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

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

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

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

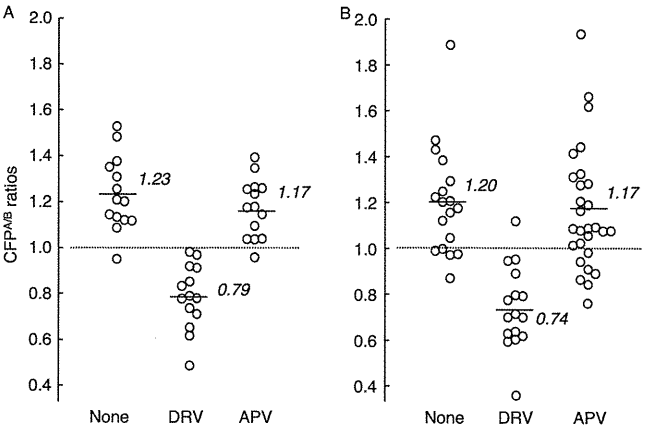

FIG. 2. DRV blocks the dimerization of both pHIV-PR_{WT}-encoded PR and pPR_{WT}-encoded PR. (A) COS7 cells were cotransfected with pHIV-PR_{WT}^{CFP} plus pHIV-PR_{WT}^{YFP} in the absence or presence of 1 μM DRV or APV. On day 3 after transfection, $CFP^{A/B}$ ratios were determined using an FV500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

RESULTS

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

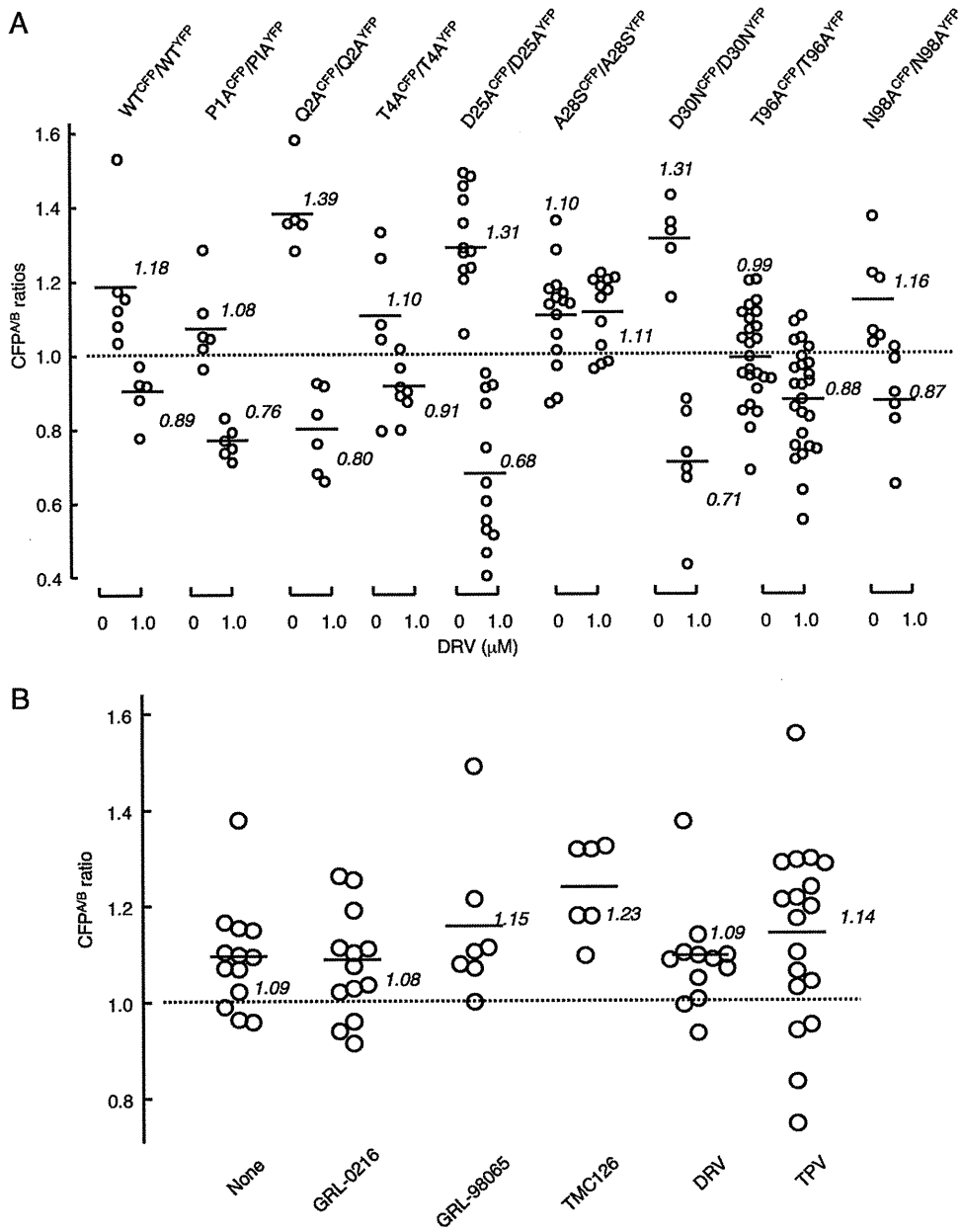

FIG. 3. Dimerization profiles of single PR mutants in the presence of DRV. (A) COS-7 cells were cotransfected with pHIV-PR_{WT}^{CFP} plus pHIV-PR_{WT}^{YFP} (shown as WT^{CFP}/WT^{YFP}) or mutated pairs such as pHIV-PR_{P1A}^{CFP} plus pHIV-PR_{P1A}^{YFP} (shown as $P1A^{CFP}/P1A^{YFP}$) in the absence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

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

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

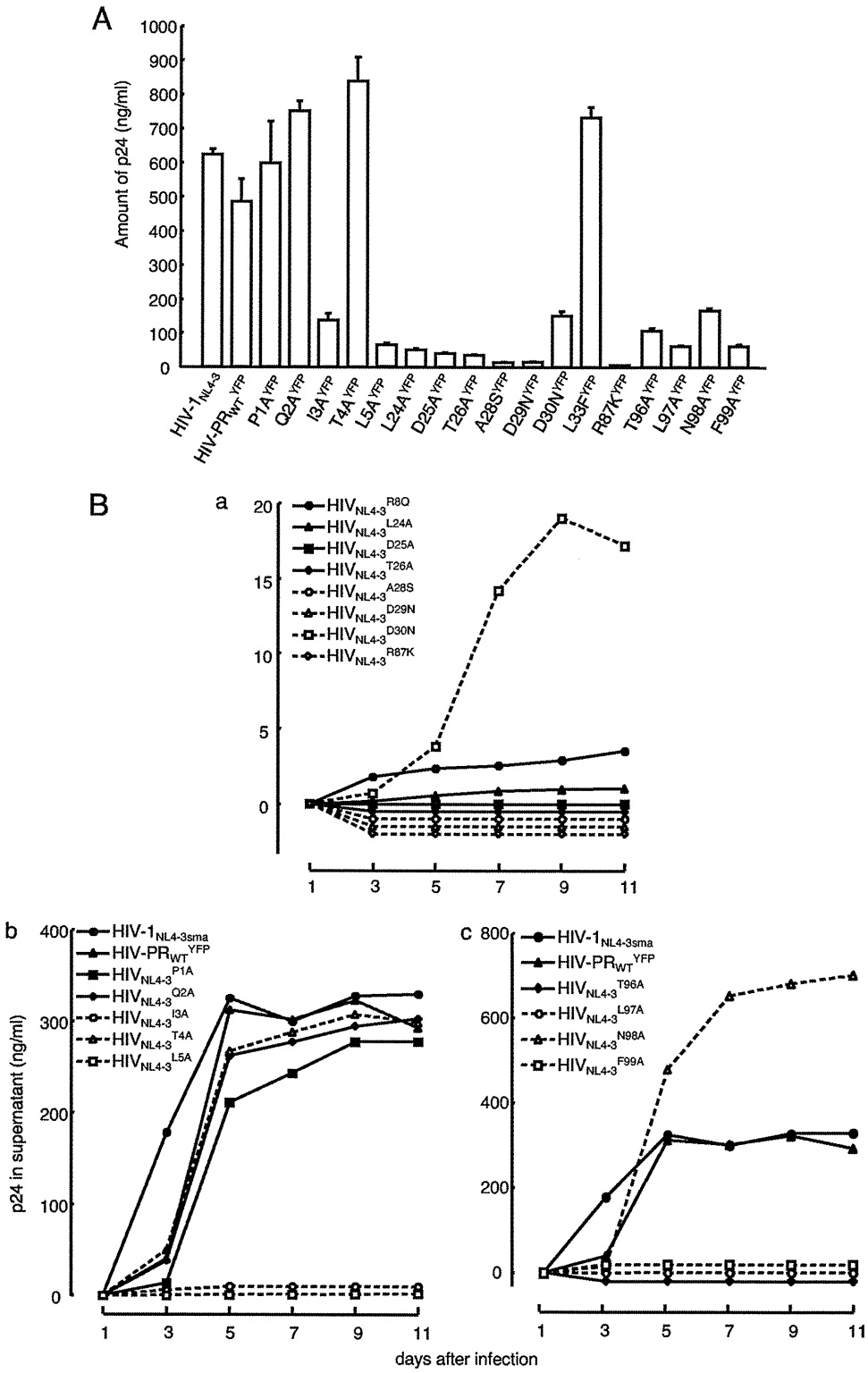

FIG. 4. Replication kinetics of HIV-PR^{YFP} with wild-type or mutated PR. (A) 293T cells were transfected with pHIV-PR$_{WT}^{YFP}$ or mutated pHIV-PR^{YFP} (if pHIV-PR$_{P1A}^{YFP}$ was used, it is shown as P1A^{YFP}), and the amounts of p24 Gag in the culture supernatants were determined 48 h after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

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

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

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

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

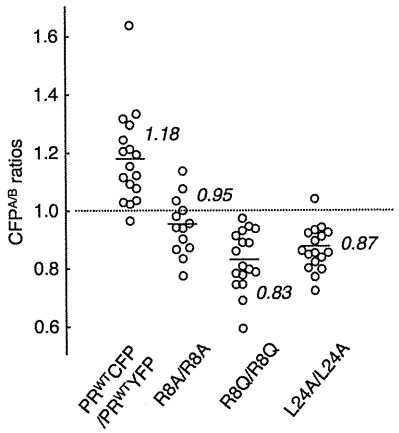

FIG. 5. Dimerization inhibition profiles of selected HIV-1 PR mutants. COS7 cells were cotransfected with a pair of HIV-PR^{CFP} and HIV-PR^{YFP} strains either wild type or carrying single amino acid (AA) substitutions, such as the R8A, R8Q, or L24A, in the absence of drug. The $CFP^{A/B}$ ratios were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

