note that the multitude of amino acid substitutions in protease observed when HIV-1_{NL4-3} or HIV-$1_{TMC126^{P9}}$ was used as a starting virus was moderate. Importantly, the two independent selection experiments described above strongly suggested that the emergence of a DRV-resistant HIV-1 variant is substantially delayed compared to the emergence of HIV-1 variants resistant to other PIs, as De Meyers et al. previously described (3), and that HIV-1 does not acquire significant levels of resistance to DRV (see Fig. S2 and S3 in the supplemental material).

Selection of DRV-resistant HIV-1 using a mixture of multi-PI-resistant HIV-1 isolates. As described above, when a single HIV-1 strain was used as a starting virus for selection against DRV, highly DRV-resistant HIV-1 variants were not obtained. Thus, we employed a mixture of 8 HIV-1 clinical isolates resistant to multiple PIs, expecting that homologous recombination from one isolate to another among them takes place in the presence of escalating doses of DRV and can expedite the emergence of highly DRV-resistant HIV-1 variants. The 8 primary HIV-1 strains were isolated from patients with AIDS who had failed various antiviral regimens after receiving 9 to 11 anti-HIV-1 drugs over 32 to 83 months, and these strains contained 9 to 14 amino acid substitutions corresponding to the protease-encoding region, which are associated with HIV-1 resistance to various PIs (25).

There were a few reportedly DRV resistance-associated mu-

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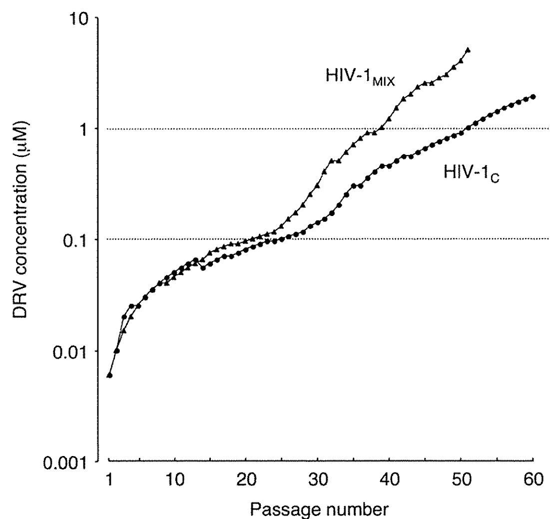

FIG. 2. *In vitro* selection of HIV-1_{MIX} and HIV-1_C resistant to DRV. A mixture of 8 HIV-1 isolates resistant to multiple PIs (triangles) or an HIV-1_C strain (circles) was passaged in the presence of increasing concentrations of DRV in MT-4 cells. The selection was carried out in a cell-free manner for a total of 60 passages, with drug concentrations escalating from 0.006 to 5.0 μM. Nucleotide sequences of proviral DNA were determined using cell lysates of HIV-1-infected MT-4 cells at the termination of each indicated passage.

tations (V11I, L33F, and G73S) in the 8 multi-PI-resistant primary isolates (Fig. 1). The mixture of these 8 isolates was obtained by propagation in PHA-PBMC as previously described (25). Each of the 8 isolates was then propagated in a mixture of an equal number of PHA-PBMC and MT-4 cells in an attempt to adapt them to replication in MT-4 cells. The cell supernatant was harvested on day 7 of coculture (PHA-PBMC and MT-4 cells), and the viruses were further propagated in fresh MT-4 cells. When a portion of culture medium was subjected to RT-PCR, molecular cloning, and nucleic acid determination immediately after a mixture of the 8 isolates was added to fresh MT-4 cells, the presence of all 8 isolates was confirmed with the presence of amino acid substitutions corresponding to the protease-encoding gene (day 0 in Fig. 1). However, by day 7, the virus population was comprised of two isolates, HIV-1_C and HIV-1_G. When examined on days 14 and 21, the predominant virus population seemed to have been derived from HIV-1_C (Fig. 1).

