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Probing Multidrug-Resistance and Protein–Ligand Interactions with Oxatricyclic Designed Ligands in HIV-1 Protease Inhibitors

Arun K. Ghosh,*[a] Chun-Xiao Xu,[a] Kalapala Venkateswara Rao,[a] Abigail Baldridge,[a] Johnson Agniswamy,[b] Yuan-Fang Wang,[b] Irene T. Weber,[b] Manabu Aoki,[c] Salcedo Gomez Pedro Miguel,[c] Masayuki Amano,[c] and Hiroaki Mitsuya[c, d]

The introduction of HIV-1 protease inhibitors (PIs) into highly active antiretroviral therapy (HAART) in 1996 had a critical impact in decreasing HIV-related morbidity and mortality.[1] Indeed, HAART regimens with PIs initially suppressed HIV-1 replication in patients to undetectable HIV-1 RNA levels in plasma.[2] However, one of the serious shortcomings of current treatments is the rapid emergence of drug-resistant HIV strains.[3] There is an urgent need for improved PIs for the treatment of the increasing number of treatment-experienced patients harboring multidrug-resistant HIV-1 strains.[4,5]

We recently reported a number of PIs that were developed by incorporating structure-based designed novel nonpeptide ligands/scaffolds that target the HIV-1 protease substrate binding site.[6–17] One of the PIs, darunavir (**1**, DRV, Figure 1), was first approved for HIV/AIDS patients harboring drug-resistant HIV who do not respond to other antiretroviral drugs.[11] Recently, DRV has received full approval for all HIV/AIDS patients, including children infected with HIV-1.[12] DRV has a stereochemically defined fused *bis*-tetrahydrofuran (*bis*-THF) moiety as the P2 ligand.[7,8] The X-ray crystal structures of the complexes of both **1**- and **2**-bound HIV-1 protease revealed extensive protein–ligand hydrogen bonding interactions involving the backbone of HIV-1 protease throughout the active site.[13,14] In particular, both oxygen atoms of the P2 *bis*-THF ligand are involved in hydrogen bonding with Asp29 and Asp30 backbone NH groups.[15] In addition, the bicyclic ligand appears to fill the hydrophobic pocket at the S2 subsite. The P2 *bis*-THF ligand is responsible for the superior drug-resistance properties of both DRV (**1**) and TMC-126 (**2**). In our preliminary investigation, we chose to incorporate *para*-methoxysulfonamide as the P2′ ligand owing to its marked hydrogen bonding interaction in the S2′ site. To combat drug resistance, our inhibitor design strategies focused on maximizing inhibitor interactions with the HIV-1 protease active site, particularly by promoting extensive hydrogen bond interactions with the protein backbone atoms.[15] We have recently shown that the enhancement of 'backbone binding' leads to the design of PIs that maintain full potency against a panel of multidrug-resistant HIV-1 variants.[17] Based on our examination of the protein–ligand crystal structure of DRV-bound HIV-1 protease, we have further speculated that the incorporation of another tetrahydrofuran ring on the *bis*-THF ligand would provide additional ligand–binding site interactions. Particularly, it appears that the ligand oxygen atoms can effectively maintain backbone hydrogen bonding with Asp29 and Asp30, and can fill the hydrophobic pocket effectively. This in turn could further improve drug-resistance properties of the PIs. Such oxatricyclic ligands could have a variety of possible stereochemical motifs, including *syn-syn-syn* (*SSS*-type) and *syn-anti-syn* (*SAS*-type) isomers. Our X-ray crystal structure-based preliminary modeling suggested that the *SAS*-type ligand-based inhibitor **4** would make enhanced interactions in the S2 subsite relative to the *SSS*-derived inhibitor **3** (Figure 1).

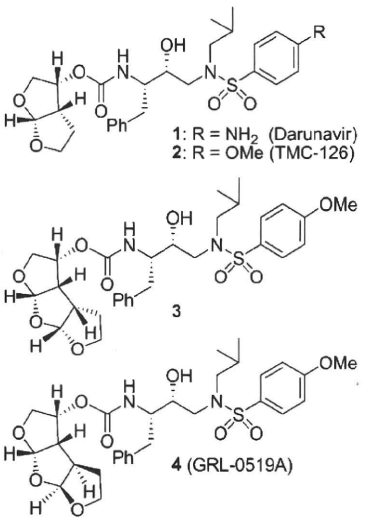

Figure 1. Structure of darunavir (DRV, **1**) and protease inhibitors **2**–**4**.

The synthesis of the oxatricyclic *tris*-THF ligand with an *SSS* configuration was carried out as shown in Scheme 1. Optically active *bis*-THF derivatives **6** and **7** were conveniently prepared in multi-gram quantities from dihydrofuran **5** in a three-step sequence followed by lipase-catalyzed optical resolution as described previously.[18,19] Optically active alcohol **7** was treated with triphenylphosphine and iodine in the presence of imidazole to provide the corresponding iodide. Treatment of the re-

[a] *Prof. Dr. A. K. Ghosh, Dr. C.-X. Xu, Dr. K. V. Rao, A. Baldridge*
Department of Chemistry and Medicinal Chemistry
Purdue University, 560 Oval Drive, West Lafayette, IN 47907 (USA)
Fax: (+1) 765-496-1612
E-mail: akghosh@purdue.edu

[b] *J. Agniswamy, Y.-F. Wang, Prof. Dr. I. T. Weber*
Department of Biology, Molecular Basis of Disease
Georgia State University, Atlanta, GA 30303 (USA)

[c] *M. Aoki, S. G. P. Miguel, M. Amano, Dr. H. Mitsuya*
Departments of Hematology and Infectious Diseases
Kumamoto University School of Medicine, Kumamoto 860-8556 (Japan)

[d] *Dr. H. Mitsuya*
Experimental Retrovirology Section, HIV and AIDS Malignancy Branch
National Cancer Institute, Bethesda, MD 20892 (USA)

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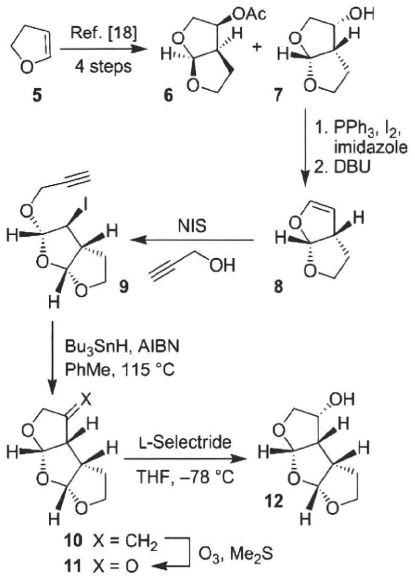
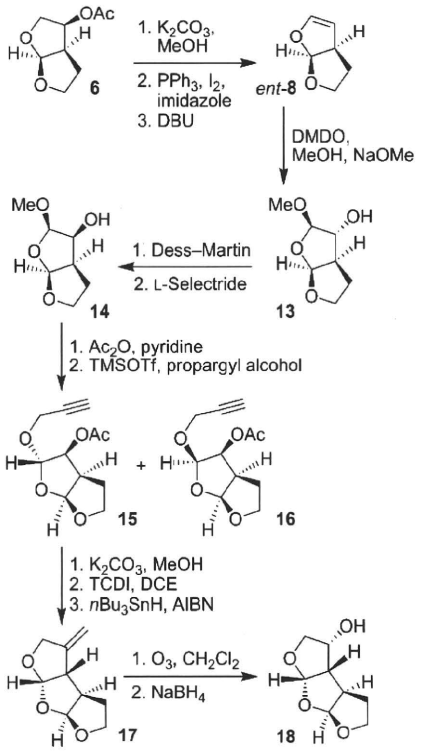


Scheme 1. Synthesis of the *syn-syn-syn tris*-THF ligand **12**.

sulting iodide with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) furnished volatile bicyclic enol ether **8**. Reaction of **8** with *N*-iodosuccinimide (NIS) and propargyl alcohol in dichloromethane at 0–23 °C afforded iodoether **9** in 58% yield over three steps.[18] Radical cyclization of **9** with tri(*n*-butyl)tin hydride in the presence of azobisisobutyronitrile (AIBN) in toluene at reflux provided tricyclic olefin **10**. The relative stereochemistry of the tricyclic core was established by extensive NMR experiments (COSY, NOESY). Ozonolysis of the olefin, followed by L-selectride reduction of the resulting ketone furnished *endo*-alcohol **12** as a single isomer (by ^{1}H NMR analysis).

The synthesis of *tris*-THF ligand with a *SAS* ring fused system is shown in Scheme 2. Optically active (3*S*,3a*R*,6a*S*)-*bis*-THF alcohol *ent*-**7** was readily obtained by saponification of acetate derivative **6**. It was converted into bicyclic enol ether *ent*-**8** as described above. This enol ether was exposed to acetone-free dimethyldioxirane (DMDO)[20] in dichloromethane at −78 °C to provide the corresponding epoxide. Treatment of the resulting epoxide with a catalytic amount of sodium methoxide (10%) in methanol provided epoxide-opened product **13** as a single diastereomer.[21] The depicted stereochemistry of **13** was fully supported by extensive NMR studies. Dess–Martin oxidation[22] of **13** followed by L-selectride reduction of the resulting ketone provided *endo*-alcohol **14** as a single isomer in 68% yield over two steps. To append the third oxacyclic ring, alcohol **14** was first acylated, and the resulting acetate was treated with trimethylsilyl trifluoromethanesulfonate (TMSOTf) and propargyl alcohol, which afforded acetals **15** and **16** as a 4:1 mixture of diastereomers. The isomers could not be separated at this stage; however, removal of the acetate provided the corresponding alcohols, which were readily separated by column chromatography. The major diastereomeric alcohol was converted into tricyclic olefin **17** in a two-step sequence involving: 1) reaction of alcohol with thiocarbonyldiimidazole (TCDI) in dichloroethane (DCE) to provide the corresponding



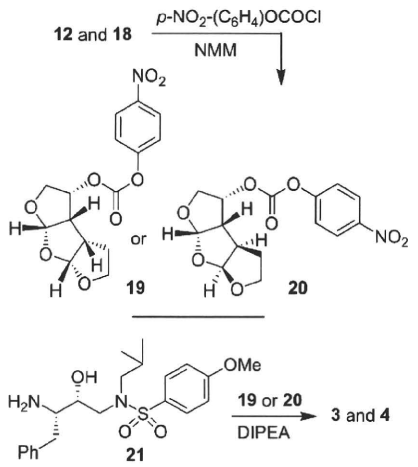
Scheme 2. Synthesis of the *syn-anti-syn tris*-THF alcohol **18**.

thiocarbamate, and 2) treatment of the resulting thiocarbamate with tri(*n*-butyl)tin hydride in the presence of AIBN in toluene at reflux to provide olefin **17** in 75% yield over two steps.[23] The stereochemistry of olefin **17** was established by ^{1}H NMR spectroscopy. Olefin **17** was converted into *endo*-alcohol **18** by ozonolysis of the olefin followed by sodium borohydride reduction of the resulting ketone to furnish **18** as a single isomer. Mosher ester analysis[24] revealed high optical purity (99% *ee*).