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

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

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

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

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

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

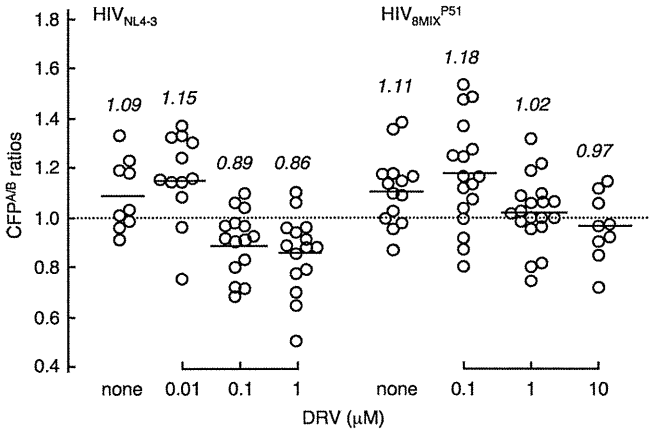

FIG. 6. DRV fails to inhibit the dimerization of the protease of a highly DRV-resistant HIV_{8MIX}^{P51} variant. COS7 cells were transfected with a pair of plasmids encoding a full-length molecular infectious HIV-1 clone (HIV_{8MIX}^{P51}) containing CFP- or YFP-tagged PR with 14 amino acid substitutions (L10I, I15V, K20R, L24I, V32I, L33F, M36I, M46L, I54M, L63P, K70Q, V82I, I84V, and L89M) in the presence or absence of 0.1, 1, or 10 μM DRV. On day 3 after transfection, $CFP^{A/B}$ ratios were determined as described in the legend to Fig. 2. HIV_{NL4-3} served as a reference. Note that 0.1 and 1 μM DRV failed to block the dimerization of the protease of HIV_{8MIX}^{P51}, while the same concentration of DRV blocked protease dimerization in HIV_{NL4-3}. The differences between the $CFP^{A/B}$ ratios in the absence of drug ($CFP^{A/B_{No\ Drug}}$) and the $CFP^{A/B}$ ratios in the presence of 0.01 μM DRV ($CFP^{A/B_{0.01\ DRV}}$), between the $CFP^{A/B}$ ratios in the presence of 0.01 μM DRV ($CFP^{A/B_{0.01\ DRV}}$) and 0.1 μM DRV ($CFP^{A/B_{0.1\ DRV}}$), and between the $CFP^{A/B}$ ratios in the presence of 0.1 μM DRV ($CFP^{A/B_{0.1\ DRV}}$) and 1.0 μM DRV ($CFP^{A/B_{1.0\ DRV}}$) had P values of 0.32, 0.0025, and 0.34 for HIV_{NL4-3}, respectively. The differences between the $CFP^{A/B_{No\ Drug}}$ and the $CFP^{A/B_{0.1\ DRV}}$, between the $CFP^{A/B_{0.1\ DRV}}$ and $CFP^{A/B_{1.0\ DRV}}$, and between the $CFP^{A/B_{1.0\ DRV}}$ and the $CFP^{A/B_{10.0\ DRV}}$ had P values of 0.42, 0.022, and 0.26, respectively, for HIV_{8MIX}^{P51}.

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

TABLE 2. Amino acid substitutions associated with DRV resistance

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

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

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

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

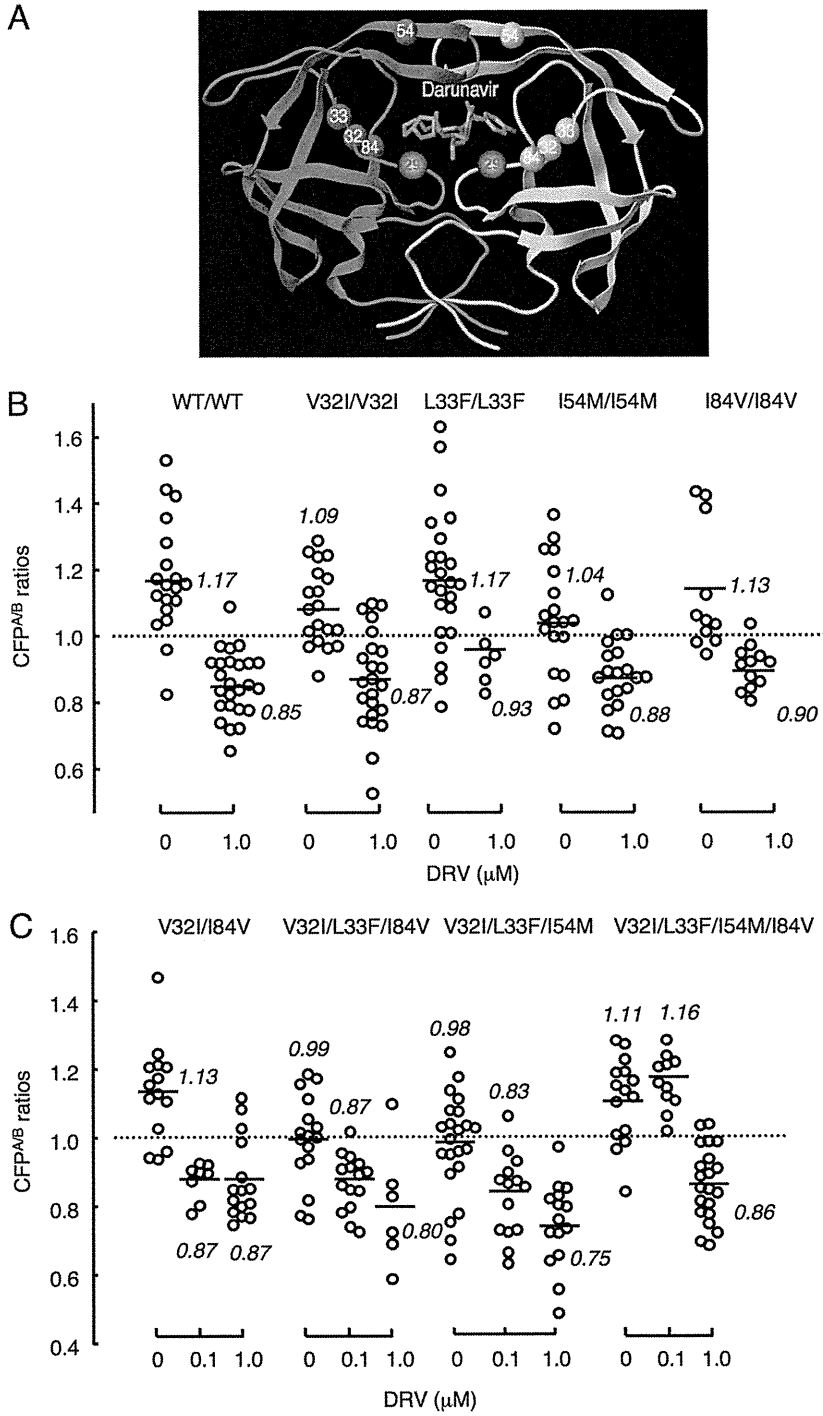

FIG. 7. Amino acid changes conferring DRV resistance on HIV. (A) Locations of amino acid substitutions V32I, L33A/F, I54M, and I84V associated with HIV's DRV resistance. The location of Asp29 (D29), which is known to be an essential amino acid for dimerization, is also shown. (B) Profiles of DRV's dimerization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

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

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

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

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

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

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

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

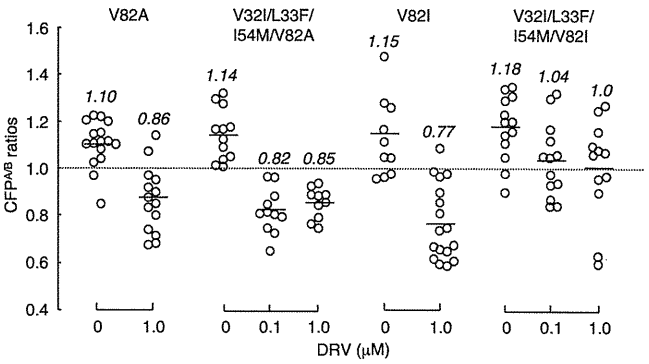

FIG. 8. Effects of V82A and V82I substitutions on DRV's activity to inhibit PR dimerization. COS7 cells were cotransfected with the $pHIV\text{-}PR^{CFP}$ and $pHIV\text{-}PR^{YFP}$ pair of plasmids carrying a single V82A or V82I substitution or four combined mutations (V32I, L33F, and I54M plus V82A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

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

DISCUSSION

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

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

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

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

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

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

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

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Novel HIV-1 Protease Inhibitors (PIs) Containing a Bicyclic P2 Functional Moiety, Tetrahydropyrano-Tetrahydrofuran, That Are Potent against Multi-PI-Resistant HIV-1 Variants▽†

Kazuhiko Ide,[1] Manabu Aoki,[1,2] Masayuki Amano,[1] Yasuhiro Koh,[1] Ravikiran S. Yedidi,[3] Debananda Das,[3] Sofiya Leschenko,[4] Bruno Chapsal,[4] Arun K. Ghosh,[4] and Hiroaki Mitsuya[1,3]*

Departments of Hematology, Rheumatology, and Infectious Diseases, Kumamoto University Graduate School of Medicine, Kumamoto 860-8556, Japan[1]; Department of Medical Technology, Kumamoto Health Science University, Kumamoto 861-5598, Japan[2]; Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892[3]; and Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, Indiana 47907[4]

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We identified GRL-1388 and -1398, potent nonpeptidic human immunodeficiency virus type 1 (HIV-1) protease inhibitors (PIs) containing a bicyclic P2 functional moiety, tetrahydropyrano-tetrahydrofuran (Tp-THF). GRL-1388 was as potent as darunavir (DRV) against various drug-resistant HIV-1 laboratory strains with 50% effective concentration (EC_{50}s) of 2.6 to 32.6 nM. GRL-1398 was significantly more potent against such variants than DRV with EC_{50}s of 0.1 to 5.7 nM. GRL-1388 and -1398 were also potent against multiple-PI-resistant clinical HIV-1 variants ($_{CL}$HIV-1_{MDR}) with EC_{50}s ranging from 2.7 to 21.3 nM and from 0.3 to 4.8 nM, respectively. A highly DRV-resistant HIV-1 variant selected *in vitro* remained susceptible to GRL-1398 with the EC_{50} of 21.9 nM, while the EC_{50} of DRV was 214.1 nM. When HIV-1_{NL4-3} was selected with GRL-1398, four amino acid substitutions—leucine to phenylalanine at a position 10 (L10F), A28S, L33F, and M46I—emerged, ultimately enabling the virus to replicate in the presence of >1.0 μM the compound beyond 57 weeks of selection. When a mixture of 10 different $_{CL}$HIV-1_{MDR} strains was selected, the emergence of resistant variants was more substantially delayed with GRL-1398 than with GRL-1388 and DRV. Modeling analyses revealed that GRL-1398 had greater overall hydrogen bonding and hydrophobic interactions than GRL-1388 and DRV and that GRL-1388 and -1398 had hydrogen bonding interactions with the main chain of the active-site amino acids (Asp29 and Asp30) of protease. The present findings warrant that GRL-1398 be further developed as a potential drug for treating individuals with HIV-1 infection.