When the mixed isolates were further propagated in a cell-free transmission manner in fresh MT-4 cells in the presence of increasing concentrations of DRV (Fig. 2), the DRV concentration with which the virus continued to replicate relatively quickly reached 0.1 μM by passage 21. The virus continued to replicate at 1 μM DRV at passage 39 and further propagated eventually in the presence of a 5 μM concentration (passage 51) (Fig. 2). The protease-encoding region of the proviral DNA isolated from infected MT-4 cells was cloned and sequenced at passages 1, 10, 30, and 51 during the DRV selection. Individual protease sequences at each passage are depicted in Fig. 3A. At passage 1, the virus had a variety of amino acid substitutions, L10I, I15V, K20R, L24I, M36I, M46L, F53S, I54V, I62V, L63P, K70Q, V82A, and L89M, compared to wild-type HIV-1_{NL4-3}. By passage 10, the virus acquired V32I substitutions, and by passage 30, the virus acquired the I84V substitution. By passage 51, the virus had also acquired the L33F, I54M, and V82I substitutions.

Since the predominant virus population seemed to have been derived from HIV-1_C when examined on day 14 (Fig. 1), another selection experiment was conducted using HIV-1_C as a starting isolate. The concentration of DRV with which HIV-1_C grew reached 0.1 μM by passage 25 and 1 μM by passage 51 (Fig. 2). It is of note that after HIV-1_C achieved its replication at a DRV concentration of 0.1 μM, the DRV concentration curve for HIV-1_C substantially diverged from that for HIV-1_{MIX} (Fig. 2). Also, HIV-1_C replicated poorly and failed to further replicate in the presence of more than 1.8 μM DRV. These data suggest that HIV-1_{MIX} had some advantages in acquiring DRV resistance.

Failure of selection of DRV-resistant variants using single HIV-1 isolates. Since it was suspected that genetic homologous recombination was mechanically involved in the emergence of HIV-$1_{MIX}{}^R$, we conducted further selection experiments using each single HIV-1 variant of HIV-1_{MIX} under the same conditions as the ones we used to obtain HIV-1_{MIX}. In the process of culture, only HIV-1_C could replicate, and 7 other HIV-1 strains were lost during cell-free transmission. When HIV-1_C was propagated in the presence of increasing concentrations of DRV (Fig. 2), HIV-1 variants resistant to DRV, which replicated at 1.0 μM, emerged by passages 51. The protease-encoding region of the proviral DNA isolated from infected MT-4 cells was cloned and sequenced at passages 1, 10, 31, and 50 upon DRV selection. An individual protease sequence at each passage is depicted in Fig. 3B. At passage 1, the virus had the L10I, I15V, K20R, L24I, M36I, M46L, F53S, I54V, I62V, L63P, K70Q, V82A, and L89M substitutions compared to the HIV-1_{NL4-3} sequence. At passage 10, HIV-1_C had the V32I substitution. By passage 31, the virus had acquired the I84V substitution. By passage 50, the virus had acquired the A71V and L89I substitutions.

We also examined whether the virus had acquired mutations in the Gag region on passages 1, 10, 31, and 50 of the selection with DRV. At passage 1, HIV-1_C had the K15R, Q28R, R76K, I82V, V84T, D93E, T122A, N124D, N125S, Q127T, V159I, S176A, H252N, T280V, S368C (the p24/p2 cleavage site substitution), P373S, I376V, I378V, K380R, T389I, K403R, R406K, D425E, A431V (the p7/p1 cleavage site substitution), L449F (the p1/p6 cleavage site substitution), P478T, L483M, A487S, S488A, R490K, and S495N substitutions. By passage 10, the V35I substitution emerged, and it persisted thereafter. By passage 50, the V128I and Q199H substitutions had additionally emerged (Fig. 3B).

HIV-1_{MIX} that was selected to be highly DRV resistant (HIV-$1_{MIX}{}^R$) likely developed DRV resistance through possible homologous recombination. Since HIV-$1_{MIX}{}^R$ apparently had some advantages in acquiring DRV resistance without significantly compromising viral fitness compared to HIV-1_C, as described above, we examined whether HIV-$1_{MIX}{}^R$ acquired amino acid substitutions in the Gag region at passages 1, 10, 30, and 51 of DRV selection (Fig. 3A). On passage 1, the virus had the K15R, Q28R, R76K, I82V, V84T, D93E, T122A, N124D, N125S, Q127T, V159I, S176A, H252N, T280V, S368C (the p24/p2 cleavage site substitution), P373S, I376V, I378V, K380R, T389I, K403R, R406K, D425E, A431V (the p7/p1