The synthesis of *tris*-THF-containing PIs is shown in Scheme 3. Optically active *tris*-THF ligand alcohols **12** and **18** were converted into the corresponding *para*-nitrophenyl carbonates **19** and **20** by treatment with *para*-nitrophenyl chloroformate and *N*-methylmorpholine (NMM). Treatment of amine **21**,[25] synthesized previously, with activated carbonate **19** or **20** in the presence of diisopropylethylamine (DIPEA) provided PIs **3** and **4** in 65% yield.

Inhibitor **3** shows potent inhibitory activity (K_i = 60 pM). In comparison, inhibitor **4** (GRL-0519A, Figure 1) has a K_i value of 5.9 pM, a 10-fold improvement over **3**. Both PIs **3** (IC_{50} = 3.5 nM) and **4** (IC_{50} = 1.8 nM) also showed excellent antiviral activity against the wild-type HIV-1_{LAV}. PI **4** (GRL-0519A) was the most potent against a laboratory HIV-1 strain, HIV-1_{LAI}, with an EC_{50} value of 1.8 nM and a favorable cytotoxicity profile (CC_{50} = 44.6 μM) as examined by using MT-2 cells as target cells, producing a selectivity index (CC_{50}/EC_{50}) of 24778.

We next examined GRL-0519A against a variety of primary HIV-1 strains, which were isolated from patients with AIDS who had failed a number of anti-HIV therapeutic regimens after re-



Scheme 3. Synthesis of inhibitors **3** and **4**.

ceiving 9–11 anti-HIV-1 drugs over the previous 32–83 months, and who proved to be highly resistant to multiple PIs.[9,26] These primary strains contain 9–14 amino acid substitutions in the protease-encoding region of the HIV-1 genome which have been reported to be associated with HIV-1 resistance against various PIs.[27] The substitutions identified include Leu10→Ile (L10I; 6 of 6 isolates), M46I/L (6 of 6), I54V (4 of 6), L63P (6 of 6), A71V/T (5 of 6), V82A (6 of 6), and L90M (4 of 6) (see the footnote of Table 1).

Darunavir (DRV) is more potent against wild-type HIV-$1_{ERS104pre}$ (EC_{50} = 0.005 μM) than amprenavir (APV),[28] which has an EC_{50} value of 0.032 μM. DRV was moderately less active against a panel of multidrug-resistant HIV-1 variants, with EC_{50} values ranging from 0.011 to 0.031 μM. The fold difference in EC_{50} values was 2–6 relative to its EC_{50} value against HIV-$1_{ERS104pre}$. APV was less active against the variants, with EC_{50} values and fold differences ranging between 0.291 and 0.521 μM, and from 9 to 16, respectively. GRL-0519A exerted the greatest potency among the three agents against wild-type HIV-$1_{ERS104pre}$, with an EC_{50} value of 0.6 nM. GRL-0519A was less potent against the variants, with EC_{50} values ranging from 0.9 to 4.3 nM, and the fold difference was 2–7 relative to wild-type HIV-1 (Table 1). Importantly, GRL-0519A is more potent than DRV by a factor of 5.9–14 in comparing absolute concentrations of EC_{50} values.

Dimerization of HIV-1 protease subunits is an essential process for the acquisition of proteolytic activity, which plays a critical role in the maturation and replication of the virus.[29] We previously demonstrated that a group of compounds, including DRV and TPV, inhibit the dimerization of HIV-1 protease monomer subunits as examined by a FRET-based HIV-1 expression assay that uses cyan (CFP) and yellow fluorescent protein (YFP)-tagged protease monomers; other conventional FDA-approved PIs such as APV failed to block dimerization.[30] As shown in Figure 2, the average $CFP^{A/B}$ ratio determined in the cells transfected and cultured in the absence of drug was 1.1 ± 0.13, indicating that protease dimerization efficiently occurred. In the same assay, in the presence of DRV at 0.1 and 1 μM, the average $CFP^{A/B}$ ratios determined were 0.93 ± 0.07 and 0.81 ± 0.11, respectively, indicating that DRV effectively inhibits protease dimerization at those concentrations. However, the average ratios were greater than 1.0 at 0.001 and 0.01 μM, indicating that no dimerization inhibition occurred at these lower DRV concentrations, in line with the data we previously reported.[30] We next examined whether GRL-0519A can block protease dimerization under exactly the same conditions in the FRET-based HIV-1 expression assay. When the ratios were determined in the cells transfected and cultured in the presence of GRL-0519A at 0.01, 0.1, and 1 μM, the average values were 0.96 ± 0.07, 0.84 ± 0.09, and 0.84 ± 0.11, respectively, indicating that GRL-0519A can block protease dimerization more potently, by at least 10-fold, relative to DRV.

Table 1. Antiviral activity of GRL-0519A (**4**), amprenavir (APV), and darunavir (DRV) against multidrug-resistant clinical isolates in PHA-PBMs.[a]

Virus	EC_{50} [μM] GRL-0519A	APV	DRV
HIV-$1_{ERS104pre}$ (wild-type: X4)	0.0006 ± 0.0002	0.032 ± 0.006	0.005 ± 0.002
HIV-$1M_{DR/B}$ (X4)	0.0043 ± 0.0012 (7)	0.521 ± 0.203 (16)	0.028 ± 0.008 (6)
HIV-$1M_{DR/C}$ (X4)	0.0009 ± 0.0002 (2)	0.357 ± 0.040 (11)	0.011 ± 0.004 (2)
HIV-$1M_{DR/G}$ (X4)	0.0027 ± 0.0012 (5)	0.485 ± 0.073 (15)	0.031 ± 0.002 (6)
HIV-$1M_{DR/TM}$ (X4)	0.0022 ± 0.0001 (4)	0.488 ± 0.009 (15)	0.031 ± 0.002 (6)
HIV-$1M_{DR/MM}$ (R5)	0.0027 ± 0.0006 (5)	0.291 ± 0.128 (9)	0.016 ± 0.006 (3)
HIV-$1M_{DR/JSL}$ (R5)	0.0028 ± 0.0001 (5)	0.419 ± 0.122 (13)	0.024 ± 0.007 (5)

[a] The amino acid substitutions identified in the protease-encoding region of HIV-$1_{ERS104pre}$, HIV-1_B, HIV-1_C, HIV-1_G, HIV-1_{TM}, HIV-$1M_M$, HIV-1_{JSL} compared with the consensus type B sequence cited from the Los Alamos database include L63P; L10I, K14R, L33I, M36I, M46I, F53I, K55R, I62V, L63P, A71V, G73S, V82A, L90M, I93L; L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, L89M; L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, L90M; L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90M, I93L; L10I, K43T, M46L, I54V, L63P, A71V, V82A, L90M, Q92K; and L10I, L24I, I33F, E35D, M36I, N37S, M46L, I54V, R57K, I62V, L63P, A71V, G73S, V82A, respectively. HIV-$1_{ERS104pre}$ served as a source of wild-type HIV-1. The EC_{50} values were determined by using PHA-PBMs as target cells, and the inhibition of p24 gag protein production by each drug was used as an endpoint. The numbers in parentheses represent the fold change in EC_{50} value for each isolate relative to the IC_{50} value for wild-type HIV-$1_{ERS104pre}$. All assays were conducted in duplicate, and the data shown represent mean values ± SD derived from the results of two or three independent experiments.

The X-ray crystal structure of wild-type HIV-1 protease co-crystallized with GRL-0519A was refined at the near-atomic resolution of 1.27 Å (PDB ID: 3OK9). The structure comprises the protease dimer and the inhibitor in two orientations related by a 180° rotation with 55/45% occupancies. The protease dimer structure is essentially identical to that in the protease–DRV complex[31] with an RMSD of 0.16 Å on Cα atoms. The inhibitor is bound in the active site cavity through a series of hydrogen bond interactions and weaker CH···O interactions with the main-chain atoms of the HIV-1 protease (Figure 3A). The