Currently available combination therapy or highly active antiretroviral therapy (HAART) has been shown to suppress the replication of human immunodeficiency virus type 1 (HIV-1) and significantly extend the life expectancy of HIV-1-infected individuals (20, 33). However, the ability to provide effective long-term antiretroviral therapy for HIV-1 infection remains a complex issue since the reverse transcriptase of HIV-1 is error-prone, and the replication rate of the virus is enormously rapid, thus enabling HIV-1 to eventually develop resistance to virtually any existing antiretroviral agents. Therefore, continuous efforts are required to develop more potent and safer therapeutics of a different structure(s), mechanism(s), and/or class(es) with a high genetic barrier against HIV-1's acquisition of drug resistance.

HIV-1 protease inhibitors (PIs) are one of the most often used classes of antiretroviral drugs in HAART. Darunavir (DRV), the latest addition among currently available PIs, contains a unique nonpeptidic P2 functional group, *bis*-tetrahydrofuranylurethane (*bis*-THF), exerts greatly potent activity against a wide spectrum of laboratory and clinical multidrug-resistant HIV-1 variants (HIV-1_{MDR}). We have previously reported that the high efficacy and genetic barrier of DRV should be associated with its dual antiviral activity: (i) protease enzymatic inhibition and (ii) protease dimerization inhibition (18, 19).

In the present work, we designed, synthesized, and evaluated nonpeptidic HIV-1 protease inhibitors, GRL-1388 and -1398, which contain a novel bicyclic P2 functional group, tetrahydropyrano-tetrahydrofuran (Tp-THF), instead of the *bis*-THF moiety of DRV, and show highly potent antiretroviral activity against not only wild-type HIV-1 but also a variety of multi-PI-resistant laboratory HIV-1 strains, including a highly DRV-resistant HIV-1 variant, selected against DRV *in vitro* as recently described (17), and various multidrug-resistant clinical isolates. Structural modeling analyses revealed that the Tp-THF moiety of GRL-1388 and -1398 forms effective and strong hydrogen bond interactions with the main chain atoms of the protease active site amino acids, as does the *bis*-THF moiety of DRV. In particular, the additional polar and hydrophobic interactions between GRL-1398 and HIV-1 protease should be an explanation of its greater potency compared to that of GRL-1388.

MATERIALS AND METHODS

Cells and viruses. $CD4^+$ MT-2 and MT-4 cell lines were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (PAA

* 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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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}, a clinical HIV-1 strain isolated from a treatment-naive AIDS patient (HIV-$1_{ERS104pre}$) (28), and six HIV-1 clinical isolates that were originally isolated from patients with AIDS, who had received long-term antiretroviral therapy using 9 to 11 anti-HIV-1 drugs (without DRV) over 32 to 83 months and were genotypically and phenotypically characterized as clinical multiple-PI-resistant HIV-1 variants ($_{CL}$HIV-1_{MDR}) (36, 37). Ten such $_{CL}$HIV-1_{MDR} strains were used as follows: $_{CL}$HIV-$1_{MDR/A}$, $_{CL}$HIV-$1_{MDR/B}$, $_{CL}$HIV-$1_{MDR/C}$, $_{CL}$HIV-$1_{MDR/EV}$, $_{CL}$HIV-$1_{MDR/G}$, $_{CL}$HIV-$1_{MDR/TM}$, $_{CL}$HIV-$1_{MDR/MM}$, $_{CL}$HIV-$1_{MDR/JSL}$, $_{CL}$HIV-$1_{MDR/SS}$, and $_{CL}$HIV-$1_{MDR/13-52}$.

Antiviral agents. Nonpeptidic PIs, GRL-1388 and -1398 (Fig. 1) (molecular weights of 591.7 and 606.7, respectively), both of which contain a polycyclic ligand (Tp-THF) in place of *bis*-THF of DRV, were synthesized. The method of synthesis of GRL-1388 and -1398 will be published elsewhere by A. K. Ghosh et al. 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 kindly provided by GlaxoSmithKline (Research Triangle Park, NC). Nelfinavir (NFV) and lopinavir (LPV) were kindly provided by Japan Energy, Inc., Tokyo, Japan. Atazanavir (ATV) was kindly provided by Bristol-Myers Squibb (New York, NY). Darunavir (DRV) was synthesized as previously described (16).

Drug susceptibility assay. The susceptibility of HIV-1_{LAI} to various drugs was determined as previously described (36), with minor modifications. Briefly, MT-2 cells (2×10^4/ml) were exposed to 100 50% tissue culture infective doses ($TCID_{50}$s) of HIV-1_{LAI} in the presence or absence of various concentrations of drugs in 96-well microtiter culture plates, followed by incubation at 37°C for 7 days. After 100 μl of the culture 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, 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 by using a kinetic microplate reader (Vmax; Molecular Devices, Sunnyvale, CA). All assays were performed in duplicate or triplicate. To determine the drug sensitivity of laboratory HIV-1 strains, MT-4 cells were used as target cells. In brief, MT-4 cells (10^5/ml) were exposed to 50 $TCID_{50}$s of HIV-1_{NL4-3} and PI-resistant HIV-1 strains in the presence or absence of various concentrations of drugs, the supernatants were harvested on day 7 of culture, and the amount of p24 Gag protein were determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio, Inc., Tokyo, Japan) (22). To determine the susceptibility of primary HIV-1 isolates to drugs, phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBMCs; 10^6/ml) were exposed to 50 $TCID_{50}$s of HIV-$1_{ERS104pre}$ or each $_{CL}$HIV-1_{MDR} isolate in the presence or absence of various concentrations of drugs and incubated for 7 days. Upon the conclusion of the culture, the amounts of p24 Gag protein in the supernatants were quantified. The drug concentrations that suppressed the production of p24 Gag protein by 50% (50% effective concentrations [EC_{50}s]) were determined. The p24 Gag amounts were compared to the amounts of those produced in drug-free control cell cultures. All assays were performed in duplicate or triplicate.

Generation of FRET-based HIV-1 expression system. The intermolecular fluorescence resonance energy transfer (FRET)-based HIV-1 expression assay (FRET/HIV-1 assay) using cyan and yellow fluorescent protein-tagged protease monomers (CFP and YFP, respectively) was performed as previously described (18). In brief, CFP- and YFP-tagged HIV-1 protease constructs were generated by 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 (7). 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 reverse transcriptase sites. The DNA fragments obtained were subsequently joined by using the PCR-mediated recombination reaction performed under the standard conditions for Ex *Taq* polymerase (Takara Bio, Inc., Otsu, Japan). 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} (10), generating pHIV-PR_{WT}^{CFP} and pHIV-PR_{WT}^{YFP} (where WT indicates wild type), respectively.

FRET procedure. COS7 cells plated on an EZVIEW glass-bottom culture plate (Iwaki, Tokyo, Japan) were cotransfected 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 test compound, cultured for 72 h, and analyzed under a Fluoview FV500 confocal laser scanning microscope (Olympus Optical Corp., Tokyo, Japan) at room temperature, as previously described (18). In the assay, each test compound was added to the culture, followed by cotransfection. The results were determined by measurement of the quenching of the CFP (donor) fluorescence and the increase in the YFP (acceptor) fluorescence (sensitized emission), since a part of CFP energy is transferred to YFP instead of being emitted. This phenomenon can be measured by bleaching YFP, which should result in an increase in CFP fluorescence (2, 3, 27, 29). The changes in the CFP and YFP fluorescence intensities in the images of the selected regions were examined and quantified by using the 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. When the $CFP^{A/B}$ ratios were <1, it was indicated that the association of the two subunits did not occur, being interpreted that protease dimerization was inhibited.

Generation of PI-resistant HIV-1 using HIV-1_{NL4-3} *in vitro*. In the experiments of the selection of 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 at an initial concentration of an EC_{50}. Viral replication was monitored with the determination of the amount of p24 Gag produced by MT-4 cells. The culture supernatants were harvested on day 7 or by up to day 14 when the amount of p24 Gag was >250 ng/ml 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 of the following round of culture was generally increased 2- to 3-fold. To determine whether amino acid substitutions associated with HIV-1 acquisition of drug resistance occurred, high-molecular-weight DNA obtained from the lysates of the cells was subjected to nucleotide sequencing.

Generation of highly GRL-1388- and -1398-resistant HIV-1 variants using a mixture of $_{CL}$HIV-1_{MDR} isolates *in vitro*. Ten $_{CL}$HIV-1_{MDR} strains ($_{CL}$HIV-$1_{MDR/A}$, $_{CL}$HIV-$1_{MDR/B}$, $_{CL}$HIV-$1_{MDR/C}$, $_{CL}$HIV-$1_{MDR/EV}$, $_{CL}$HIV-$1_{MDR/G}$, $_{CL}$HIV-$1_{MDR/TM}$, $_{CL}$HIV-$1_{MDR/MM}$, $_{CL}$HIV-$1_{MDR/JSL}$, $_{CL}$HIV-$1_{MDR/SS}$, and $_{CL}$HIV-$1_{MDR/13-52}$) were isolated from patients with AIDS who had failed existing anti-HIV regimens after receiving 9 to 12 anti-HIV-1 drugs, not including DRV (17, 30). These strains contained 9 to 21 amino acid substitutions in protease, which have reportedly been associated with HIV-1 resistance to various PIs. The mixture of the viruses was propagated initially in MT-4 cells and PHA-PBMCs as previously described (37). The mixture was transferred to the culture with fresh MT-4 cells on day 7, and the culture supernatant was harvested and used to infect fresh MT-4 cells to continue the selection. This cycle of cell-free transmission was repeated every 7 to 14 days, each time increasing the drug concentration by a factor of 2 or 3.

Determination of nucleotide sequences. Molecular cloning and the determination of nucleotide sequences of HIV-1 passaged in the presence of each agent were performed as previously described (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 primers used for the first-round PCR amplification of the entire Gag- and protease-encoding regions of the HIV-1 genome were LTR F1 (5′-GAT GCT ACA TAT AAG CAG CTG C-3′) and PR12 (5′-CTC GTG ACA AAT TTC TAC TAA TGC-3′). The first-round PCR mixture consisted of 1 μl of proviral DNA solution, 2.0 U of premix *Taq* (Ex Taq version; Takara Bio, Inc., Otsu, Japan), and 12.5 pmol of each of the first-round PCR primers in a total volume of 50 μl. The PCR conditions used were an initial 2-min step at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 3 min at 72°C, with a final 8 min of extension at 72°C. The first-round PCR products (1 μl) were used directly in the second round of PCR with primers LTR F2 (5′-GAG ACT CTG GTA ACT AGA GAT C-3′) and Ksma2.1 (5′-CCA TCC CGG GCT TTA ATT TTA CTG GTA C-3′) under the same PCR conditions described above. The second-round PCR products were purified with spin columns (MicroSpin S-400 HR; Amersham Biosciences Corp., Piscataway, NJ), cloned, and subjected to sequencing with a model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Determination of replication kinetics of GRL-1398-resistant HIV-1 variants and HIV-1_{NL4-3}. We determined the replication kinetics of two HIV-1 variant populations, which were selected in the presence of up to 1 μM GRL-1398. One population was selected for 53 weeks in experiment I (designated HIV-$1_{GRL1398\text{-}1\mu M^{Exp.I}}$) and the other for 57 weeks in experiment II (HIV-$1_{GRL1398\text{-}1\mu M^{Exp.II}}$) (see Fig. 4). MT-4 cells (3×10^5) were exposed to an HIV-