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A Protease

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                    10         20         30         40         50         60         70         80         90        99
pNL4-3      PQITLWQRPL VTIKIGGQLK EALLDTGADD TVLEEMNLPG RWKPKMIGGI GGFIKVRQYD QILIEICGHK AIGTVLVGPT PVNIIGRNLL TQIGCTLNF
 1P         .........I ....V....R ...I...... .....I.... .....L.... ..SV...... .VP......Q .......... .A......M. .........
10P         .........I ....V....R ...I...... .I...I.... .....L.... .......... ..P....... .......... .A......M. .........
30P         .........I ....V....R ...I...... .I...I.... .....L.... .......... ..P......Q .......... .A.V....M. .........
51P         .........I ....V....R ...I...... .IF..I.... .....L.... ...M...... ..P......Q .......... .I.V....M. .........
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Gag

```
pNL4-3   1 MGARASVLSGGELDKWEKIRLRPGGKKQYKLKHIVWASRELERFAVNPGLLETSEGCRQILGQLQPSLQTGSEELRSLYNTIAVLYCVHQRIDVKDTKEA  100
 1P      1 ..............R............R...............................................K.....V.T........E.......  100
10P      1 ..............R............R...............................................K.....V.T........E.......  100
30P      1 ..............R............R......L........................................K.....V.T........E.......  100
51P      1 ..........E...R.....M......R......L........................................K.....V.T........E.......  100

pNL4-3 101 LDKIEEEQNKSKKKAQQAAADTGNNSQVSQNYPIVQNLQGQMVHQAISPRTLNAWVKVVEEKAFSPEVIPMFSALSEGATPQDLNTMLNTVGGHQAAMQM  200
 1P    101 .....................A.DS.T...............................I................A........................  200
10P    101 .....................A.DS.T...............................I................A........................  200
30P    101 .................V...A.DS.T...............................I................A........................  200
51P    101 .................V...A.DS.TI..............................I................A........................  200

pNL4-3 201 LKETINEEAAEWDRLHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQIGWMTHNPPIPVGEIYKRWIILGLNKIVRMYSPTSILDIRQGPKEPFRDYVDRF  300
 1P    201 ...................................................N...........................V....................  300
10P    201 ..................Q...V.......................V....S...........................V....................  300
30P    201 ..................Q...V.......................V....S...........................V....................  300
51P    201 ..................Q...V.......................V....N...........................V....................  300

pNL4-3 301 YKTLRAEQASQEVKNWMTETLLVQNANPDCK-TILKALGPGATLEEMMTACQGVGGPGHKARVLAEAMSQVTNPATIMIQKGNFRNQRKTVKCFNCGKEGH  400
 1P    301 ...............................-....................................C....S..V.V.R........I...........  400
10P    301 ...............................P....................................C....S..V.V.R........I...........  401
30P    301 ...............................-....................................C....S..V.V.R........I...........  400
51P    301 ...............................-....................................C....S..V.V.R........I...........  400

pNL4-3 401 IAKNCRAPRKKGCWKCGKEGHQMKDCTERQANFLGKIWPSHKGRPGNFLQSRPEPTAPPEESFRFGEETTTPSQKQEPIDKELYPLASLRSLFGSDPSSQ  500
 1P    401 ..R..K..................E.....V.................F............................T....M...SA.K....N.....  500
10P    402 ..R..K..................E.....V.................F............................T....M...SA.K....N.....  501
30P    401 ..R..K..................E.....V.................F............................T....M...SA.K....N.....  500
51P    401 ..R..K..................E.....V.................F............................T....M...SA.K....N.....  500
```

B Protease

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                    10         20         30         40         50         60         70         80         90        99
pNL4-3      PQITLWQRPL VTIKIGGQLK EALLDTGADD TVLEEMNLPG RWKPKMIGGI GGFIKVRQYD QILIEICGHK AIGTVLVGPT PVNIIGRNLL TQIGCTLNF
 1P         .........I ....V....R ...I...... .....I.... .....L.... ..SV...... .VP......Q .......... .A......M. .........
10P         .........I ....V....R ...I...... .I...I.... .....L.... ...V...... .VP......Q .......... .A......M. .........
31P         .........I ....V....R ...I...... .I...I.... .....L.... ...L...... .VP......Q .......... .A.V....M. .........
50P         .........I ....V...XR ...I...... .I...I.... .....L.... ...L...... .VP......Q V......... .A.V....I. .........
                              L/I
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Gag