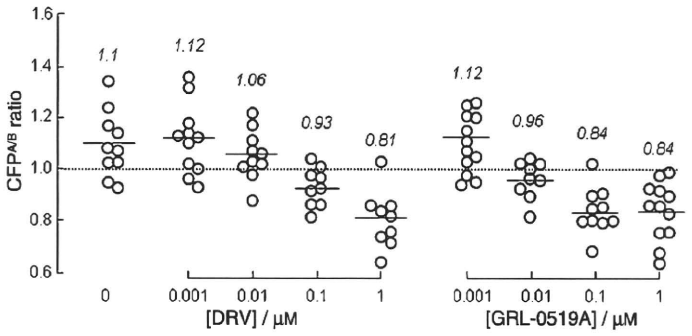


Figure 2. Inhibition of HIV-1 protease dimerization by GRL-0519A. Changes in emission intensity ratios in the presence of DRV or GRL-0519A are shown. COS7 cells were co-transfected with a pair of HIV-PR^{CFP} and HIV-PR^{YFP}, and CFP$^{A/B}$ ratios were determined. The mean values of the ratios are shown as horizontal bars. The results of statistical evaluation of the changes in the CFP$^{A/B}$ ratios determined in the presence or absence of DRV or GRL-0519A are as follows: the CFP$^{A/B}$ ratios in the absence of drug (CFP$^{A/B}_{No\,Drug}$) vs. the CFP$^{A/B}$ ratios in the presence of 0.001 μM DRV (CFP$^{A/B}_{0.001\,DRV}$), $p=0.94$; CFP$^{A/B}_{No\,Drug}$ vs. CFP$^{A/B}_{0.01\,DRV}$, $p=0.15$; CFP$^{A/B}_{No\,Drug}$ vs. CFP$^{A/B}_{0.1\,DRV}$, $p=0.0042$; CFP$^{A/B}_{No\,Drug}$ vs. CFP$^{A/B}_{1.0\,DRV}$, $p=0.0004$; CFP$^{A/B}_{No\,Drug}$ vs. CFP$^{A/B}_{0.001\,GRL\text{-}519}$, $p=0.947$; CFP$^{A/B}_{No\,Drug}$ vs. CFP$^{A/B}_{0.01\,GRL\text{-}519}$, $p=0.0077$; CFP$^{A/B}_{No\,Drug}$ vs. CFP$^{A/B}_{0.1\,GRL\text{-}519}$, $p=0.0003$; CFP$^{A/B}_{No\,Drug}$ vs. CFP$^{A/B}_{1.0\,GRL\text{-}519}$, $p=0.0002$; CFP$^{A/B}_{0.01\,DRV}$ vs. CFP$^{A/B}_{0.01\,GRL\text{-}519}$, $p=0.013$. Panel A: HIV$_{WT}$ vs. rHIV$_{WTpro}^{75/219gag}$, $p=0.53$; HIV$_{WT}$ vs. rHIV$_{WTpro}^{12/75/219/309/409gag}$, $p=0.0080$; HIV$_{WT}$ vs. rHIV$_{WTpro}^{219/409gag}$, $p=0.22$; rHIV$_{WTpro}^{75/219gag}$ vs. rHIV$_{WTpro}^{12/75/219/309/409gag}$, $p=0.0065$; rHIV$_{WTpro}^{75/219gag}$ vs. rHIV$_{WTpro}^{219/409gag}$, $p=0.15$; rHIV$_{WTpro}^{12/75/219/309/409gag}$ vs. rHIV$_{WTpro}^{219/409gag}$, $p=0.0018$. Panel B: HIV$_{WT}$ vs. rHIV$_{WTpro}^{75/219gag}$, $p=0.65$; HIV$_{WT}$ vs. rHIV$_{WTpro}^{12/75/219/309/409gag}$, $p<0.0001$; HIV$_{WT}$ vs. rHIV$_{WTpro}^{219/409gag}$, $p<0.0001$.

major conformation of the inhibitor forms hydrogen bonding interactions of its urethane NH group with the carbonyl oxygen of Gly27. The oxymethyl oxygen of the *tris*-THF interacts with the amide of Asp30, and the second THF oxygen forms hydrogen bonds with the amides of Asp29, Asp30, and the carboxylate side chain of Asp30. In addition, water-mediated interactions connect the inhibitor carbonyl oxygen and sulfonamide oxygen with the amides of Ile50 and 50′ in the flaps, and link the inhibitor P2′ aromatic ring with the amide of Asp30′. Similar interactions are shared by the protease–DRV complex, and are considered responsible for the high affinity of DRV for HIV protease and its potency against drug-resistant HIV-1 variants.[31]

The majority of differences are confined in the interactions of the third THF moiety in GRL-0519A. The third THF ring forms CH···O interactions with Gly48 in the protease flap and water-mediated hydrogen bonds with conserved residues at the dimer interface as shown in Figure 3B. The third THF ring forms CH···O and water-mediated interactions with the carbonyl and amide of Gly48 that may stabilize the flexible flap conformation. The oxygen at the opposite side of the THF ring forms a hydrogen bond with a conserved water molecule and stabilizes a network of hydrogen bonds and ionic interactions connecting Arg8′ from the other subunit with the side chains of Asp29 and Arg87, as well as with the main-chain carbonyl oxygen atoms of Thr26 and Gly27 in the characteristic catalytic triplet (Asp25-Thr26-Gly27) of aspartic proteases. The ionic and hydrogen bonding interactions of Arg8′ with Asp29 and Arg87 are conserved among HIV protease structures and are important components of the dimer interface.[32,33] Also, the oxygen of the third THF ring interacts with a semicircular network of three conserved water molecules that surround the guanidine side chain of Arg8′. The hydrogen bond and van der Waals interactions of the third THF restrain the Arg8′ side chain in a single conformation, thus preventing the formation of an alternate conformation observed in the DRV complex. Furthermore, the third THF ring packs neatly against the P1 phenyl group of the inhibitor with better internal hydrophobic contacts than are possible for *bis*-THF in DRV. The ring stereochemistry (*SSS*-type) in the *tris*-THF ligand of **3** would not be able to make similar hydrophobic contacts. Thus, the *tris*-THF ring of GRL-0519A (**4**) fills the substrate binding cavity better than the *bis*-THF ligand of DRV, anchors Arg87 at the dimer interface, and forms new CH···O and conserved water-mediated interactions with the flaps and the base of the substrate binding cavity.

In conclusion, we designed and synthesized novel oxatricyclic [3(*R*), 3a*S*, 4a*S*, 6a*R*, 7a*S*] and [3(*R*), 3a*S*, 4a*R*, 6a*S*, 7a*S*] ligands and incorporated these ligands in the (*R*)-hydroxyethyl sulfonamide isostere.[16] The orientation, ring size, and stereochemistry are all critical to the potency of the oxatricyclic ligand-derived inhibitors. The ligands were synthesized with defined stereochemistry in optically active forms. Incorporation of a *syn-syn-syn*-fused *tris*-THF led to inhibitor **3**, which has significantly decreased enzyme inhibitory and antiviral potency relative to DRV. However, the *tris*-THF ligand with a *syn-anti-syn* fusion provided inhibitor GRL-0519A (**4**), which has remarkable enzyme inhibitory and antiviral activity. Of particular interest, GRL-0519A displayed potent activity against a variety of multidrug-resistant clinical HIV-1 strains, with EC_{50} values ranging from 0.6 to 4.3 nM, a nearly 10-fold improvement over darunavir. Moreover, GRL-0519A blocked protease dimerization at least 10-fold more potently than DRV. An X-ray crystal structure of GRL-0519A-bound HIV-1 protease revealed molecular insight into the ligand–binding site interactions responsible for its potency against wild-type and mutant viruses. It appears that the first and second THF rings of the *tris*-THF ligand with a *syn-anti-syn* configuration maintain key backbone hydrogen bonding interactions similar to the *bis*-THF ligand of darunavir. The third THF ring oxygen makes water-mediated hydrogen bonds with Asp29, Arg8′, and Gly27. In addition, the ring fills the hydrophobic pocket in the S2 subsite and also stacks nicely behind the P1 phenylmethyl substituent at the S1 subsite. Therefore, our basic design strategy has shown to be extremely powerful and is well worth further experimentation.

Acknowledgements

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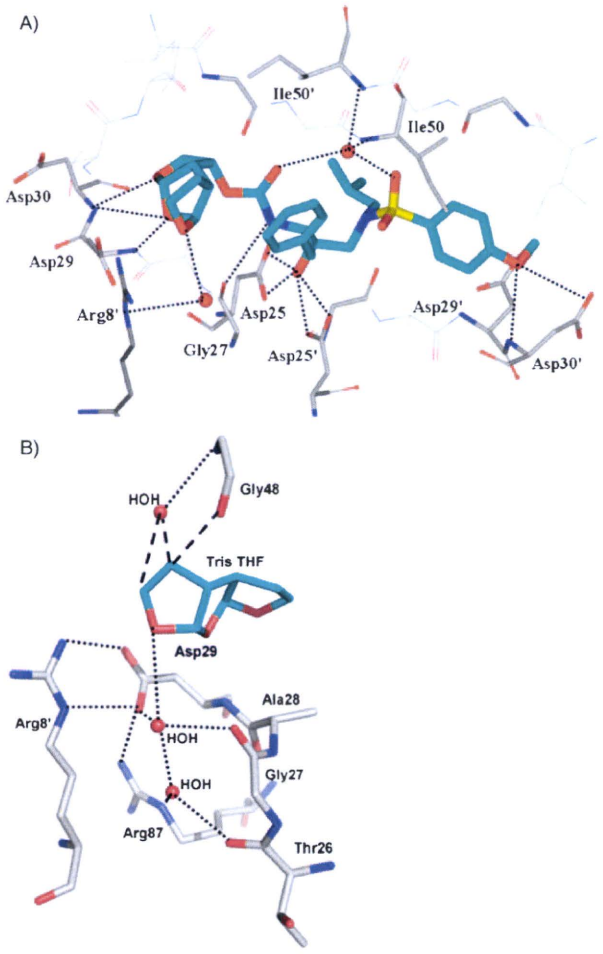

Figure 3. A) X-ray crystal structure of the HIV-1 protease–GRL-0519A complex. The major orientation of the inhibitor is shown. The inhibitor carbon atoms are shown in cyan, water molecules are red spheres, and the hydrogen bonds are indicated by dotted lines. B) Polar interactions of the third THF group with the protease; hydrogen bonds are indicated by dotted lines, and CH···O interactions by dashed lines.

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Keywords: dimerization inhibitors · HIV-1 protease · multidrug resistance · oxatricyclic ligands · X-ray crystallography

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COMMUNICATIONS

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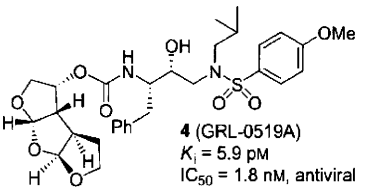
Probing Multidrug-Resistance and Protein–Ligand Interactions with Oxatricyclic Designed Ligands in HIV-1 Protease Inhibitors



4 (GRL-0519A)
K_i = 5.9 pM
IC_{50} = 1.8 nM, antiviral

A healthier HAART: We report the design, synthesis, biological evaluation, and X-ray crystallographic analysis of a new class of HIV-1 protease inhibitors. Compound **4** proved to be an extremely potent inhibitor toward various multidrug-resistant HIV-1 variants, representing a near 10-fold improvement over darunavir (DRV). Compound **4** also blocked protease dimerization with at least 10-fold greater potency than DRV.

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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▽†

Yasuhiro Koh,[1] Masayuki Amano,[1] Tomomi Towata,[1] Matthew Danish,[1] Sofiya Leshchenko-Yashchuk,[2] Debananda Das,[3] Maki Nakayama,[1] Yasushi Tojo,[1] Arun K. Ghosh,[2] and Hiroaki Mitsuya[1,3]*

Departments of Infectious Diseases and Hematology, Kumamoto University Graduate School of Medical and Pharmaceutical Sciences, Kumamoto 860-8556, Japan[1]; Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, Indiana 47907[2]; and Experimental Retrovirology Section, HIV and AIDS, Malignancy Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892[3]

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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.