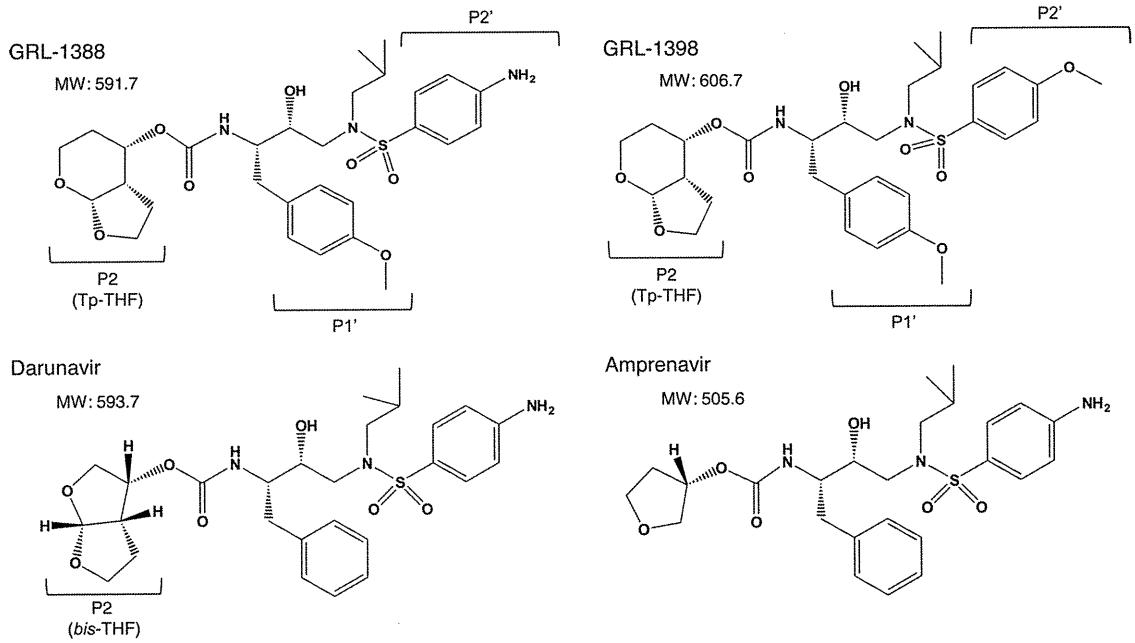

FIG. 1. Structures of GRL-1388, GRL-1398, darunavir, and amprenavir.

$1_{GRL1398\text{-}1\mu M^{Exp.I}}$, HIV-$1_{GRL1398\text{-}1\mu M^{Exp.II}}$ or wild-type HIV-$1_{NL4\text{-}3}$ preparation that contained 30 ng of p24 in six-well culture plates for 3 h, and each MT-4 cell population was divided into three fractions. Each fraction was propagated in the presence of 0, 0.1, or 1 μM GRL-1398. The amounts of p24 were measured every 2 days in culture for up to 8 or 10 days.

Modeling and analysis of GRL-1388 and -1398 interactions with wild-type HIV-1 protease. The structures of GRL-1388 and -1398 were modeled into the active site cavity of the wild-type HIV-1 protease using the extra precision Glide 5.5-ligand docking method from the Schrödinger suite of programs (8, 9, 26). Crystal structures of protease complexed with DRV (PDB code 2IEN [31]) and protease complexed with brecanavir (PDB code 2FDE [23]) obtained from the RCSB Protein Data Bank (http://www.rcsb.org) were used as template protease structures. All of the ions and glycerol-related atoms were deleted from the structures. The hydrogen atoms in both structures, 2IEN and 2FDE, were energy minimized (MacroModel, version 9.7) by using constraints on the heavy atoms of the structures. Crystallographic waters were deleted from both of the minimized structures (except the conserved water molecule that forms a tetracoordinated hydrogen bond interaction between the protease flaps and the inhibitor). These structures were then processed using the protein preparation wizard of MAESTRO (version 9.1), and the resultant structures were used to generate the receptor grid. Molecular models for GRL-1388 and -1398 were prepared by using the structure of DRV as a template. The correct stereochemistries of GRL-1388 and -1398 were assigned, and a set of diverse conformers was generated for the latter compound by using ConfGen (version 2.1). These conformers were then docked against the receptor grid. Among the docked conformers, the best binding conformations and the interactions with wild-type HIV-1 protease were analyzed. The structural model for GRL-1388 was generated by energy minimization from the optimized model of GRL-1398. Hydrogen bonds were calculated by using MAESTRO (with a 3.0-Å distance cutoff and angle constraints of 90° [donor] and 60° [acceptor], respectively), and the hydrophobic contacts of the inhibitor were obtained by probing all of the protease atoms within a radius of 4 Å using the CCP4 suite of programs (4). The programs MacroModel (version 9.7), ConfGen (version 2.1), and Glide (version 5.5) from Schrödinger LLC (New York, NY) were used in generating the final models described above. All of the graphics were prepared by using PyMOL molecular graphics program (version 0.99 DeLano Scientific LLC: http://pymol.org/).

RESULTS

Antiviral activity of GRL-1388 and -1398 against HIV-1_{LAI}. We designed and synthesized ~100 novel PIs containing the *bis*-THF component, which we reported plays a major role in the potent antiviral activity of DRV (15, 19) and its related moieties, including polycyclic ligands (11–14). Among such novel PIs, we identified GRL-1388 and -1398 (Fig. 1) as potent anti-HIV-1 agents, which contain Tp-THF. As shown in Table 1, GRL-1388 and -1398 were highly potent *in vitro* against a laboratory wild-type HIV-1 strain, HIV-1_{LAI}, with EC_{50}s of 3.6 ± 1.8 and 0.2 ± 0.2 nM, respectively, as examined in the MTT assay with $CD4^+$ MT-2 cells. Of note, the antiviral activity of GRL-1388 against HIV-1_{LAI} was comparable to or more potent than four representative U.S. Food and Drug Administration (FDA)-approved PIs: SQV, APV, ATV, and DRV. GRL-1398 was significantly more potent against HIV-1_{LAI} than these four PIs by a factor of 16.5 to 98. Both GRL-1388 and -1398 had favorable cytotoxicity profiles with 50% cytotoxicity concentrations [CC_{50}s] of more than 100 and 37.7 μM, giving selectivity index (SI) values of more than 27,800 and 188,500, respectively (Table 1).

TABLE 1. Antiviral activities of GRL-1388 and -1398 against HIV-1_{LAI} and their cytotoxicities *in vitro*[a]

Drug	Mean ± SD		Selectivity index[b]
	EC_{50} (nM)	CC_{50} (μM)	
SQV	6.2 ± 1.9	19.7 ± 6.1	3,200
APV	19.6 ± 3.9	>100	>5,100
ATV	5.0 ± 1.9	27.6 ± 0.7	5,500
DRV	3.3 ± 0.9	>100	>30,300
GRL-1388	3.6 ± 1.8	>100	>27,800
GRL-1398	0.2 ± 0.2	37.7 ± 2.7	188,500

[a] All assays were conducted in duplicate, and the data shown represent mean values (±1 standard deviation) derived from the results of three independent experiments.

[b] Each selectivity index denotes a ratio of CC_{50} to EC_{50} against HIV-1_{LAI}.

GRL-1388 and -1398 are potent against various PI-selected laboratory HIV-1 variants. We also examined whether GRL-1388 and -1398 were active against a variety of HIV-1 variants that had been selected *in vitro* with each of six FDA-approved PIs: SQV, RTV, NFV, LPV, ATV, and APV (Table 2). Each HIV-1 variant was selected *in vitro* by propagating HIV-1_{NL4-3} in the presence of increasing concentrations of each PI (up to 5 μM) in MT-4 cells and was confirmed to have acquired multiple amino acid substitutions in protease of the virus, which have reportedly been associated with viral resistance to PIs (see footnote *a* of Table 2). Each of the variants (HIV-$1_{SQV\text{-}5\mu M}$, HIV-$1_{RTV\text{-}5\mu M}$, HIV-$1_{NFV\text{-}5\mu M}$, HIV-$1_{LPV\text{-}5\mu M}$, and HIV-$1_{ATV\text{-}5\mu M}$) was highly resistant to the corresponding PI, with which the variant was selected, having the EC_{50}s of >1 μM, and the fold differences in the EC_{50}s relative to the EC_{50} of each drug against HIV-1_{NL4-3} ranged from >25 to >400 (Table 2). The activity of GRL-1388 against all five variants was well maintained, with the fold changes being 1 to 11. GRL-1398 was significantly more potent against five such HIV-1 variants than DRV with an EC_{50} of 0.1 to 5.7 nM. Of note, GRL-1388 and -1398 were moderately active against HIV-$1_{APV\text{-}5\mu M}$, with EC_{50}s of 475.7 and 49.0 nM, presumably due to the structural resemblance of GRL-1388 and -1398 to APV (Fig. 1).

GRL-1388 and -1398 exert potent activity against multiple-PI-resistant clinical HIV-1 strains ($_{CL}$HIV-1_{MDR}). We also sought to determine whether GRL-1388 and -1398 were active against $_{CL}$HIV-1_{MDR} isolates, including $_{CL}$HIV-$1_{MDR/B}$, $_{CL}$HIV-$1_{MDR/C}$, $_{CL}$HIV-$1_{MDR/G}$, $_{CL}$HIV-$1_{MDR/TM}$, $_{CL}$HIV-$1_{MDR/MM}$, and $_{CL}$HIV-$1_{MDR/JSL}$, which contained 9 to 14 PI-resistance-associated amino acid substitutions in protease (see footnote *a* of Table 3). The EC_{50}s of LPV against these multi-PI-resistant clinical HIV-1 isolates were mostly >1 μM, and the activity of other three PIs (SQV, ATV, and APV) had also been significantly compromised, as determined using PHA-PBMCs as the target cells and p24 production inhibition as the endpoint (Table 3). Both GRL-1388 and DRV remained active against all of the clinical variants examined and the fold differences between the EC_{50}s against HIV-$1_{ERS104pre}$ and those against each clinical variant ranged from as low as 1 to 7. The fold changes seen with GRL-1398 ranged from 1 to 16; however, the absolute EC_{50}s remained substantially lower, ranging from 0.3 to 4.8 nM, than those of DRV (Fig. 2).

GRL-1398 is active against DRV-resistant variants. We determined antiviral activity against DRV-resistant HIV-1 variants that had been selected *in vitro* by propagating a mixture of eight $_{CL}$HIV-1_{MDR} strains in the presence of increasing concentrations of DRV in MT-4 cells (17). Two such variants, HIV-1_{DRV^RP10} and HIV-1_{DRV^RP20}, containing a set of amino acid substitutions at passages 10 and 20, respectively (Table 4), were resistant to DRV with EC_{50}s of 29.1 and 214.1 nM and to GRL-1388 with EC_{50}s of 24.6 and 150.8 nM. In contrast, GRL-1398 had substantially lower absolute EC_{50}s—3.3 and 21.9 nM against HIV-1_{DRV^RP10} and HIV-1_{DRV^RP20}, respectively(Table 4).