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pNL4-3   1 MGARASVLSGGELDKWEKIRLRPGGKKQYKLKHIVWASRELERFAVNPGLLETSEGCRQILGQLQPSLQTGSEELRSLYNTIAVLYCVHQRIDVKDTKEA  100
 1P        ..............R............R...............................................K.....V.T........E.......
10P        ..............R............R......I........................................K.....V.T........E.......
31P        ..............R............R......I........................................K.....V.T........E.......
50P        ...........................X......I........................................K.....V.T........E......X
                                     K/R                                                                        V/A

pNL4-3 101 LDKIEEEQNKSKKKAQQAAADTGNNSQVSQNYPIVQNLQGQMVHQAISPRTLNAWVKVVEEKAFSPEVIPMFSALSEGATPQDLNTMLNTVGGHQAAMQM  200
 1P        .....................A.DS.T...............................I................A........................
10P        .....................A.DS.T...............................I................A........................
31P        .....................A.DS.T...............................I................A......................X.
                                                                                                           Q/H
50P        .....................A.DS.TI..............................I................A......................H.

pNL4-3 201 LKETINEEAAEWDRLHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQIGWMTHNPPIPVGEIYKRWIILGLNKIVRMYSPTSILDIRQGPKEPFRDYVDRF  300
 1P        ...................................................N...........................V....................
10P        ...................................................N...........................V....................
31P        ...................................................N...........................V....................
50P        ...................................................N...........................V....................

pNL4-3 301 YKTLRAEQASQEVKNWMTETLLVQNANPDCKTILKALGPGATLEEMMTACQGVGGPGHKARVLAEAMSQVTNPATIMIQKGNFRNQRKTVKCFNCGKEGH  400
 1P        ...................................................................C....S..V.V.R........I...........
10P        ...................................................................C....S..V.V.R........I...........
31P        ...................................................................C....S..V.V.R........I...........
50P        ...................................................................C....S..V.V.R........I...........