* Corresponding author. Mailing address: Department of Infectious Diseases and Department of Hematology, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan. Phone: (81) 96-373-5156. Fax: (81) 96-363-5265. E-mail: hmitsuya@helix.nih.gov.

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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

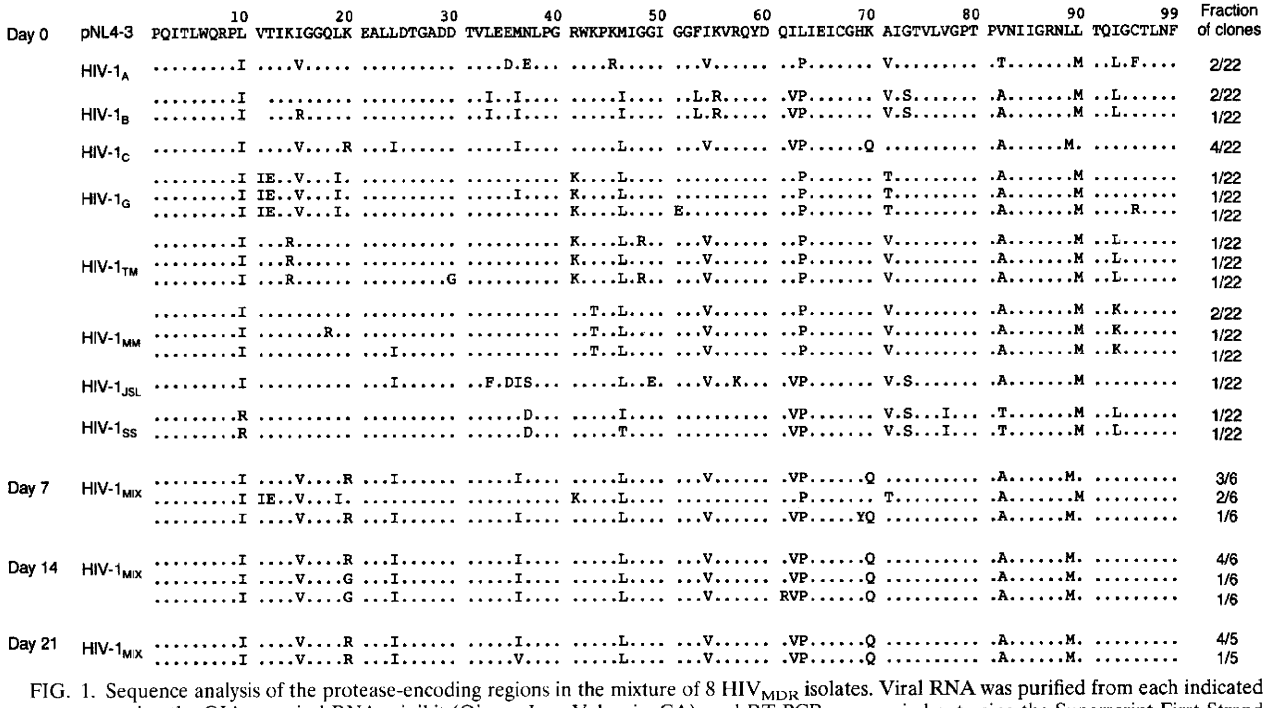

FIG. 1. Sequence analysis of the protease-encoding regions in the mixture of 8 HIV_{MDR} isolates. Viral RNA was purified from each indicated supernatant using the QIAamp viral RNA minikit (Qiagen Inc., Valencia, CA), and RT-PCR was carried out using the Superscript First-Strand synthesis system for 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-$1_{NL4\text{-}3}$ 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-

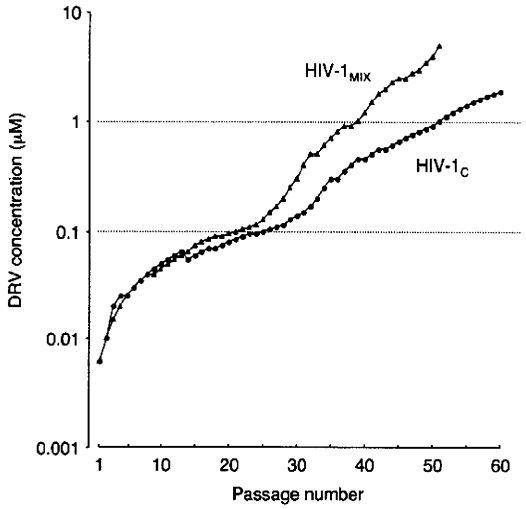

FIG. 2. *In vitro* selection of HIV-1_{MIX} and HIV-1_C resistant to DRV. A mixture of 8 HIV-1 isolates resistant to multiple PIs (triangles) or an HIV-1_C strain (circles) was passaged in the presence of increasing concentrations of DRV in MT-4 cells. The selection was carried out in a cell-free manner 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

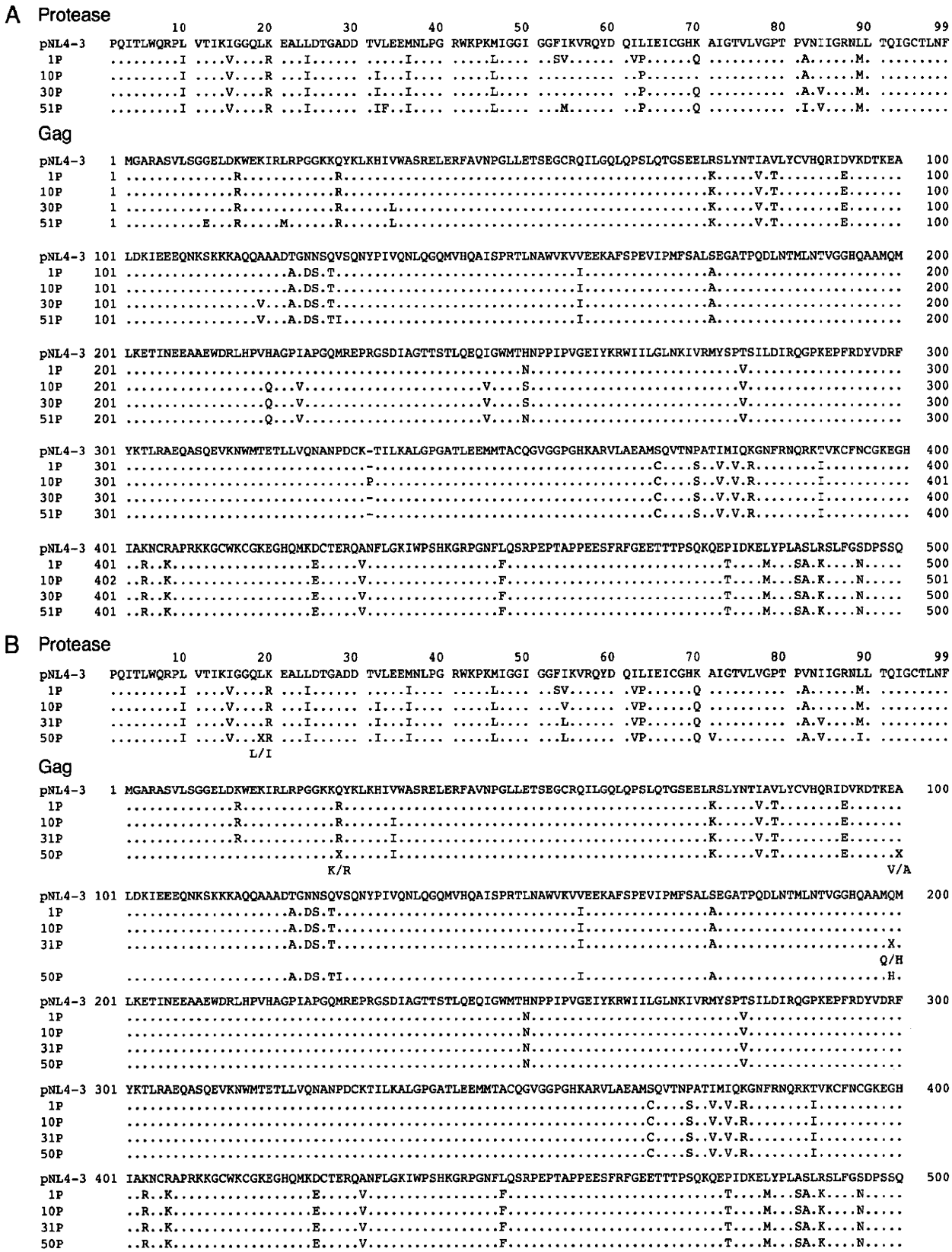

FIG. 3. Amino acid sequences of HIV-1_{MIX} and HIV-1_C passaged in the presence of DRV. (A) The amino acid sequences of the Gag-protease-encoding region from 4 different passages of each strain derived from HIV-1_{MIX} passaged in the presence of DRV are indicated. The top line shows the Gag-protease 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.

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-$1_{C^{P5I}}$ (replicative at 1 μM DRV), HIV-$1_{MIX^{P39}}$ (replicative at 1 μM DRV), and HIV-$1_{MIX^{P5I}}$ (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-$1_{C^{P5I}}$, HIV-$1_{MIX^{P39}}$, and HIV-$1_{MIX^{P5I}}$ 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-$1_{C^{P5I}}$, HIV-$1_{MIX^{P39}}$, and HIV-$1_{MIX^{P5I}}$ 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-$1_{C^{P5I}}$, HIV-$1_{MIX^{P39}}$, and HIV-$1_{MIX^{P5I}}$, 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-$1_{MIX^{P5I}}$ 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.