GRL-1388 and -1398 moderately disrupt the dimerization of HIV-1 protease. We previously reported that DRV effectively disrupts the dimerization of HIV-1 protease monomer subunits as determined with the FRET/HIV-1 expression assay (18). We therefore sought to determine whether GRL-1388

TABLE 2. Antiviral activities of GRL-1388 and -1398 against laboratory PI-selected HIV-1variants[a]

Virus	Mean EC_{50} in nM ± SD (fold change)								
	SQV	RTV	NFV	LPV	ATV	APV	DRV	GRL-1388	GRL-1398
HIV-1_{NL4-3}	5.2 ± 1.7	40.4 ± 4.9	30.2 ± 3.0	27.8 ± 5.3	2.5 ± 0.8	34.3 ± 1.3	3.2 ± 0.3	3.1 ± 0.2	0.2 ± 0.1
HIV-$1_{SQV\text{-}5\mu M}$	>1,000 (192)	>1,000 (>25)	>1,000 (>33)	>1,000 (>36)	415.0 ± 15.1 (166)	353.7 ± 12.4 (10)	30.4 ± 0.5 (10)	32.6 ± 7.5 (11)	5.7 ± 1.3 (29)
HIV-$1_{RTV\text{-}5\mu M}$	66.3 ± 8.9 (13)	>1,000 (>25)	386.4 ± 84.7 (13)	698.3 ± 87.4 (25)	32.5 ± 5.9 (13)	334.5 ± 42.5 (10)	19.9 ± 13.5 (6)	28.8 ± 1.8 (9)	3.1 ± 0.2 (16)
HIV-$1_{NFV\text{-}5\mu M}$	24.1 ± 6.3 (5)	46.5 ± 9.4 (1)	>1,000 (>33)	37.2 ± 3.3 (1)	12.3 ± 2.2 (5)	71.3 ± 10.5 (2)	2.7 ± 0.4 (1)	2.6 ± 0.4 (1)	0.1 ± 0.1 (1)
HIV-$1_{LPV\text{-}5\mu M}$	33.5 ± 0.9 (6)	>1,000 (>25)	401.6 ± 11.0 (13)	>1,000 (>36)	17.0 ± 7.7 (7)	320.7 ± 23.2 (9)	4.0 ± 0.9 (1)	29.9 ± 12.5 (10)	3.5 ± 0.4 (18)
HIV-$1_{ATV\text{-}5\mu M}$	134.3 ± 22.8 (26)	>1,000 (>25)	>1,000 (>33)	>1,000 (>36)	>1,000 (>400)	521.0 ± 124.1 (15)	6.8 ± 0.6 (2)	21.9 ± 7.7 (7)	3.1 ± 0.4 (16)
HIV-$1_{APV\text{-}5\mu M}$	47.3 ± 2.1 (9)	>1,000 (>25)	>1,000 (>33)	633.2 ± 21.0 (23)	453.6 ± 12.2 (181)	>1,000 (>29)	423.9 ± 6.0 (132)	475.7 ± 38.1 (153)	49.0 ± 1.1 (245)

[a] The amino acid substitutions identified in protease of HIV-$1_{SQV\text{-}5\mu M}$, HIV-$1_{RTV\text{-}5\mu M}$, HIV-$1_{NFV\text{-}5\mu M}$, HIV-$1_{LPV\text{-}5\mu M}$, HIV-$1_{ATV\text{-}5\mu M}$, and HIV-$1_{APV\text{-}5\mu M}$ compared to the consensus type B sequence cited from the Los Alamos database include L10I/N37D/G48V/I54V/L63P/G73C/I84V/L90M, L10I/M46L/I54V/V82A, L10F/K20T/D30N/K45I/A71V/V77I, L10F/M46I/I54V/V82A, L23I/E34Q/K43I/M46I/I50L/G51A/L63P/A71V/V82A/T91A, and L10F/V32I/L33F/M46L/I54M/A71V, respectively. Numbers in parentheses represent fold changes in EC_{50}s for each isolate compared to the EC_{50}s for wild-type 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. Antiviral activity of GRL-1388 and -1398 against multidrug-resistant clinical isolates in PHA-PBMCs[a]

Virus (syncytium formation)	Mean EC_{50} in nM ± SD (fold change)						
	SQV	LPV	ATV	APV	DRV	GRL-1388	GRL-1398
$_{CL}$HIV-$1_{ERS104pre}$ (SI)	4.4 ± 1.8	41.0 ± 6.0	1.9 ± 1.2	35.1 ± 4.3	3.5 ± 0.6	3.2 ± 0.3	0.3 ± 0.1
$_{CL}$HIV-$1_{MDR/B}$ (SI)	206.9 ± 81.2 (47)	>1,000 (>24)	228.0 ± 82.6 (120)	328.8 ± 138.7 (9)	19.4 ± 3.6 (6)	4.7 ± 3.6 (1)	4.8 ± 0.3 (16)
$_{CL}$HIV-$1_{MDR/C}$ (SI)	38.8 ± 16.2 (9)	>1,000 (>24)	25.4 ± 7.5 (13)	265.2 ± 79.8 (8)	5.2 ± 1.5 (1)	4.0 ± 1.0 (1)	1.1 ± 0.1 (4)
$_{CL}$HIV-$1_{MDR/G}$ (SI)	26.3 ± 2.4 (6)	319.3 ± 31.0 (8)	16.3 ± 4.5 (9)	260.1 ± 55.8 (7)	5.1 ± 2.3 (1)	6.2 ± 0.6 (2)	1.6 ± 1.1 (5)
$_{CL}$HIV-$1_{MDR/TM}$ (SI)	110.6 ± 48.6 (25)	658.1 ± 115.9 (16)	22.4 ± 3.8 (12)	197.7 ± 65.0 (6)	3.7 ± 1.3 (1)	2.7 ± 0.7 (1)	0.3 ± 0.1 (1)
$_{CL}$HIV-$1_{MDR/MM}$ (NSI)	190.7 ± 75.9 (43)	>1,000 (>24)	68.9 ± 27.8 (36)	402.2 ± 193.9 (11)	21.4 ± 1.7 (6)	21.3 ± 5.0 (7)	2.6 ± 1.4 (9)
$_{CL}$HIV-$1_{MDR/JSL}$ (NSI)	281.8 ± 35.7 (64)	>1,000 (>24)	347.0 ± 56.9 (183)	306.6 ± 124.4 (9)	16.0 ± 4.4 (5)	18.8 ± 9.0 (6)	3.1 ± 0.1 (10)

[a] Amino acid substitutions identified in protease compared to the consensus type B sequence cited from the Los Alamos database include L63P in $_{CL}$HIV-$1_{ERS104pre}$; L10I, K14R, L33I, M36I, M46I, F53I, K55R, I62V, L63P, A71V, G73S, V82A, L90M, and I93L in $_{CL}$HIV-$1_{MDR/B}$; L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, and L89M in $_{CL}$HIV-$1_{MDR/C}$; L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, and L90M in $_{CL}$HIV-$1_{MDR/G}$; L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90M, and I93L in $_{CL}$HIV-$1_{MDR/TM}$; L10I, K43T, M46L, I54V, L63P, A71V, V82A, L90M, and Q92K in $_{CL}$HIV-$1_{MDR/MM}$; L10I, L24I, I33F, E35D, M36I, N37S, M46L, I54V, R57K, I62V, L63P, A71V, G73S, and V82A in $_{CL}$HIV-$1_{MDR/JSL}$. $_{CL}$HIV-$1_{ERS104pre}$ served as a source of wild-type HIV-1. Numbers in parentheses represent the fold changes of EC_{50}s against each isolate compared to the EC_{50}s against wild-type $_{CL}$HIV-$1_{ERS104pre}$. All assays were conducted in duplicate or triplicate, and the data shown represent mean values (±1 standard deviation) derived from results of three independent experiments. PHA-PBMCs were derived from a single donor in each experiment.

and -1398 exerted such protease dimerization inhibition activity. In the absence of agents, the mean $CFP^{A/B}$ ratio obtained in the assay was 1.12, indicating that protease dimerization clearly occurred (Fig. 3). However, when COS7 cells were cotransfected in the presence of 1 μM GRL-1388 and -1398, protease dimerization still occurred, with the mean $CFP^{A/B}$ ratios being 1.02 and 1.10, respectively. However, the mean $CFP^{A/B}$ ratios obtained in the presence of 10 and 20 μM GRL-1388 and -1398 were all <1.0, indicating that both compounds blocked the dimerization. Of note, DRV blocked the dimerization at as low as 1.0 μM under the same conditions, suggesting that GRL-1388 and -1398 had relatively moderate activity to disrupt the dimerization of HIV-1 protease compared to DRV.

***In vitro* selection of HIV-1 variants resistant to GRL-1388 and -1398.** We attempted to select HIV-1 variants resistant to GRL-1388 and -1398 by propagating HIV-1_{NL4-3} in MT-4 cells in the presence of increasing concentrations of each compound. Considering that drug resistance-associated amino acid substitutions occur stoichiometrically at random, we conducted the selection with GRL-1398 on two different occasions. When selected in the presence of increasing concentrations of RTV and APV, the virus quickly became resistant to the drugs and started replicating in the presence of 5 μM at 18 and 26 weeks of selection (Fig. 4) and had acquired L10I/M46L/I54V/V82A and L10F/V32I/L33F/M46L/I54M/A71V, respectively (Fig. 4). In contrast, the acquisition of resistance to GRL-1398 was significantly delayed and it took 53 and 57 weeks in the selection until the virus started replicating in the presence of 1 μM in experiments I and II, respectively (Fig. 4). The virus preparations selected with GRL-1398 in experiments I and II had acquired no amino acid substitutions in protease by 20 and 16 weeks of culture; however, they acquired apparently resistance-associated substitutions by 26 and 22 weeks with L10F/M46I/I50V and L10F, respectively. The virus had acquired A28S by 28 weeks of culture, along with L10F and M46M/I in experiment II ($HIV_{1398}^{II\text{-}Wk28}$); thereafter, $HIV_{1398}^{II\text{-}Wk28}$ appeared to start developing resistance at a faster rate, although the virus in experiment I did not acquire A28S but had L10F/M46I/I50V and developed resistance. In contrast, when HIV-1_{NL4-3} was selected with GRL-1388 under the same conditions, the virus remained wild-type over 45 weeks of selection, whereas HIV-1_{NL4-3}, selected with DRV, had acquired V82I by 14 weeks. All amino acid substitutions identified during the selection are illustrated in Fig. S1 in the supplemental material.

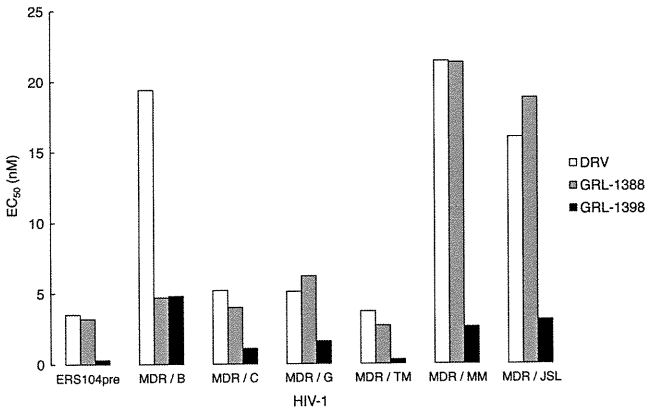

FIG. 2. Antiviral activity of GRL-1388, GRL-1398, and darunavir against multidrug-resistant clinical HIV-1 isolates. The EC_{50}s of darunavir (white), GRL-1388 (gray), and GRL-1398 (black) against $_{CL}$HIV-$1_{ERS104pre}$, which served as a wild-type HIV-1, and six $_{CL}$HIV-1_{MDR} isolates were determined as described in the legend to Table 3 and Materials and Methods. Note that the EC_{50}s of GRL-1398 against all of these isolates were substantially lower than those of DRV.