pNL4-3 401 IAKNCRAPRKKGCWKCGKEGHQMKDCTERQANFLGKIWPSHKGRPGNFLQSRPEPTAPPEESFRFGEETTTPSQKQEPIDKELYPLASLRSLFGSDPSSQ  500
 1P        ..R..K..................E.....V.................F............................T....M...SA.K....N.....
10P        ..R..K..................E.....V.................F............................T....M...SA.K....N.....
31P        ..R..K..................E.....V.................F............................T....M...SA.K....N.....
50P        ..R..K..................E.....V.................F............................T....M...SA.K....N.....
```

FIG. 3. Amino acid sequences of HIV-1_{MIX} and HIV-1_C passaged in the presence of DRV. (A) The amino acid sequences of the Gag-protease-encoding region from 4 different passages of each strain derived from HIV-1_{MIX} passaged in the presence of DRV are indicated. The top line shows the Gag-protease sequence of the wild-type pNL4-3 clone. Identity of each amino acid with that from pNL4-3 (top) at an individual amino acid position is indicated by a dot. (B) The amino acid sequences of the Gag-protease-encoding region from 4 different passages of each strain derived from HIV-1_C passaged in the presence of DRV are indicated.

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cleavage site substitution), L449F (the p1/p6 cleavage site substitution), P478T, L483M, A487S, S488A, R490K, and S495N substitutions. By passage 10 and beyond, the H219Q (within the CypA-binding loop), I223V (within the CypA-binding loop), and I247V substitutions emerged and persisted. By passage 30 and beyond, the V35L and A118V substitutions emerged. By passage 51, the G11E and L21M substitutions emerged.

It is of note that HIV-1_{MIX} had acquired the two amino acid substitutions H219Q and I223V, located in the CypA binding loop of the Gag protein, as early as by passage 10 (Fig. 3A). Since certain amino acid substitutions are known to affect the viral fitness of HIV-1 (7) and it was possible that HIV-1_{MIX} might have acquired these two substitutions through homologous recombination, we examined the amino acid sequences of each isolate of HIV-1_{MIX}. Although HIV-1_{MIX^R} was thought to have derived predominantly from HIV-1_C, HIV-1_C was devoid of H219Q and I223V (see Fig. S4 in the supplemental material). Since HIV-1_C selected to be DRV resistant (HIV-1_{C^R}) was still devoid of the two substitutions, it was thought more likely that HIV-1_{MIX^R} acquired H219Q and I223V from other HIV-1 variants within HIV-1_{MIX} through homologous recombination rather than spontaneously acquiring the substitutions during the DRV selection (Fig. 2). Indeed, HIV-1_A and HIV-1_B had I223V; HIV-1_G carried both H219Q and I223V (see Fig. S4). Thus, it was thought that although all three strains (HIV-1_A, HIV-1_B, and HIV-1_G) apparently disappeared during selection with DRV, either or all of the three strains served as a donor(s) to provide the two substitutions to HIV-1_{MIX^R}.

The role of another amino acid substitution, I247V, which has been identified in HIV-1 isolates (13 of 156 different HIV-1 isolates in the HIV Sequence Compendium 2008/Los Alamos HIV Sequence Database) remains to be elucidated, although it is possible that I247V was incorporated into HIV-1_{MIX^R} alongside H219Q and I223V through homologous recombination. These data suggest that at least these two substitutions contributed the favorable replicative ability of HIV-1_{MIX^R} compared to that of HIV-1_{C^R}. We generated four different clones that contained a variety of amino acid substitutions identified in the protease- and Gag-encoding genes of the DRV-selected mixture population (see Table S2 in the supplemental material); however, all such recombinant HIV-1 clones we generated failed to replicate (data not shown).

H219Q and I223V substitutions reduce the virion content of CypA. One salient difference between HIV-1_{MIX^R} and HIV-1_{C^R} was the presence of the H219Q and I223V substitutions as described above. These two substitutions are located in the CypA binding loop of the Gag protein, which regulates the intravirion content of CypA, which is believed to play an essential role early in the HIV-1 replication cycle. CypA perhaps destabilizes the capsid (p24 Gag protein) shell during viral entry and uncoats (6) and/or performs an additional chaperon function, facilitating correct capsid condensation during viral maturation (12).