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

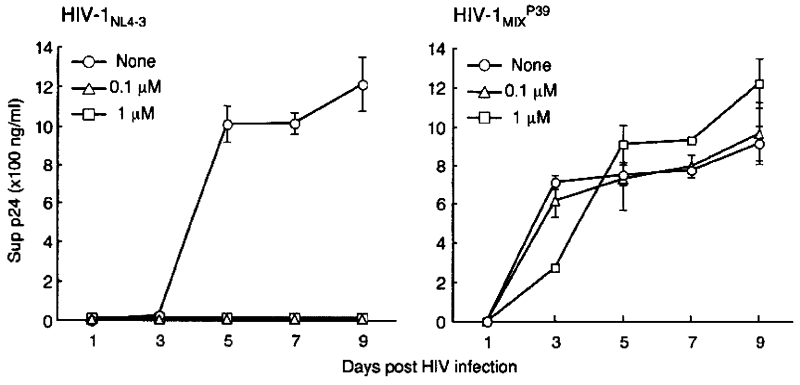

FIG. 4. Replication kinetics of HIV-$1_{MIX}{}^{P39}$ in the presence of DRV. MT-4 cells were exposed to HIV-1_{NL4-3} or HIV-$1_{MIX}{}^{P39}$ and cultured in the presence or absence of 0.1 or 1.0 μM DRV. Viral replication was monitored by measuring p24 Gag protein in the culture supernatant.

blocked the dimerization of HIV-1 protease in the fluorescence resonance energy transfer (FRET)-based HIV-1 expression assay (15). Since HIV-$1_{MIX^{PSI}}$ can propagate in the presence of 5 μM DRV, it is likely that DRV is no longer capable of inhibiting the dimerization of the protease with the mutations seen in HIV-$1_{MIX^{PSI}}$. Considering that conventional protease inhibitors, such as SQV, RTV, NFV, APV, and LPV, failed to block the dimerization of HIV-1 protease (15), it appears that the activity inhibiting the proteolytic function of protease is independent from that inhibiting the dimerization of HIV-1 protease, although it should be determined what mutations (a single mutation or combined mutations) seen in HIV-$1_{MIX^{PSI}}$ are responsible for the viral acquisition of the ability to escape from DRV's protease dimerization inhibition. It is also yet to be determined whether and what mutations seen in HIV-$1_{MIX^{PSI}}$ can confer resistance to TPV on HIV-1.

During reverse transcription, reverse transcriptase is known to frequently switch a template from one genomic RNA strand to another, yielding recombinant proviral DNA, which represents a mosaic consisting of multiple parent genomic components. Indeed, there is firm evidence that a single $CD4^+$ target cell can be infected with multiple HIV-1 virions both *in vitro* and *in vivo* (2, 13). If resultant proviral DNA acquires mutations in one strand that confer HIV-1 resistance to one drug and mutations in the other strand that are associated with HIV-1 resistance to the other drug, such proviral DNA-containing daughter virions will be resistant to both drugs, a process called homologous recombination (1, 20, 26). Homologous recombination is thus highly likely to accelerate the emergence of multidrug and multiclass drug resistance in infected individuals. In the present work, the nucleic acid sequence of the protease-encoding region of HIV-$1_{MIX^{PSI}}$ was virtually identical to that of HIV-1_{C^R}, suggesting that HIV-1_C had resistance to DRV, immediately predominated over other 7 HIV-1 isolates, and continued to propagate in the presence of DRV, suggesting a lack of involvement of homologous recombination in the emergence of highly DRV-resistant HIV-1 variants. Thus, we further conducted a selection experiment using HIV-1_C as a starting HIV-1 isolate. However, HIV-1_C's DRV resistance acquisition was substantially delayed compared to DRV resistance acquisition of HIV-$1_{MIX^{PSI}}$ in two independent selection experiments (Fig. 2 and data not shown). Therefore, we determined the nucleic acid sequence of the *gag* gene in both HIV-$1_{MIX^{PSI}}$ and HIV-1_{C^R} and readily found that the former had two mutations (H219Q and I223V) in the CypA-binding loop and I247V by passage 10 while the latter lacked all three mutations (Fig. 3A and B). Among the 8 isolates used, only the original HIV-1_G contained these three mutations (see Fig. S4 in the supplemental material). The H219Q substitution in the viral CypA binding loop, a polymorphic amino acid change, has been shown to confer a replication advantage on HIV-1 in CypA-rich target cells (8). As expected, both HIV-$1_{MIX^{P39}}$ and HIV-$1_{MIX^{PSI}}$ had substantially less CypA content within virions (see Fig. S5).

When 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), only recombinant HIV_{p1} ($rHIV_{p1}$) replicated, while other recombinant HIV-1 clones failed to replicate, although all the amino acid substitutions identified in the protease- and Gag-encoding genes of the replicative mixture HIV-1 population were introduced to such recombinant clones. When we generated recombinant HIV-1 virions with two amino acid substitutions mutated back (H219Q/I223V to H219/I223; designated $rHIV_{p530\text{-}MB}$), these virions also failed to replicate. Suspecting that other amino acid substitutions residing in a minor HIV-1 population helped the mixture population replicate through homologous recombination in the mixture population, we added such an amino acid substitution, A196T or A196S, to $rHIV_{p530\text{-}MB}$ (designated $rHIV_{p530\text{-}MB196T}$ and $rHIV_{p530\text{-}MB196T}$). However, such recombinant clones also failed to replicate. It is unclear at this time how these recombinant HIV-1 virions failed to replicate; however, it is possible that other unidentified yet critical amino acid substitutions are required for the replication of the DRV-selected mixture population. It should be noted that in general, the population size of HIV-1 in patients is relatively larger, the magnitude of replication is greater, and the duration of HIV-1 replication is longer than in a test tube. It is also of note that the appearance of mutations can be largely affected by stochastic phenomena, i.e., rates and orders of appearance of mutations *in vitro* compared to the *in vivo* situation.

In summary, the present results demonstrated the first successful *in vitro* selection of highly DRV-resistant HIV-1 variants and a new method for efficiently selecting drug-resistant HIV-1 variants in a test tube when such variants are hardly generated *in vitro* and *in vivo*. The present data also suggest that DRV would not easily permit HIV-1 to develop significant resistance; however, HIV-1 can develop high levels of DRV resistance with robust viral fitness comparable to the fitness of wild-type HIV-1 when a variety of PI-resistant HIV-1 strains are generated, as seen in patients experiencing sequential PI failure, and ensuing homologous recombination occurs.

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Novel Protease Inhibitors (PIs) Containing Macrocyclic Components and 3(*R*),3a(*S*),6a(*R*)-*bis*-Tetrahydrofuranylurethane That Are Potent against Multi-PI-Resistant HIV-1 Variants *In Vitro*[▽]

Yasushi Tojo,[1] Yasuhiro Koh,[1] Masayuki Amano,[1] Manabu Aoki,[1] Debananda Das,[2] Sarang Kulkarni,[3] David D. Anderson,[3] Arun K. Ghosh,[3] and Hiroaki Mitsuya[1,2]*

Departments of Hematology and Infectious Diseases, Kumamoto University Graduate School of Biomedical Sciences, Kumamoto 860-8556, Japan[1]; Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, Bethesda, Maryland 20892[2]; and Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, Indiana 47907[3]

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Natural products with macrocyclic structural features often display intriguing biological properties. Molecular design incorporating macrocycles may lead to molecules with unique protein-ligand interactions. We generated novel human immunodeficiency virus type 1 (HIV-1) protease inhibitors (PIs) containing a macrocycle and *bis*-tetrahydrofuranylurethane. Four such compounds exerted potent activity against HIV-1_{LAI} and had 50% effective concentrations (EC_{50}s) of as low as 0.002 μM with minimal cytotoxicity. GRL-216 and GRL-286 blocked the replication of HIV-1_{NL4-3} variants selected by up to 5 μM saquinavir, ritonavir, nelfinavir, lopinavir, or atazanavir; they had EC_{50}s of 0.020 to 0.046 μM and potent activities against six multi-PI-resistant clinical HIV-1 (HIV_{mPI^r}) variants with EC_{50}s of 0.027 to 0.089 μM. GRL-216 and -286 also blocked HIV-1 protease dimerization as efficiently as darunavir. When HIV-1_{NL4-3} was selected by GRL-216, it replicated progressively more poorly and failed to replicate in the presence of >0.26 μM GRL-216, suggesting that the emergence of GRL-216-resistant HIV-1 variants is substantially delayed. At passage 50 with GRL-216 (the HIV isolate selected with GRL-216 at up to 0.16 μM [$HIV_{216\text{-}0.16\ \mu M}$]), HIV-$1_{NL4-3}$ containing the L10I, L24I, M46L, V82I, and I84V mutations remained relatively sensitive to PIs, including darunavir, with the EC_{50}s being 3- to 8-fold-greater than the EC_{50} of each drug for HIV-1_{NL4-3}. Interestingly, $HIV_{216\text{-}0.16\ \mu M}$ had 10-fold increased sensitivity to tipranavir. Analysis of the protein-ligand X-ray structures of GRL-216 revealed that the macrocycle occupied a greater volume of the binding cavity of protease and formed greater van der Waals interactions with V82 and I84 than darunavir. The present data warrant the further development of GRL-216 as a potential antiviral agent for treating individuals harboring wild-type and/or HIV_{mPI^r}.

Currently available combination chemotherapy for human immunodeficiency virus type 1 (HIV-1) infection and AIDS typically uses two reverse transcriptase (RT) inhibitors (RTIs) and boosted protease inhibitors (PIs) or an integrase inhibitor, also known as highly active antiretroviral therapy (HAART) (6, 29, 30, 33), and has been shown to suppress the replication of HIV-1 and significantly extend the life expectancy of HIV-1-infected individuals. Indeed, several recent analyses have revealed that the mortality rates for HIV-infected persons have become much closer to the general mortality rates since the introduction of HAART and that first-line HAART with boosted PI-based regimens resulted in less resistance within and across drug classes (2, 20, 21, 35).

However, the ability to provide effective long-term antiretroviral therapy for HIV-1 infection has become a complex issue since those who initially achieved favorable viral suppression to undetectable levels have experienced treatment failure (10, 20, 27). In addition, it is evident that even with these anti-HIV-1 drugs, only partial immunologic reconstitution is attained in patients with advanced HIV-1 infection, and it is likely that HIV-1 will eventually acquire resistance to virtually any antiviral agent. Thus, it appears that the development of potent and drug-resistance-deferring antiviral agents will continue to be required for successful long-term control of HIV-1 infection and AIDS (3, 11).

A number of natural-product-derived small molecules are known to interact with human proteins, and a variety of such molecules have been identified to fulfill significant biochemical functions, permitting the development of a wide range of pharmaceuticals. Macrocycles are representatives of natural-product-derived small molecules. Of note, any molecule containing a ring of seven or more atoms is defined to be a macrocycle (8).