Selection with GRL-1388 and -1398 of a mixture of 10 $_{CL}$HIV-1_{MDR} variants. Considering that amino acid substitutions in HIV-1 stoichiometrically and randomly occur and the resulting amino acid substitutions can vary from one experiment to another as seen above (Fig. 4), we performed an additional selection experiment with MT4 cells and a mixture of 10 $_{CL}$HIV-1_{MDR} variants as a starting virus population with DRV, GRL-1388, and -1398. In line with our previous observations that a highly DRV-resistant HIV-1 variants emerged when selected with DRV using a mixture of eight $_{CL}$HIV-1_{MDR} variants (17), HIV-1 started replicating in the presence of 5 μM DRV and GRL-1388 by 21 and 17 weeks of selection, respectively (Fig. 5). In contrast to the results of selection experiments using MT-4 cells and HIV-1_{NL4-3}, in which HIV-1 started replicating with GRL-1398, but not with GRL-1388 or DRV (Fig. 4), the emergence of HIV-1 capable of replicating

TABLE 4. Antiviral activity of GRL-1388 and -1398 against laboratory darunavir-resistant HIV-1[a]

Virus	Amino acid substitution(s) in protease	Mean EC_{50} in nM ± SD (fold change)		
		DRV	GRL-1388	GRL-1398
$cHIV\text{-}1_{ERS104pre}$	L63P	4.0 ± 1.0	2.5 ± 0.4	0.4 ± 0.1
$HIV\text{-}1_{DRV^{R}P10}$	L10I, I15V, K20R, L24I, V32I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, L88M	29.1 ± 0.9 (7)	24.6 ± 6.9 (10)	3.3 ± 0.3 (8)
$HIV\text{-}1_{DRV^{R}P20}$	L10I, I15V, K20R, L24I, V32I, M36I, M46L, L63P, A71T, V82A, L88M	214.1 ± 47.9 (54)	150.8 ± 46.7 (60)	21.9 ± 5.7 (54)

[a] DRV-resistant HIV-1 variants were selected *in vitro* by propagating a mixture of eight $_{CL}HIV\text{-}1_{MDR}$ isolates in the presence of increasing concentrations of DRV in MT-4 cells. Six of the eight isolates were the same as those used for drug susceptibility assay (Table 3). Amino acid substitutions identified in protease of the other two isolates compared to the consensus type B sequence cited from the Los Alamos database include L10I, I15V, E35D, N37E, K45R, I54V, L63P, A71V, V82T, L90M, I93L, and C95F in $_{CL}HIV\text{-}1_{MDR/A}$ and L10R, N37D, M46I, I62V, L63P, A71V, G73S, V74I, V82T, L90M, and I93L in $_{CL}HIV\text{-}1_{MDR/SS}$. Numbers in parentheses represent the fold changes of EC_{50}s against each isolate compared to the EC_{50}s against $HIV\text{-}1_{ERS104pre}$. 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. PHA-PBMCs were derived from a single donor.

in the presence of GRL-1398 was significantly delayed compared to the cases with GRL-1388 and DRV (Fig. 5). Sequence analyses of the protease-encoding gene of the viruses in these selection experiments revealed that the HIV-1 replicating in the presence of GRL-1398 had acquired A28S by 24 weeks of selection (see Fig. S2 in the supplemental material), when the virus appeared to obtain robust replication fitness despite the presence of GRL-1398. There was no A28S substitution in those selected with GRL-1388 or DRV, as seen in the selection experiments discussed above in Fig. 4. All amino acid substitutions identified during the selection are illustrated in Fig. S2 in the supplemental material.

Effects of amino acid substitutions in the protease of GRL-1398-resistant variants on the antiviral activity of GRL-1388 and DRV. We also examined whether GRL-1398-resistant variants had cross-resistance with GRL-1388 and DRV. As described above, we selected two HIV-1 variant populations in the presence of up to 1 μM GRL-1398 (Fig. 4). One population was selected for 53 weeks in experiment I (designated as $HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.I}}$) and the other for 57 weeks in experiment II ($HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.II}}$). These two populations were resistant to GRL-1398 with EC_{50}s of 505.1 and 552.8 nM (Table 5). GRL-1388 and DRV were moderately active against $HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.I}}$, whereas both GRL-1388 and DRV essentially lost their activity against $HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.II}}$, having EC_{50}s of >1,000 nM (Table 5).

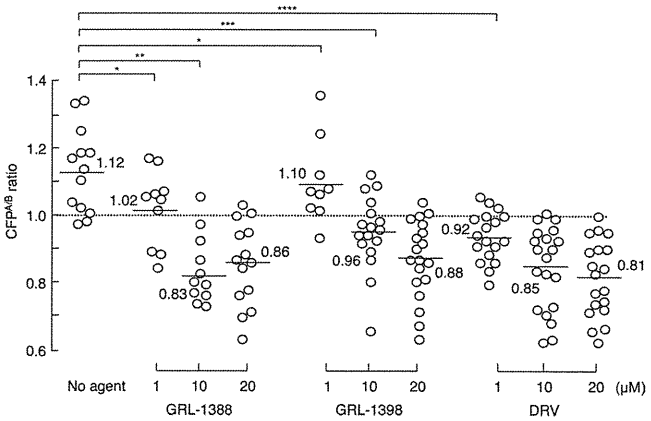

FIG. 3. Inhibition of HIV-1 protease dimerization. COS7 cells were exposed to each of the agents (GRL-1388, GRL-1398, and DRV) in various concentrations (1, 10, and 20 μM) and subsequently cotransfected with plasmids encoding full-length molecular infectious HIV-1 ($HIV_{NL4\text{-}3}$) clones producing CFP- or YFP-tagged protease. After 72 h, cultured cells were examined in the FRET-based HIV-1 expression assay and the $CFP^{A/B}$ ratios (*y* axis) were determined. The mean values of the ratios obtained are shown as horizontal bars. A $CFP^{A/B}$ ratio that is >1 signifies that protease dimerization occurred, whereas a ratio that is <1 signifies the disruption of protease dimerization. All of the experiments were conducted in a blind fashion. *, Not significant; **, $P < 0.0001$; ***, $P = 0.0005$; ****, $P = 0.0001$.

Moderately compromised replication fitness of GRL-1398-resistant HIV-1 variants. We have conducted a replication kinetics assay and determined the fitness of $HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.I}}$, $HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.II}}$, and $HIV\text{-}1_{NL4\text{-}3}$ with or without GRL-1398 (0, 0.1, 1 μM). It is of note that the resistant viruses were selected and adapted in MT-4 cells, and MT-4 cells were used in the assay. Although $HIV\text{-}1_{NL4\text{-}3}$ failed to replicate in the presence of 0.1 and 1 μM GRL-1398, both $HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.I}}$ and $HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.II}}$ replicated despite the presence of GRL-1398 (Fig. 6). It is also noteworthy that when $HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.I}}$ and $HIV\text{-}1_{GRL1398\text{-}1\mu M^{Exp.II}}$ were propagated in the presence or absence of GRL-1398, their replication activities were found to be moderately compromised compared to that of $HIV\text{-}1_{NL4\text{-}3}$ without GRL-1398 (Fig. 6).

GRL-1398 forms greater interactions with protease than GRL-1388. Analysis of the molecular complexes of GRL-1388 and -1398 with wild-type HIV-1 protease, generated by docking simulations, revealed that GRL-1398 has a greater number of hydrophobic interactions with protease compared to GRL-1388 and DRV (Fig. 7). The bicyclic structure of Tp-THF representing the P2 group in both GRL-1388 and -1398 shows hydrogen bond interactions with the backbone amide nitrogen atoms of Asp29 and Asp30, similar to that of the *bis*-THF of DRV. The oxygen atoms of the pyran and the furan rings showed two and one hydrogen bonds with the amide nitrogen atoms of Asp29 and Asp30, respectively. The transition state mimetic hydroxyl group in both compounds shows two hydrogen bonds with the catalytic Asp25, in addition, GRL-1398 shows a hydrogen bond with the catalytic Asp25′ residue of protease. The P2′ amine group in GRL-1388 has a potential to form contacts with the delta oxygen atoms on the side chains of Asp29′ and Asp30′, respectively. The oxygen atom from the O-methoxy (P2′) functional group of GRL-1398 has a potential to form hydrogen bonds with the two backbone amide nitrogen atoms of Asp29′ and Asp30′, respectively. Both GRL-1388 and -1398 form hydrogen bonds with the conserved bridg-

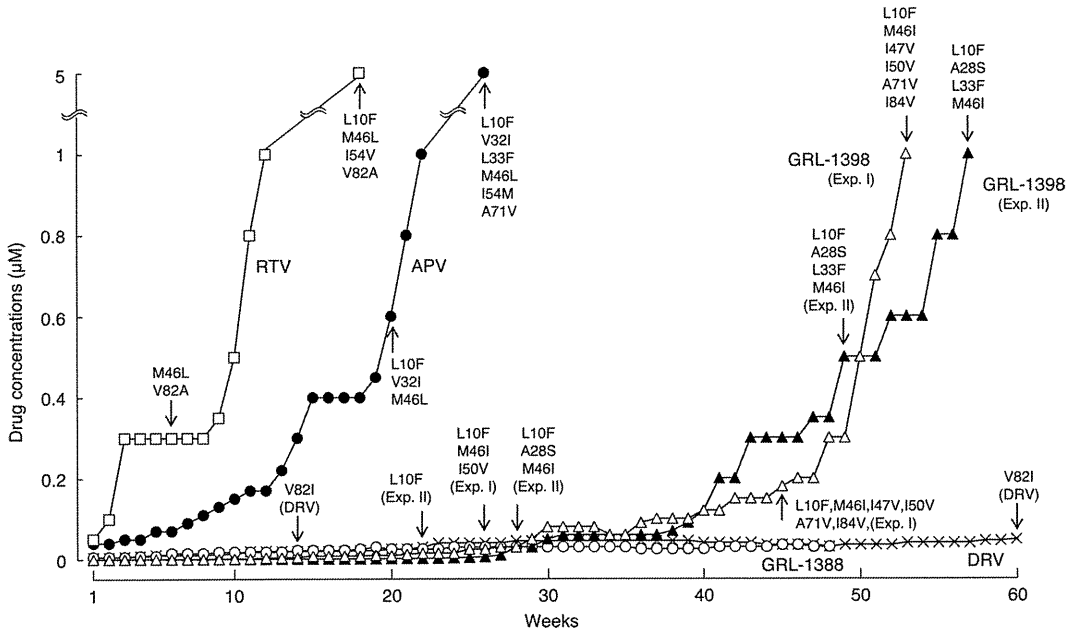

FIG. 4. *In vitro* selection of HIV-1 variants against GRL-1388 and -1398. HIV-1_{NL4-3} was propagated in MT-4 cells in the presence of increasing concentrations of ritonavir (□), amprenavir (●), darunavir (×), GRL-1388 (○), or GRL-1398 (experiment I △ and experiment II ▲). Each passage of the virus was carried out in a cell-free manner. Amino acid substitutions identified in the protease of each HIV-1 at each indicated time of the selection are shown. Note that, by week 48, no amino acid mutations were detected in the protease of GRL-1388-resistant isolate. All amino acid substitutions identified during the selection are illustrated in Fig. S1 in the supplemental material.