Also, based on the data from crystal structure analyses by Gamble et al. (6) of the p24 Gag protein complexed with CypA, showing that His^{219} binds to Asn^{71} and Gln^{111} of CypA through a hydrogen bond and a hydrophobic contact(s), respectively, we postulated that the H219Q and I223V substitutions cancel or weaken such interactions, resulting in the reduction of binding of p24 to CypA and of CypA incorporation into daughter virions in CypA-rich MT-4 cells. Thus, we determined the CypA content of the cells where HIV-1 was propagated and of virions used in the present study. As shown in Fig. S5A and B in the supplemental material, the CypA contents in 10^5 MT-2 cells (relative density, 100%), 10^5 H9 cells (91.0%), and 10^5 MT-4 cells (66.6%) appeared to be greater than those in 10^5 PHA-PBMC (21.6%), suggesting that MT-2 and H9 cells contained 3 to 5 times as much CypA per cell as PHA-PBM, in agreement with our previous findings (7). We subsequently determined the virion-associated CypA amounts in HIV-1_{NL4-3}, HIV-$1_{C^{P51}}$ (replicative at 1 μM DRV), HIV-$1_{MIX^{P39}}$ (replicative at 1 μM DRV), and HIV-$1_{MIX^{P51}}$ (replicative at 5 μM DRV), employing Western blotting using anti-p24 Gag and anti-CypA antisera. The virions in each supernatant were pelleted by ultracentrifugation and subsequently subjected to SDS-PAGE.

As shown in Fig. S5B in the supplemental material, p24 signal densities in HIV-1_{NL4-3}, HIV-$1_{C^{P51}}$, HIV-$1_{MIX^{P39}}$, and HIV-$1_{MIX^{P51}}$ were 100, 75.4, 101.2, and 83.3%, respectively, compared with that in HIV-1_{NL4-3} (serving as a standard at 100%). CypA signal densities in HIV-1_{NL4-3}, HIV-$1_{C^{P51}}$, HIV-$1_{MIX^{P39}}$, and HIV-$1_{MIX^{P51}}$ were 100, 103.4, 68.4, and 61.9%, respectively, compared with that in HIV-1_{NL4-3} (serving as a standard at 100%) (see Fig. S5C). Ratios of densities of the CypA signal relative to each p24 Gag signal were 1.0, 1.37, 0.68, and 0.74 for HIV-1_{NL4-3}, HIV-$1_{C^{P51}}$, HIV-$1_{MIX^{P39}}$, and HIV-$1_{MIX^{P51}}$, respectively. These data suggest that both the H219Q and I223V substitutions improved HIV-1_{MIX^R} replication in CypA-rich MT4 cells by reducing CypA incorporation into daughter virions. The impact of the H219Q and I223V substitutions on the structure of the CypA-binding loop of the Gag protein was also examined using molecular dynamic simulation (see Fig. S6).

Significantly reduced susceptibilities of HIV-1_{MIX^R} to DRV and other PIs. We also examined the susceptibilities of HIV-1_{MIX^R} to a variety of FDA-approved PIs, including DRV in MT-4 cells (Table 1). HIV-1_{MIX} harvested on passage 1 (HIV-$1_{MIX^{P1}}$) was already resistant to APV with an EC_{50} (0.28 μM) 28-fold greater than the EC_{50} with the wild-type clinical isolate HIV-$1_{ERS104pre}$, to NFV with a 41-fold greater EC_{50} (0.66 μM), and to LPV with a 17-fold greater EC_{50} (0.26 μM). The EC_{50}s of RTV and IDV against HIV-1_{MIX^R} were both >1 μM. In contrast, SQV, ATV, and TPV relatively maintained their antiviral activity against HIV-1_{MIX} at early stages of DRV selection. However, HIV-$1_{MIX^{P39}}$ was found to be highly resistant to DRV (EC_{50} >333-fold greater than that against HIV-$1_{ERS104pre}$). HIV-$1_{MIX^{P51}}$ was highly resistant to APV, IDV, NFV, RTV, LPV, and ATV (all with EC_{50}s of >1 μM) and also had significant resistance against SQV (33-fold increases in the EC_{50}) and TPV (18-fold increases in the EC_{50}) (Table 1). We also determined replication kinetics of HIV-1_{NL4-3} along with that of HIV-$1_{MIX^{P39}}$, which turned out to be capable of replicating in the presence of 1 μM DRV. As shown in Fig. 4, when HIV-$1_{MIX^{P39}}$ was propagated in MT-4 cells in the presence or absence of 0.1 or 1 μM DRV, there was no discernible difference observed in the replication kinetics of HIV-$1_{MIX^{P39}}$ compared to that of HIV-1_{NL4-3} in the absence of DRV.

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TABLE 1. High levels of HIV-1_{MIX}^{R} resistance to DRV and other PIs

Virus	EC_{50} (μM) of drug[a]								
	SQV	APV	IDV	NFV	RTV	LPV	ATV	TPV	DRV
HIV-$1_{ERS104pre}$ (wild type)	0.009	0.025	0.021	0.016	0.030	0.015	0.005	0.10	0.003
HIV-1_{MIX}^{P1}	0.034 (4)	0.28 (11)	>1 (>48)	0.66 (41)	>1 (>33)	0.26 (17)	0.021 (4)	0.060 (0.6)	0.005 (2)
HIV-1_{MIX}^{P10}	0.026 (3)	0.45 (18)	>1 (>48)	>1 (>63)	>1 (>33)	0.22 (14)	0.035 (7)	0.023 (0.2)	0.013 (4)