In the present study, we designed and synthesized PIs containing functionalities that interact with the amino acid backbones of the catalytic site of HIV-1 protease along with a flexible macrocyclic group involving P1′-P2′ ligands for effective repacking of the altered PI-binding cavity of protease that emerges upon side chain mutations in PI-resistant HIV-1 variants. We identified a group of novel potent macrocyclic PIs (16). GRL-216, which possesses a dual anti-HIV-1 mechanism, inhibits the catalytic activity and dimerization of HIV-1 pro-

* Corresponding author. Mailing address: Department of Infectious Diseases and Department of Hematology, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan. Phone: (81) 96-373-5156. Fax: (81) 96-363-5265. E-mail: hmitsuya@helix.nih.gov.

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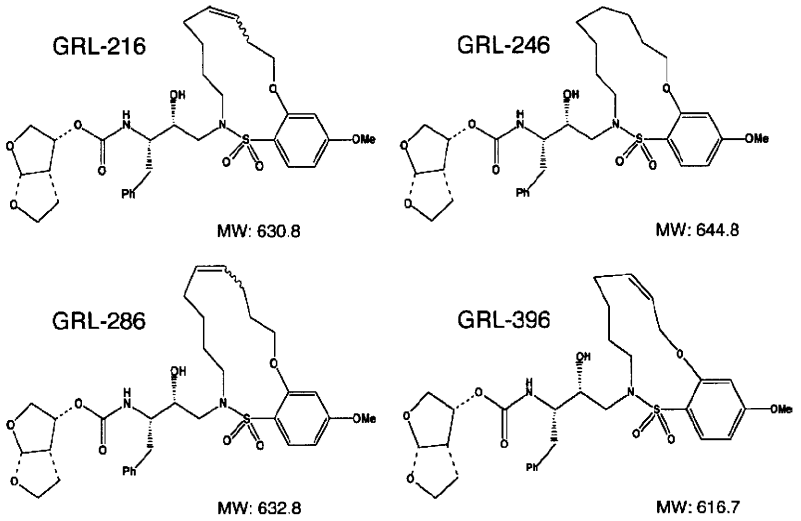

FIG. 1. Structures of macrocyclic PIs GRL-216, GRL-246, GRL-286, and GRL-396.

tease and exerts potent activity against wild-type HIV-1 as well as a wide spectrum of PI-resistant HIV-1 variants *in vitro*.

MATERIALS AND METHODS

Cells and viruses. Human $CD4^+$ MT-2 and MT-4 cell lines were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Linz, Austria) plus 50 U of penicillin and 100 μg of kanamycin per ml. The following HIV-1 strains were used for the drug susceptibility assay: HIV-1_{LAI}, HIV-1_{NL4-3}, HIV-2_{EHO}, and HIV-2_{ROD} (5, 32, 36). In addition, we used clinical HIV-1 strains from drug-naive patients with AIDS (HIV-$1_{ERS104pre}$) (32) and six HIV-1 clinical isolates that were originally isolated from patients with AIDS who had received anti-HIV-1 therapy heavily (for 32 to 83 months) and that were genotypically and phenotypically characterized as multiple-PI-resistant HIV-1 variants (36, 37). To determine whether each clinical HIV-1 isolate used in the present study was a syncytium-inducing (SI) virus or a non-syncytium-inducing (NSI) virus strain, MT-2 cells (10^5) were exposed to an aliquot of viral stock supernatant containing 100 50% tissue culture infectious doses ($TCID_{50}$s) of the virus and cultured in six-well culture plates. The cultures were maintained for 4 weeks and were examined under an inverted microscope to determine the syncytium-inducing or non-syncytium-inducing nature of the virus, as described previously (36, 37).

Antiviral agents. In this work, 40 novel nonpeptidic PIs which contained 3(*R*),3a(*S*),6a(*R*)-bis-tetrahydrofuranylurethane (*bis*-THF) (14, 15, 24) and a macrocyclic ring (16) were designed and synthesized. Among them, four PIs, GRL-216, GRL-246, GRL-286, and GRL-396 (Fig. 1), with molecular weights of 630.8, 644.8, 632.8, and 616.7, respectively, were chosen for detailed analysis, primarily on the basis of their potent antiviral activity. The method of the synthesis of these four PIs has been described elsewhere by Ghosh and coworkers (16). 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) and lopinavir (LPV) were kindly provided by Japan Energy, Inc., Tokyo, Japan. Indinavir (IDV) was kindly provided by Merck Research Laboratories (Rahway, NJ). Atazanavir (AZV) was a kind gift from Bristol-Myers Squibb (New York, NY). Darunavir (DRV) was synthesized as described previously (17). Tipranavir (TPV) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health.

Drug susceptibility assay. The susceptibilities of HIV-1_{LAI} and primary HIV-1 isolates to various drugs were determined as described previously (24), with minor modifications. Briefly, MT-2 cells (2×10^4/ml) were exposed to 100 $TCID_{50}$s of HIV-1_{LAI} in the presence or absence of various concentrations of drugs in 96-well microculture plates, and the plates were incubated at 37°C for 7 days. After 100 μl of the medium was removed from each well, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10 μl, 7.5 mg/ml in phosphate-buffered saline) was added to each well in the plate, followed by incubation at 37°C for 3 h. After incubation to dissolve the formazan crystals, 100 μl of acidified isopropanol containing 4% (vol/vol) Triton X-100 was added to each well, and the optical density was measured using a kinetic microplate reader (Vmax; Molecular Devices, Sunnyvale, CA). All assays were performed in duplicate or triplicate. To determine the susceptibilities of primary HIV-1 isolates to the drugs, phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBMCs; 10^6/ml) were exposed to 50 $TCID_{50}$s of each isolate. The target cells were exposed to HIV-$1_{ERS104pre}$ or drug-resistant HIV-1 in the presence or absence of various concentrations of drugs and were incubated for 7 days. Upon the conclusion of the culture, the amounts of p24 Gag protein in the supernatants were determined using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo, Japan) (28).

To determine the drug susceptibilities of certain laboratory HIV-1 strains, MT-4 cells were used as target cells. In brief, MT-4 cells (10^5/ml) were exposed to 100 $TCID_{50}$s of drug-resistant HIV-1 strains in the presence or absence of various concentrations of drugs, and on day 7 of culture, the supernatant was harvested and the amounts of p24 Gag protein were determined. The drug concentrations that suppressed the production of p24 Gag protein by 50% (50% effective concentrations [EC_{50}s]) were determined by comparison of the levels produced by drug-treated cell cultures with the levels produced in drug-free control cell cultures. All assays were performed in duplicate or triplicate.

Generation of PI-resistant HIV-1 *in vitro*. In the experiments for selecting drug-resistant variants, MT-4 cells were exploited as target cells, since HIV-1 in general replicates at greater levels in MT-4 cells than in MT-2 cells. MT-4 cells (10^5/ml) were exposed to HIV-1_{NL4-3} (500 $TCID_{50}$s) and cultured in the presence of various PIs, each at an initial concentration of its 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 of the HIV genome.

Determination of nucleotide sequences. Molecular cloning and determination of the nucleotide sequences of HIV-1 strains passaged in the presence of anti-HIV-1 agents were performed as described previously (36, 37). In brief, high-molecular-weight DNA was extracted from HIV-1-infected MT-4 cells by using the InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) and was subjected to molecular cloning, followed by sequence determination. The first-round PCR mixture consisted of 1 μl of proviral DNA solution, 10 μl of Premix *Taq* (Ex *Taq* version; Takara Bio, Inc., Otsu, Japan), and 10 pmol of each of the first PCR primers in a total volume of 20 μl. The PCR conditions used were an initial 5 cycles of 30 s at 95°C, 2 min at 55°C, and 2 min at 72°C, followed by 15 cycles of 30 s at 95°C, 20 s at 55°C, and 2 min at 72°C. The first-round PCR products were used directly in the second round of PCR. The second-round PCR products were purified with spin columns (MicroSpin S-400 HR columns; Amersham Biosciences Corp., Piscataway, NJ), cloned directly, and subjected to sequencing

TABLE 1. Activities of mcPIs against HIV-1_{LAI}, HIV-2_{EHO}, and HIV-2_{ROD} and cytotoxicities[a]

Drug	EC_{50} (μM)			CC_{50} (μM)	Selectivity index
	HIV-1_{LAI}	HIV-2_{EHO}	HIV-2_{ROD}		
APV	0.024 ± 0.008	0.12 ± 0.03	0.42 ± 0.10	>100	>4,170
LPV	0.039 ± 0.006	0.035 ± 0.025	0.028 ± 0.004	>100	>2,560
TPV	0.17 ± 0.005	0.30 ± 0.15	0.33 ± 0.07	54.1 ± 2.1	320
DRV	0.003 ± 0.0004	0.008 ± 0.007	0.010 ± 0.004	>100	>33,300
GRL-216	0.002 ± 0.001	0.010 ± 0.007	0.017 ± 0.001	48.8 ± 1.3	24,400
GRL-246	0.022 ± 0.005	ND	ND	33.3 ± 0.8	1,510
GRL-286	0.004 ± 0.001	0.018 ± 0.013	0.025 ± 0.002	33.1 ± 2.5	8,280
GRL-396	0.014 ± 0.001	ND	ND	44.9 ± 4.1	3,200

[a] MT-2 cells (2×10^3) were exposed to 100 $TCID_{50}$s of HIV-1_{LAI}, HIV-2_{EHO}, or HIV-2_{ROD} and cultured in the presence of various concentrations of each PI. The EC_{50}s were determined by using the MTT assay. All assays were conducted in duplicate, and the data shown represent mean values (±1 standard deviation) derived from the results of three independent experiments. Each selectivity index denotes the ratio of the CC_{50} to the EC_{50} against HIV-1_{LAI}. ND, not done.

with a model 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Generation of FRET-based HIV-1 expression system. The intermolecular fluorescence resonance energy transfer (FRET)-based HIV-1 expression assay employing cyan and yellow fluorescent protein-tagged protease monomers (CFP and YFP, respectively) was performed as described previously (23). In brief, CFP- and YFP-tagged HIV-1 protease constructs were generated using BD Creator DNA cloning kits (BD Biosciences, San Jose, CA). For the generation of full-length molecular infectious clones containing CFP- or YFP-tagged protease, the PCR-mediated recombination method was used (9). A linker consisting of five alanines was inserted between the protease and the fluorescent proteins. The phenylalanine-proline site that HIV-1 protease cleaves was also introduced between the fluorescent protein and RT sites. Thus, the DNA fragments obtained were subsequently joined by using the PCR-mediated recombination reaction performed under the standard conditions for ExTaq polymerase (Takara Bio, Inc.). The amplified PCR products were cloned into the pCR-XL-TOPO vector, according to the manufacturer's instructions (Gateway cloning system; Invitrogen, Carlsbad, CA). PCR products were generated with the pCR-XL-TOPO vector and used as templates, followed by digestion by both ApaI and SmaI; and the ApaI-SmaI fragment was introduced into pHIV-1_{NLSma} (12), generating pHIV-PR_{WT}^{CFP} and pHIV-PR_{WT}^{YFP} (where WT indicates wild type), respectively.