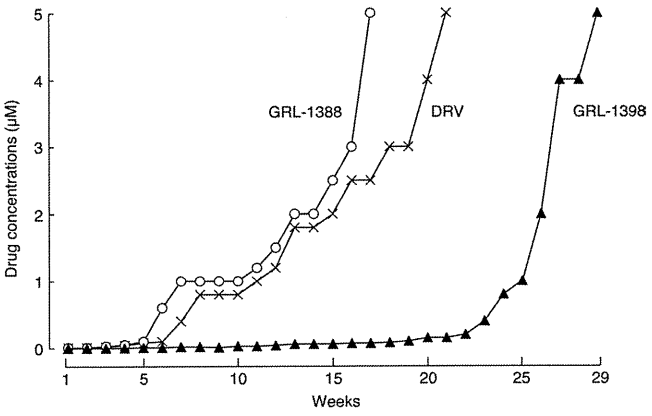

FIG. 5. *In vitro* selection multidrug-resistant clinical isolates against GRL compounds and DRV. A mixture of 10 $_{CL}$HIV-1_{MDR} variants were propagated in MT-4 cells in the presence of increasing concentrations of darunavir (×), GRL-1388 (○), or GRL-1398 (▲). Each selection was conducted in a cell-free fashion. The amino acid substitutions in protease of eight $_{CL}$HIV-1_{MDR} isolates are given in the footnotes for Tables 3 and 4. The amino acid substitutions identified in protease were L10V, T12E, G16A, L19I, K20R, L33F, E35D, M36I, N37S, M46I, I50V, F53L, I54V, K55R, R57K, D60E, I62V, L63P, A71V, V82A, and L90M in $_{CL}$HIV-$1_{MDR/EV}$ and N47D, I54V, D60E, L63P, A71V, V74I, V82A, L90M, and I93L in $_{CL}$HIV-$1_{MDR/13-52}$. HIV-1 strains, capable of replicating in the presence of each agent at 5 μM, had acquired L10V, T12E, G16A, L19I, K20R, V32I, L33F, E34K, E35D, M36I, N37S, M46I, I50V, F53L, I54V, K55R, R57K, D60E, I62V, L63P, A71V, G73S,V82A, and L90M in $HIV_{MIX-DRV}^{WK21}$; L10V, T12E, G16A, L19I, K20R, V32I, L33F, E35D, M36I, N37S, M46I, I50V, F53L, I54V, K55R, R57K, D60E, I62V, L63P, A71V, G73S, V82A, and L90M in $HIV_{MIX-1388}^{WK17}$; and L10I, I15V, A28S, L33I, M36I, M46I, I50V, F53L, K55R, I62V, L63P, A71V, G73S, L90M, and I93L in $HIV_{MIX-1398}^{WK29}$. The amino acid substitutions identified in each HIV-1 during the selection are illustrated in Fig. S2 in the supplemental material.

ing water molecule that connects to the protease flaps via hydrogen bonds with the amide nitrogen atoms of I50 and I50′. As shown in Table 6, the hydrophobic contacts were examined for both compounds and were compared to each other as well as to those of DRV. GRL-1388 has a similar interaction profile as DRV, whereas GRL-1398 has an overall greater number of interactions.

DISCUSSION

We have previously designed and synthesized a series of PIs possessing a *bis*-THF moiety that interacts with the backbone atoms of the catalytic site amino acids, Asp29 and Asp30, of HIV-1 protease (1, 19, 32). In the present study, we report two newly generated PIs, GRL-1388 and -1398 (Fig. 1), containing a polycyclic ligand, Tp-THF, in place of the *bis*-THF moiety, which displayed potent anti-HIV-1 activity against wild-type HIV-1 and a wide spectrum of laboratory and primary multi-PI-resistant HIV-1 strains with favorable cytotoxicity profiles *in vitro*. Structurally, GRL-1388 has a methoxybenzene moiety and an aminobenzene moiety at the P1′ and P2′ sites, respectively, while GRL-1398 has a methoxybenzene moiety at each of the P1′ and P2′ sites, respectively. The activity of GRL-1388 against HIV-1_{LAI} was comparable to or more potent than four representative FDA-approved PIs, SQV, APV, ATV, and DRV, whereas that of GRL-1398 was significantly more potent than these four PIs by factors of 16.5 to 98 (Table 1). It should be noted that the cytotoxicity of GRL-1398 is relatively greater, with a CC_{50} value of 37.7 μM and a SI of 188,500, compared to GRL-1388 with a CC_{50} value of >100 μM and an SI of >27,800. The mechanism of the relatively greater cytotoxicity of GRL-1398 compared to that of GRL-1388 is unknown at

TABLE 5. Antiviral activity of darunavir and GRL-1388 against GRL-1398-resistant HIV-1[a]

Virus	Amino acid substitutions in protease	Mean EC_{50} (nM) ± SD		
		DRV	GRL-1388	GRL-1398
$HIV\text{-}1_{NL4\text{-}3}$		3.1 ± 0.4	3.0 ± 0.3	0.2 ± 0.1
$HIV\text{-}1_{GRL1398\text{-}1\mu M}{}^{Exp.I}$	L10F, M46I, I47V, I50V, A71V, I84V	67.3 ± 12.0	151.9 ± 10.5	505.1 ± 168.0
$HIV\text{-}1_{GRL1398\text{-}1\mu M}{}^{Exp.II}$	L10F, A28S, L33F, M46I	>1,000	>1,000	552.8 ± 213.3

[a] GRL-1398-resistant HIV-1 was selected *in vitro* by propagating $HIV\text{-}1_{NL4\text{-}3}$ in the presence of increasing concentrations of GRL-1398 in MT-4 cells (Fig. 4). All assays to determine the EC_{50}s were conducted in duplicate or triplicate, and the data shown represent mean values (±1 standard deviation) derived from the results of three independent assays.

this time. Nevertheless, the CC_{50} values of two currently widely used FDA-approved PIs, SQV and ATV, were 19.7 and 27.6 μM, with their SI values of 3,200 and 5,500, respectively, when assessed under the same conditions together with GRL-1388 and -1398 (Table 1). Thus, even if GRL-1398 is relatively more cytotoxic than GRL-1388, one can assume that the level of toxicity of GRL-1398 is likely to be reasonably favorable, although the actual safety issue of GRL-1388 and -1398 has to be carefully examined in the setting of preclinical and clinical trials as needed.

GRL-1388 was also potently active against HIV-1 variants, which were selected to be resistant *in vitro* to each of five FDA-approved PIs, SQV, RTV, NFV, LPV, and ATV. GRL-1398 was significantly more potent against such five HIV-1variants with EC_{50}s of as low as 0.1 to 5.7 nM (Table 2). It is noteworthy that both GRL-1388 and -1398 were moderately active against $HIV\text{-}1_{APV\text{-}5\mu M}$, with EC_{50}s of 475.7 and 49.0 nM, which is explained by the fact that the two compounds have a resemblance to APV, as does DRV (Fig. 1). Moreover, GRL-1388 was also potent against all six highly multi-PI-resistant clinical HIV-1 isolates examined with the observed fold differences between EC_{50}s against a wild-type clinical isolate $HIV\text{-}1_{ERS104pre}$ and those against each clinical variant, ranging from as low as 1 to 7. The fold changes seen with GRL-1398 similarly ranged from 1 to 16; however, the absolute EC_{50}s of GRL-1398 remained substantially low, ranging from 0.3 to 4.8 nM, compared to 3.7 to 21.4 nM for DRV (Table 3 and Fig. 2). These data strongly suggest that these two new PIs could serve as good candidates for further development as potential anti-HIV-1 therapeutics, but it was noted that, of the two compounds, GRL-1398 could be more promising since it is such a potent PI against both wild-type HIV-1 and various multi-PI-resistant HIV-1 variants.

We previously demonstrated that DRV effectively disrupts the dimerization of HIV-1 protease monomer subunits, as determined with a FRET/HIV-1 expression assay (18); this might explain the reason for the highly favorable clinical efficacy and high-level genetic barrier against HIV-1 acquisition of resistance to DRV in clinical settings (6, 21, 25). However, the protease dimerization inhibition activity of both GRL-1388 and -1398 was modest compared to that of DRV, suggesting that the protease dimerization activity of GRL-1388 and -1398 does not appear to significantly contribute to the potency of the two compounds. Indeed, none or only one of the set of four amino acid substitutions (V32I, L33F, I54M, and I84V), which appears to reduce the activity of DRV to disrupt HIV-1 protease dimerization (17), was seen in the protease of HIV-1 that replicated in the presence of high concentrations of GRL-1398, although both V32I and L33F were seen in $HIV_{MIX\text{-}DRV^{WK19}}$ and $HIV_{MIX\text{-}DRV^{WK21}}$ (see Fig. S2 in the supplemental material). Of interest, when $HIV\text{-}1_{NL4\text{-}3}$ was propagated in the presence of GRL-1398, the A28S substitu-

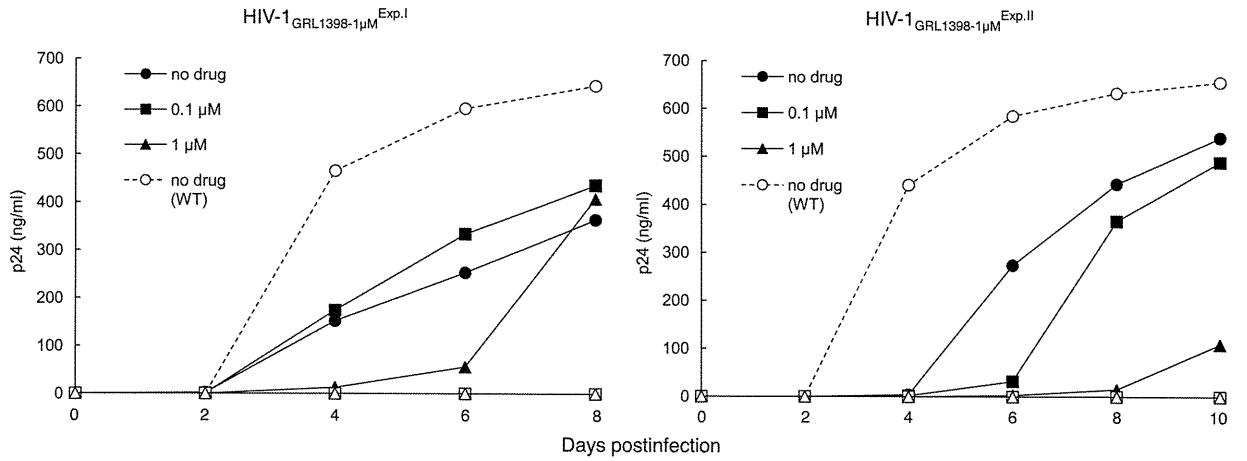

FIG. 6. Replication kinetics of GRL-1398-resistant HIV-1 variants and $HIV\text{-}1_{NL4\text{-}3}$. MT-4 cells were exposed to $HIV\text{-}1_{GRL1398\text{-}1\mu M}{}^{Exp.I}$ (left panel, closed symbols), $HIV\text{-}1_{GRL1398\text{-}1\mu M}{}^{Exp.II}$ (right panel, closed symbols), or wild-type $HIV\text{-}1_{NL4\text{-}3}$ (WT, ○) for 3 h, and each MT-4 cell population was cultured in the presence of 0.1 μM (■) or 1 μM (▲) of GRL-1398 or without the agent (●). The amounts of p24 were measured every 2 days for up to 8 or 10 days. Note that $HIV\text{-}1_{NL4\text{-}3}$ failed to replicate in the presence of 0.1 and 1 μM GRL-1398 (□ and △, respectively), both $HIV\text{-}1_{GRL1398\text{-}1\mu M}{}^{Exp.I}$ and $HIV\text{-}1_{GRL1398\text{-}1\mu M}{}^{Exp.II}$ replicated in the presence of GRL-1398.