HIV-1_{MIX}^{P20}	0.27 (30)	>1 (>40)	>1 (>48)	>1 (>63)	>1 (>33)	0.39 (26)	0.39 (78)	0.18 (2)	0.12 (40)
HIV-1_{MIX}^{P30}	0.30 (33)	>1 (>40)	>1 (>48)	>1 (>63)	>1 (>33)	>1 (>67)	>1 (>200)	0.33 (3)	0.31 (100)
HIV-1_{MIX}^{P39}	0.35 (39)	>1 (>40)	>1 (>48)	>1 (>63)	>1 (>33)	>1 (>67)	>1 (>200)	0.41 (4)	>1 (>333)
HIV-1_{MIX}^{P51}	0.30 (33)	>1 (>40)	>1 (>48)	>1 (>63)	>1 (>33)	>1 (>67)	>1 (>200)	1.79 (18)	>1 (>333)

[a] HIV-1_{MIX} was propagated in the presence of increasing concentrations of DRV, harvested at passages 1, 10, 20, 30, 39, and 51 (HIV-1_{MIX}^{P1}, HIV-1_{MIX}^{P10}, HIV-1_{MIX}^{P20}, HIV-1_{MIX}^{P30}, HIV-1_{MIX}^{P39}, and HIV-1_{MIX}^{P51}, respectively), and examined for susceptibilities to DRV and other PIs listed. In the assay, MT-4 cells (10^4) were exposed to each HIV-1 preparation (100 $TCID_{50}$s), and to determine EC_{50}s of each drug against HIV-1, the inhibition of p24 Gag protein production by each drug was used as an endpoint. The numbers in parentheses represent fold changes of EC_{50}s against each HIV-1 preparation compared to EC_{50}s against a wild-type clinical strain, HIV-$1_{ERS104pre}$. All assays were conducted in triplicate, and the mean values are shown.

DISCUSSION

Darunavir (DRV) potently inhibits the enzymatic activity and dimerization of HIV-1 protease (15, 16) and exhibits a high genetic barrier to HIV-1 development of resistance to DRV (4, 5). In a relatively small percentage of heavily drug-experienced patients, a variety of amino acid substitutions potentially related to HIV-1 resistance to DRV have been reported (4, 19); however, the mechanism of development of HIV-1 resistance to DRV still remains to be elucidated. For determining the mechanism of development of HIV-1 resistance to DRV, it is critical to acquire highly DRV-resistant HIV-1 variants, which should be of great utility.

In the present study, we attempted to select DRV-resistant HIV-1 variants by propagating a mixture of HIV-1 variants (HIV-1_{MIX}) isolated from 8 patients with AIDS who had received antiretroviral therapy over 32 to 83 months and were not responding to antiretroviral regimens in the presence of DRV. DRV-resistant HIV-1 at passage 51 (HIV-1_{MIX}^{P51}) replicated in the presence of 5 μM DRV and contained 14 mutations (L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54M, L63P, K70Q, V82I, I84V, and L89M) encoded in the protease-encoding region.

A set of mutations (V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, and L89V) was identified in HIV-1 isolated from those failing DRV-containing regimens that were associated with a diminished virological response to DRV boosted with a low dose of ritonavir at week 24 in the POWER studies (4). The most common mutations identified were V32I, L33F, I54M/L, I84V, and L89V (17, 21). In the present study, HIV-1 variants resistant to DRV, which replicated in the presence of 1 and 5 μM DRV, emerged by passages 39 and 51, respectively. The protease-encoding region of the proviral DNA isolated from infected MT-4 cells was cloned and sequenced at passages 1, 10, 30, and 51 upon DRV selection. Individual protease sequences at each passage are depicted in Fig. 3A. On passage 1, the virus had L10I, I15V, K20R, L24I, M36I, M46L, F53S, I54V, I62V, L63P, K70Q, V82A, and L89M substitutions compared to wild-type HIV-1_{NL4-3}. By passage 10 and beyond, the virus additionally acquired a V32I substitution. By passage 30 and beyond, the virus contained an I84V substitution. By passage 51, the virus had acquired L33F, I54M, and V82I substitutions and was found to contain 14 mutations, L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54M, L63P, K70Q, V82I, I84V, and L89M, corresponding to the protease-encoding region. It is of note that the four mutations (V32I, L33F, I54M, and I84V) HIV-1 acquired in the present study were the ones identified in highly DRV-resistant HIV-1 variants.

With respect to HIV-1's acquisition of cross-resistance to TPV and DRV, our recent results showed both compounds

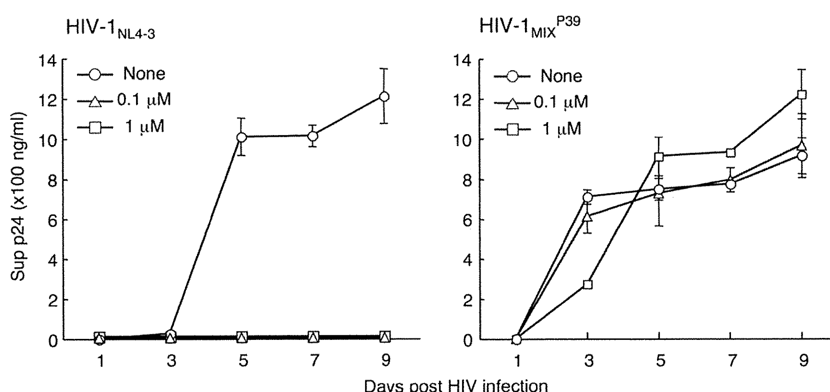

FIG. 4. Replication kinetics of HIV-1_{MIX}^{P39} in the presence of DRV. MT-4 cells were exposed to HIV-1_{NL4-3} or HIV-1_{MIX}^{P39} and cultured in the presence or absence of 0.1 or 1.0 μM DRV. Viral replication was monitored by measuring p24 Gag protein in the culture supernatant.

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