FRET procedure. COS7 cells plated on an EZ-view cover, glass bottom culture plate (Iwaki, Tokyo) were transfected with pHIV-PR_{WT}^{CFP} and pHIV-PR_{WT}^{YFP} 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, Japan) at room temperature, as described previously (23). When the effect of each compound was analyzed by FRET, the test compounds were added to the culture medium simultaneously with the transfected plasmids.

The results of FRET were determined by measurement of the quenching of the CFP (donor) fluorescence and the increase in the YFP (acceptor) fluorescence (sensitized emission), since part of the energy of CFP is transferred to YFP instead of being emitted. The changes in the CFP and YFP fluorescence intensities in the images of the selected regions were examined and quantified using an FV500 Image software system (Olympus Optical Corp.). The ratios of the intensities of the CFP fluorescence after photobleaching to the CFP fluorescence before photobleaching ($CFP^{A/B}$ ratios) were determined. $CFP^{A/B}$ ratios less than 1 indicated that the association of the two subunits did not occur, and it was interpreted that protease dimerization was inhibited (23).

Structural analysis of interaction of GRL-216 and darunavir. Counterions were deleted from the crystal structures of HIV-1 protease complexed with GRL-216 (Protein Data Bank [PDB] accession number 3I6O) (16) and darunavir (PDB accession number 2IEN) (13). Bond orders were properly assigned to the inhibitor molecules. Hydrogens were added to all the heavy atoms, and their positions were optimized in an OPLS2005 force field with constraints on the heavy atom positions. A cutoff distance of 3.0 Å between a polar hydrogen and an oxygen or nitrogen atom was used to determine the presence of hydrogen bonds. The structures were analyzed using the Maestro (version 9.0) program (Schrödinger, LLC, New York, NY, 2009).

RESULTS

Generation of mcPIs and their activities against HIV-1_{LAI}. We designed and synthesized ~40 PIs containing a variety of macrocyclic components (16). Among those, we identified GRL-216, GRL-246, GRL-286, and GRL-396 (Fig. 1) to be PIs with potent activities against HIV-1 *in vitro*. As shown in Table 1, GRL-216 and -286 were highly potent against a laboratory wild-type HIV-1 strain, HIV-1_{LAI}, with EC_{50}s of 0.002 ± 0.001 and 0.004 ± 0.001 μM, respectively, determined with the MTT assay using MT-2 cells. Of note, GRL-216 and -286 were more potent against HIV-1_{LAI} than three representative FDA-approved PIs (APV, LPV, and TPV), although the potencies of these two compounds were comparable to the potency of DRV, whose EC_{50} was 0.003 ± 0.0004 μM. The cytotoxicity of GRL-216 and -286 was seen only at high concentrations, with the 50% cytotoxic concentrations (CC_{50}s) being 48.8 and 33.1 μM, respectively, resulting in reasonably good selectivity indices of 24,400 and 8,280 for these two compounds, respectively. GRL-246 and -396 were also potent and had EC_{50}s of 0.022 ± 0.005 and 0.014 ± 0.001 μM, respectively, and selectivity indices of 1,510 and 3,200, respectively. Thus, in a further study, we primarily examined the pharmacological and virological profiles of the two most potent compounds with greater selectivity indices, GRL-216 and -286.

Activities of GRL-216 and GRL-286 against HIV-2 strains *in vitro*. The activities of GRL-216 and GRL-286 against two HIV-2 strains, HIV-2_{EHO} and HIV-2_{ROD}, were determined using the MTT assay and MT-2 cells as the target cells, as described previously (1). While the activity of APV against HIV-2_{EHO} and HIV-2_{ROD} was slightly less potent than that of APV against HIV-1_{LAI} (EC_{50}s for HIV-2_{EHO}, HIV-2_{ROD}, and HIV-1_{LAI}, 0.12 μM [3.53-fold less potent], 0.42 μM [12.35-fold less potent], and 0.034 μM, respectively (Table 1), three other PIs (LPV, TPV, and DRV) were comparably potent against both HIV-2 strains and HIV-1_{LAI}. GRL-216 was relatively less potent against HIV-2_{EHO} and HIV-2_{ROD} than against HIV-1_{LAI} (EC_{50}s for HIV-2_{EHO}, HIV-2_{ROD}, and HIV-1_{LAI}, 0.010 μM [5-fold less potent], 0.017 μM [8.5-fold less potent], and 0.002 μM, respectively) (Table 1). Similarly, GRL-286 exerted relatively less activity against the two HIV-2 strains (Table 1) than against HIV-1_{LAI}.

GRL-216 and GRL-286 are potent against PI-selected laboratory HIV-1 variants. We also examined whether GRL-216 and GRL-286 were active against a variety of HIV-1 variants that had been selected *in vitro* with each of five FDA-approved PIs (SQV, NFV, APV, LPV and AZV) (Table 2). These HIV-1 variants were obtained by propagating a wild-type laboratory HIV-1 strain, HIV-1_{NL4-3}, in the presence of increasing concentrations of each PI in MT-4 cells over 37 to 60 passages *in vitro*, and the variants thus obtained were confirmed to have acquired various PI resistance-associated amino acid substitutions in the protease-encoding region of the viral genome (see footnote *a* of Table 2). Each of the variants (HIV-$1_{SQV\text{-}5\ \mu M}$, HIV-$1_{NFV\text{-}5\ \mu M}$, HIV-$1_{APV\text{-}5\ \mu M}$, HIV-$1_{LPV\text{-}5\ \mu M}$, and HIV-$1_{AZV\text{-}5\ \mu M}$ [where the subscripts indicate the drug-drug concentration with which the variant was selected]) (22) was highly resistant to the corresponding PI with which the variant was selected, with the EC_{50}s being >1 μM; and the fold differences in the EC_{50}s compared to the EC_{50} of each drug against HIV-1_{NL4-3} ranged from >22 to >250. The activities of GRL-216 against all these variants except HIV-$1_{APV\text{-}5\ \mu M}$ were well maintained, with the fold differences being 4 to 8. GRL-216 was virtually inert against HIV-$1_{APV\text{-}5\ \mu M}$, with the EC_{50} being >1 μM. GRL-286 had potent activity against HIV-$1_{SQV\text{-}5\ \mu M}$, HIV-$1_{NFV\text{-}5\ \mu M}$, and HIV-$1_{AZV\text{-}5\ \mu M}$; but the compound was less potent against HIV-$1_{LPV\text{-}5\ \mu M}$ and was virtually inert against HIV-$1_{APV\text{-}5\ \mu M}$.(24). The observation that both GRL-216 and GRL-286 were essentially inert against HIV-$1_{APV\text{-}5\ \mu M}$ was presumably due to the structural resemblance among GRL-216A, GRL-286A, and APV, all of which contain a sulfonamide isostere (Fig. 1).

GRL-216 and GRL-286 exert potent activities against highly MDR clinical HIV-1 strains. In our previous work, we isolated highly multiple-drug (PI)-resistant (MDR) clinical HIV-1 strains (HIV-1_{MDR}), including HIV-$1_{MDR/B}$, HIV-$1_{MDR/C}$, HIV-$1_{MDR/G}$, HIV-$1_{MDR/TM}$, HIV-$1_{MDR/MM}$, and HIV-$1_{MDR/JSL}$, from patients with AIDS who had failed the then-existing anti-HIV regimens after receiving 9 to 11 anti-HIV-1 drugs over 32 to 83 months (36, 37). These clinical strains contained 9 to 14 amino acid substitutions in the protease-encoding region which have reportedly been associated with HIV-1 resistance to various PIs (see footnote *a* of Table 3). The EC_{50}s of IDV and LPV for these multidrug-resistant clinical HIV-1 isolates were mostly >1 μM, and the activities of the other three PIs (SQV, APV, and AZV) were also found to be significantly compromised, determined using PHA-PBMCs as the target cells and p24 production inhibition as the end point (Table 3). Both TPV and DRV well maintained their activities, and the fold differences between their EC_{50}s against HIV-$1_{ERS104pre}$ (wild-type isolate) and their EC_{50}s against multidrug-resistant clinical isolates ranged from 1 to 9, while it was noteworthy that the highest EC_{50} of DRV was much lower (0.027 μM) than that of TPV (0.38 μM). GRL-216 and -286 exerted potent activities against HIV-$1_{ERS104pre}$, with the EC_{50}s being as low as 0.005 and 0.007 μM, respectively (Table 3). The potencies of GRL-216 and -286 against five PI-resistant variants (HIV-$1_{MDR/B}$, HIV-$1_{MDR/C}$, HIV-$1_{MDR/G}$, HIV-$1_{MDR/TM}$, and HIV-$1_{MDR/MM}$) were well maintained, with the fold differences in the EC_{50}s compared to their EC_{50}s against HIV-$1_{ERS104pre}$ ranging from 4 to 13. However, both GRL-216 and -286 were less potent against HIV-$1_{MDR/JSL}$, with the

TABLE 2. Activities of GRL-216 and GRL-286 against laboratory PI-resistant HIV-1 variants[a]