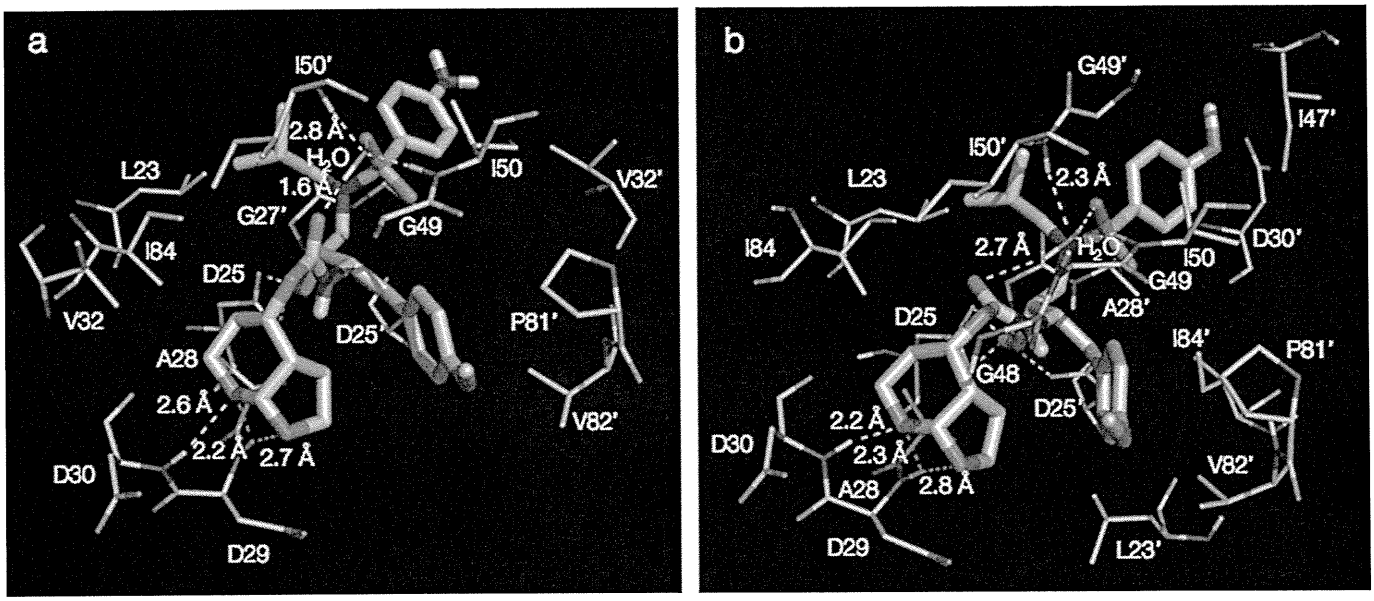

FIG. 7. Structural analysis of GRL-1388 and -1398 modeled into the active-site cavity of wild-type HIV-1 protease. GRL-1388 (a) and GRL-1398 (b) are shown as stick models (white color). Protease residues involved in either hydrogen bonding or hydrophobic interactions are highlighted in green. The distances for selected hydrogen bonds are shown. The novel Tp-THF functional group in both the compounds shows strong hydrogen bonding interactions with the backbone of residues Asp29 and Asp30.

tion was seen in two sets of selection experiments (Fig. 4 and 5; see Fig. S1 and S2 in the supplemental material), whereas A28S did not appear throughout the selection experiments with GRL-1388 or DRV. Although A28S substitution has been seen in HIV-1 selected with TMC-126 (36), GRL-98065 (1), and brecanavir (GW640385) (35), all of which contain the *bis*-THF moiety, the appearance of A28S has not been reported in the case of HIV-1 exposed to DRV *in vitro* (17) or *in vivo* (6, 24). Considering that both TMC-126 and GRL-1398 contain a methoxybenzene moiety but both DRV and GRL-1388 contain an aminobenzene moiety in the P2′ site, the presence of the methoxybenzene might be associated with the development of A28 substitution (Fig. 7b).

Amino acid substitutions in HIV-1 stoichiometrically and randomly occur, and the resulting amino acid substitutions in *in vitro* selection can vary from one experiment to another (34, 36). Thus, the results of a single selection experiment have to be confirmed with repeated selection experiments. In the present study, the selection experiment using HIV-1_{NL4-3} as a starting HIV-1 strain was conducted twice, and different profiles of amino acid substitutions were observed: the A28S substitution developed in the experiment II with GRL-1398, while it did not in the experiment I of the same compound (Fig. 4). Another selection experiment with GRL-1398 was planned; however, the selection experiment is, in general, labor-intensive and time-consuming. In fact, the selection experiment in the present study illustrated in Fig. 4 took more than 50 weeks each time. We thus performed the third selection experiment with a mixture of 10 $_{CL}$HIV-1_{MDR} variants as a starting virus population, expecting that highly GRL-1398-resistant HIV-1 variants would develop much sooner than when a single HIV-1 strain was used as a starting virus

TABLE 6. Interactions between wild type HIV-1 protease and GRL-1388 or -1398[a]

Compound	Amino acid residues and a bridging water involved in H bonding (no. of H bonds)	Total no. of H bonds	Amino acid residues involved in hydrophobic interactions (no. of hydrophobic interactions)	Total no. of hydrophobic interactions
DRV	D25 (1), G27 (1), D29 (2), D30 (1), D29′ (1), D30′ (1), water (2)	9	L23 (1), G27 (1), A28 (2), V32 (2), P81 (5), V82 (1), I84 (4), L23′ (1), D29′ (1), G49′ (3), G50′ (3), V82′ (1), I84′ (1)	26
GRL-1388	D25 (2), D29 (2), D30 (1), water (2)	7	L23 (1), A28 (4), D29 (1), V32 (2), G49 (5), I50 (1), I84 (2), G27′ (1), A28′ (6), V32′ (1), I50′ (1), P81′ (4), V82′ (2)	31
GRL-1398	D25 (2), D29 (2), D30 (1), D25′ (2), water (2)	8	L23 (1), D25 (1), A28 (1), D29 (2), D30 (3), G48 (1), G49 (3), I84 (2), L23′ (1), D25′ (1), G27′ (2), A28′ (3), D30′ (1), I47′ (1), G49′ (1), P81′ (4), V82′ (4), I84′ (2)	34

[a] The hydrogen bonding, as well as the hydrophobic interaction profiles of GRL-1388 and -1398 formed within the hydrophobic cavity of wild type HIV-1 protease, are shown along with those of DRV. The protease amino acid residues are listed, along with the numbers (in parentheses) of corresponding interactions. Note that GRL-1398 has relatively more hydrophobic interactions than GRL-1388, which should be one explanation for the greater potency of GRL-1398 against HIV-1. The two identical subunits that HIV-1 protease consists of were distinguished from each other by the use of a prime sign (′) at the upper right of each amino acid number.

population since multiple $_{CL}$HIV-1_{MDR} variants would undergo homologous recombination and also acquire amino acid substations *de novo* under the pressure of GRL-1398, resulting in quicker development of GRL-1398 resistance. In fact, using a mixture of eight multi-PI resistant clinical isolates, we successfully selected a highly DRV-resistant HIV-1 variant by 51 passages (68 weeks), although our group (17) and other groups (5) had failed to select such a variant using a single strain as a starting virus population. Indeed, in the third selection experiment, HIV-1 variants that replicated in the presence of 5 μM GRL-1388, DRV, and GRL-1398 appeared in 17, 21, and 29 weeks of selection periods, respectively.

One can assume that the amino acid positions, where secondary and further mutations occur, are affected under the influence of primary mutations. In the selection assay starting with 10 $_{CL}$HIV-1_{MDR} isolates, certain HIV-1 isolates most likely had already possessed various sets of amino acid substitutions in protease, some of which might have readily given replication advantages to the resulting GRL-1388- and DRV-resistant variants but not to GRL-1398-resistant variant. This should explain why the emergence of GRL-1398 resistance-associated mutations was delayed compared to the viruses selected with GRL-1388 or DRV. Moreover, homologous recombination among these 10 $_{CL}$HIV-1_{MDR} isolates might have given certain advantages in the speed for gaining resistance.

HIV-$1_{GRL1398\text{-}1\mu M}^{Exp.I}$ was more susceptible to DRV and GRL-1388 than was HIV-$1_{GRL1398\text{-}1\mu M}^{Exp.II}$ (Table 5). This susceptibility difference should stem from the difference in the amino acid substitutions obtained differently by the two variant populations. Both populations contained L10F and M46I substitutions; however, the former additionally had four amino acid substitutions and the latter had two substitutions. Both populations had compromised replication fitness compared to wild-type $HIV_{NL4\text{-}3}$, whereas both of them had a similarly significant advantages in replication in the presence of GRL-1398 (Fig. 6), proving that each set of amino acid substitutions conferred on the population significant levels of resistance to GRL-1398.

Modeling studies of GRL-1388 and -1398 docked against wild-type HIV-1 protease showed that the overall binding conformation of both compounds is similar (albeit not identical) to that of DRV. GRL-1398 shows more hydrogen bonds and hydrophobic interactions compared to GRL-1388. Structural analysis of GRL-1398 suggested that the potential extra hydrogen bonds with amide nitrogen atoms of Asp29′ and Asp30′ residues of the protease backbone could contribute to its greater potency. Based on this analysis, we postulate that these two hydrogen bonds with the backbone should be tolerant against mutations in the side chains from a wide spectrum of multidrug-resistant HIV-1 variants that we examined in comparison to either GRL-1388 or DRV. Due to the strong hydrogen bonds formed by GRL-1398 with the protease backbone, the overall binding profile of this compound is different from that of GRL-1388. This unique binding conformation of GRL-1398 is explained by the difference in the profiles of both GRL-1388 and -1398 with respect to their hydrophobic interactions in the protease active site.

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