Virus	EC_{50} (μM)								
	SQV	NFV	APV	LPV	AZV	TPV	DRV	GRL-216	GRL-286
HIV-1_{NL4-3}	0.005 ± 0.002	0.045 ± 0.032	0.034 ± 0.001	0.046 ± 0.025	0.004 ± 0.0004	0.32 ± 0.07	0.003 ± 0.0006	0.005 ± 0.001	0.009 ± 0.0006
HIV-$1_{SQV\text{-}5\ \mu M}$	>1 (>200)	0.51 ± 0.081 (11)	0.18 ± 0.10 (5)	0.12 ± 0.02 (2)	0.21 ± 0.065 (53)	0.022 ± 0.009 (0.1)	0.003 ± 0.0002 (1)	0.020 ± 0.009 (4)	0.046 ± 0.021 (5)
HIV-$1_{NFV\text{-}5\ \mu M}$	0.003 ± 0.002 (1)	>1 (>22)	0.028 ± 0.005 (1)	0.037 ± 0.004 (1)	0.027 ± 0.005(7)	0.039 ± 0.041 (0.1)	0.006 ± 0.0002 (2)	0.040 ± 0.017 (8)	0.041 ± 0.006 (5)
HIV-$1_{APV\text{-}5\ \mu M}$	0.026 ± 0.010 (5)	0.30 ± 0.03 (7)	>1 (>29)	>1 (>22)	0.006 ± 0.002 (2)	0.22 ± 0.089 (0.7)	0.29 ± 0.0018 (97)	>1 (>200)	>1 (>111)
HIV-$1_{LPV\text{-}5\ \mu M}$	0.028 ± 0.007 (6)	0.32 ± 0.12 (7)	0.26 ± 0.07 (8)	>1 (>22)	0.036 ± 0.001 (9)	0.31 ± 0.030 (1)	0.025 ± 0.0066 (8)	0.035 ± 0.005 (7)	0.35 ± 0.019 (39)
HIV-$1_{AZV\text{-}5\ \mu M}$	0.031 ± 0.005 (6)	>1 (>22)	0.28 ± 0.06 (8)	>1 (>22)	>1 (>250)	0.41 ± 0.047 (1)	0.009 ± 0.0025 (3)	0.023 ± 0.005 (5)	0.030 ± 0.006 (3)

[a] The amino acid substitutions identified in the protease-encoding regions of HIV-$1_{SQV\text{-}5\ \mu M}$, HIV-$1_{NFV\text{-}5\ \mu M}$, HIV-$1_{APV\text{-}5\ \mu M}$, HIV-$1_{LPV\text{-}5\ \mu M}$, and HIV-$1_{AZV\text{-}5\ \mu M}$ compared to the consensus type B sequence cited from the Los Alamos database include L10I/G48V/I54V/A71V/I84V/L90 M, L10F/D30N/K45I/A71V/T74S, L10F/M46I/I50V/A71V/I84V/L90 M, L10F/M46I/I54V/V82A, and L23I/E34Q/K43N/M46I/I50L/G51A/L63P/A71V/V82A/T91A, respectively. MT-4 cells (10^4) were exposed to 100 $TCID_{50}$s of each HIV-1 isolate, and inhibition of p24 Gag protein production by each drug was used as the end point. Numbers in parentheses represent the *n*-fold changes in the EC_{50}s for each isolate compared to the EC_{50}s for wild-type isolate HIV-1_{NL4-3}. All assays were conducted in duplicate or triplicate, and the data shown represent mean values (±1 standard deviation) derived from the results of three independent experiments.

TABLE 3. Activities of GRL-216 and GRL-286 against multi-drug-resistant clinical isolates in PHA-PBMs[a]

Virus (syncytium formation)	EC_{50} (μM)								
	SQV	IDV	APV	LPV	AZV	TPV	DRV	GRL-216	GRL-286
HIV-$1_{ERS104\ pre}$ (wild type; SI)	0.008 ± 0.005	0.043 ± 0.004	0.030 ± 0.005	0.034 ± 0.002	0.002 ± 0.001	0.12 ± 0.03	0.003 ± 0.0002	0.005 ± 0.003	0.007 ± 0.002
HIV-$1_{MDR/B}$ (SI)	0.27 ± 0.073 (34)	>1 (>23)	>1 (>33)	>1 (>29)	0.20 ± 0.10 (100)	0.18 ± 0.009 (2)	0.019 ± 0.012 (6)	0.037 ± 0.016 (7)	0.089 ± 0.016 (13)
HIV-$1_{MDR/C}$ (SI)	0.032 ± 0.002 (11)	>1 (>23)	0.37 ± 0.011 (12)	>1 (>29)	0.065 ± 0.008 (33)	0.38 ± 0.079 (3)	0.008 ± 0.006 (3)	0.044 ± 0.002 (9)	0.029 ± 0.001 (4)
HIV-$1_{MDR/G}$ (SI)	0.030 ± 0.002 (4)	0.34 ± 0.14 (5)	0.43 ± 0.004 (14)	0.26 ± 0.04 (8)	0.033 ± 0.024 (17)	0.24 ± 0.08 (2)	0.023 ± 0.006 (5)	0.057 ± 0.012 (11)	0.028 ± 0.004 (4)
HIV-$1_{MDR/TM}$ (SI)	0.26 ± 0.04 (33)	>1 (>23)	0.32 ± 0.007 (11)	>1 (>29)	0.065 ± 0.008 (33)	0.38 ± 0.05 (3)	0.004 ± 0.001 (1)	0.027 ± 0.001 (6)	0.072 ± 0.014 (10)
HIV-$1_{MDR/MM}$ (NSI)	0.19 ± 0.05 (24)	>1 (>23)	0.21 ± 0.222 (7)	>1 (>29)	0.18 ± 0.021 (89)	0.35 ± 0.06 (3)	0.011 ± 0.002 (4)	0.033 ± 0.010 (7)	0.055 ± 0.025 (8)
HIV-$1_{MDR/JSL}$ (NSI)	0.30 ± 0.02 (37)	>1 (>23)	0.62 ± 0.02 (21)	>1 (>29)	0.43 ± 00.036 (215)	0.23 ± 0.049 (2)	0.027 ± 0.011 (9)	0.073 ± 0.07 (15)	0.21 ± 0.032 (30)

[a] The amino acid substitutions identified in the protease-encoding region compared to the consensus type B sequence cited from the Los Alamos database include L63P in HIV-$1_{ERS104pre}$; L10I, K14R, L33I, M36I, M46I, F53I, K55R, I62V, L63P, A71V, G73S, V82A, L90M, and I93L in HIV-$1_{MDR/B}$; L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, and L89M in HIV-$1_{MDR/C}$; L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, and L90 M in HIV-$1_{MDR/G}$; L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90M, and I93L in HIV-$1_{MDR/TM}$; L10I, K43T, M46L, I54V, L63P, A71V, V82A, L90M, and Q92K in HIV-$1_{MDR/MM}$; and L10I, L24I, I33F, E35D, M36I, N37S, M46L, I54V, R57K, I62V, L63P, A71V, G73S, and V82A in HIV-$1_{MDR/JSL}$. HIV-$1_{ERS104pre}$ served as a source of wild-type HIV-1. EC_{50}s were determined by using PHA-PBMCs as target cells, and inhibition of p24 Gag protein production by each drug was used as the end point. Numbers in parentheses represent the *n*-fold changes in the EC_{50}s for each isolate compared to the EC_{50}s for wild-type isolate HIV-$1_{ERS104pre}$. All assays were conducted in duplicate or triplicate, and the data shown represent the mean values (±1 standard deviation) derived from results of three independent experiments.

differences in the EC_{50}s being 15- and 30-fold, respectively. It was noted that HIV-$1_{MDR/JSL}$ was the most resistant to all other PIs examined except TPV and DRV (Table 3).

GRL-216 and GRL-286 block the dimerization of HIV-1 protease. Since the structurally related *bis*-THF-containing PI DRV effectively blocked the dimerization of HIV-1 protease, determined with the FRET-based HIV-1 expression system as described previously (23), we asked whether GRL-216 and GRL-286 had such protease dimerization-inhibitory activity. In the FRET-based HIV-1 expression system, COS7 cells were transfected with pHIV-PR_{WT}^{CFP} and pHIV-PR_{WT}^{YFP} and exposed to various concentrations of either of the drugs, and the $CFP^{A/B}$ ratios were determined at the end of 72 h of culture. In the absence of drug, virtually all the $CFP^{A/B}$ ratios were above 1.0, with the average figures being 1.29 and 1.13 for GRL-216 and GRL-286, respectively (Fig. 2), indicating that protease dimerization occurred in the system. However, when the transfected COS7 cells were exposed to GRL-216 at a concentration greater than 0.1 μM, all the average $CFP^{A/B}$ ratios were less than 1.0, indicating that GRL-216 effectively blocked HIV-1 protease dimerization (Fig. 2). GRL-286 also effectively blocked dimerization at the same concentration range.

***In vitro* selection of HIV-1 variants resistant to four mcPIs.** We attempted to select out HIV-1 variants resistant to GRL-216, GRL-246, GRL-286, and GRL-396 by propagating HIV-1_{NL4-3} in MT-4 cells in the presence of increasing concentrations of each PI, as described previously (1). We also selected HIV-1 variants in the presence of increasing concentrations of APV. As shown in Fig. 3, HIV-1 variants that replicated in the presence of 1 μM APV, GRL-396, GRL-246, and GRL-286 emerged by passages 20, 33, 33, and 37, respectively; however, the virus exposed to GRL-216 continued to be fairly susceptible to GRL-216 even after 50 passages. Beyond approximately 50 passages with GRL-216 exposure, the virus replicated extremely poorly and virtually failed to replicate after exposure to GRL-216 at >0.26 μM, demonstrating that the emergence of a GRL-216-resistant HIV-1 variant is substantially delayed compared to the time to the emergence of resistance to APV and the other mcPIs examined here.

Determination of the nucleotide sequence of the protease-encoding region disclosed that the variants resistant to APV (5 μM, passage 20) had acquired previously reported mutations, such as L10I, V32I, M46I, and I84V (Fig. 4). By passage 5 with GRL-216 exposure, the wild-type protease-encoding sequence in the virus was seen. However, by passage 10 and beyond, the virus was seen to contain the V82I substitution. As the passage proceeded, more amino acid substitutions were acquired. By passage 30, the virus had acquired L10I and I84V substitutions. By passage 50 (P50) with 0.16 μM GRL-216 ($HIV_{216\text{-}P50}$), the virus had further acquired L24I and M46I substitutions. By passage 60, the virus had gained L63P substitutions as well.

HIV-1 exposed to GRL-246 (1 μM) had acquired L10F, M46I, and T91S in the protease-encoding region by passage 33. By passage 37, HIV-1 exposed to GRL-286 (1 μM) had acquired L10F, M46L, I50V, and A71V in the protease-encoding region. By passage 33, the HIV-1 isolate selected for resistance to GRL-396 (1 μM) had acquired L10F, M46I, Q61K, V82I, and I84V in the protease-encoding region. We also examined whether the virus acquired mutations in the Gag region after