TABLE 1. Sensitivities of infectious HIV-1 clones with Gag mutations to various PIs

Infectious HIV-1 clone	IC_{50}[a] (μM) of:				
	APV	SQV	IDV	RTV	NFV
HIV_{WT}	0.031 ± 0.0008	0.021 ± 0.002	0.032 ± 0.002	0.032 ± 0.0005	0.028 ± 0.002
$rHIV_{WTpro}^{75/219gag}$	0.031 ± 0.003	0.017 ± 0.003	0.032 ± 0.003	0.031 ± 0.0007	0.029 ± 0.003
$rHIV_{WTpro}^{219/409gag}$	0.029 ± 0.003	0.020 ± 0.01	0.032 ± 0.001	0.031 ± 0.004	0.028 ± 0.002
$rHIV_{WTpro}^{12/75/219/390/409gag}$	0.032 ± 0.0001	0.023 ± 0.005	0.032 ± 0.003	0.032 ± 0.0001	0.028 ± 0.002

[a] Data shown are mean values (with 1 standard deviation) derived from the results of three independent experiments conducted in triplicate. The IC_{50}s were determined by employing MT-4 cells exposed to each infectious HIV-1 clone (50 $TCID_{50}$) in the presence of each PI, with the inhibition of p24 Gag protein production as an end point.

$rHIV_{WTpro}^{12/75/219/390/409gag}$, affected the susceptibility of HIV-1 to any of five PIs (APV, SQV, IDV, RTV, and NFV). Indeed, the IC_{50}s for HIV_{WT} were highly comparable to those for any of the three recombinant clones carrying combined Gag mutations.

Replication rate difference is not the cause of the contrasting resistance acquisition patterns. Our observations of the contrasting resistance acquisition patterns, in which $rHIV_{WTpro}^{12/75/219/390/409gag}$ acquired resistance to APV more rapidly than HIV_{WT} when selected with APV (Fig. 3) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ significantly delayed the acquisition of resistance to other PIs compared to HIV_{WT} (Fig. 5), prompted us to ask whether the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} were differentially affected by the presence of PIs. We therefore compared the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} in the presence or absence of APV, SQV, IDV, RTV, or NFV by using the CHRA (21). As shown in Fig. 6, $rHIV_{WTpro}^{12/75/219/390/409gag}$ outgrew HIV_{WT} regardless of the absence or presence of PIs. Comparing the divergence patterns of the curves for $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} in the absence and presence of APV (Fig. 6A and B) revealed that those for growth in the presence of APV diverged more quickly than those for growth in the absence of APV (Fig. 6B). However, similar divergence patterns were seen with SQV, IDV, RTV, and NFV (Fig. 6C, D, E, and F), suggesting that the replication advantage of $rHIV_{WTpro}^{12/75/219/390/409gag}$ seen in the CHRA was not the cause for the observed contrasting resistance acquisition patterns.

NFV resistance-conferring protease mutations increase HIV-1 susceptibility to APV. There have been reports that an

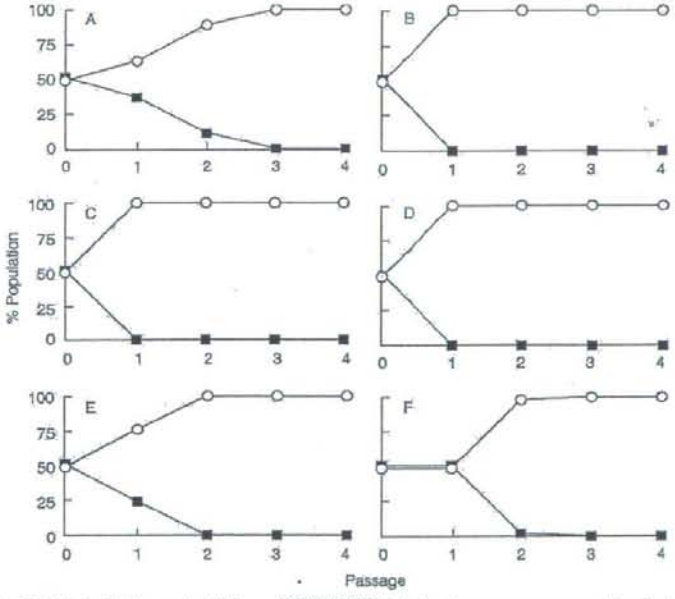

FIG. 6. Results from the CHRA for HIV_{WT} and $rHIV_{WTpro}^{12/75/219/390/409gag}$ in the absence or presence of each drug. Replication profiles of HIV_{WT} (■) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (○) in the absence (A) or presence of 0.03 μM APV (B), 0.02 μM SQV (C), 0.03 μM IDV (D), 0.03 μM RTV (E), or 0.03 μM NFV (F) were examined by the CHRA. The cell-free supernatant was transferred to fresh MT-4 cells every 7 days. High-molecular-weight DNA extracted from infected cells at the end of each passage was subjected to nucleotide sequencing, and the proportions of Arg and Lys at position 409 in Gag were determined.

TABLE 2. Phenotypic sensitivities of recombinant HIV-1 clones passaged with NFV*

Infectious HIV-1 clone	IC_{50} (μM) ± SD (change, *n*-fold) of:	
	APV	NFV
HIV_{WT}	0.031 ± 0.0008 (1)	0.028 ± 0.002 (1)
$rHIV_{N88Spro}{}^{WTgag}$	0.0015 ± 0.0007 (0.05)	0.028 ± 0.001 (1)
$rHIV_{D30Npro}{}^{WTgag}$	0.0031 ± 0.0001 (0.1)	0.045 ± 0.001 (1.6)
$rHIV_{10/30/45/71pro}{}^{WTgag}$	0.014 ± 0.0021 (0.45)	0.26 ± 0.03 (9)
$rHIV_{10/30/45/71pro}{}^{12/75/219/390/409gag}$	0.020 ± 0.002 (0.64)	0.32 ± 0.03 (11)
$rHIV_{30/46/77pro}{}^{WTgag}$	0.0069 ± 0.0024 (0.22)	0.25 ± 0.04 (9)
$rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$	0.0046 ± 0.0019 (0.15)	0.21 ± 0.06 (8)

* Recombinant HIV clones $rHIV_{10/30/45/71pro}{}^{WTgag}$ and $rHIV_{10/30/45/71pro}{}^{12/75/219/390/409gag}$ were generated to have a set of four protease mutations (L10F, D30N, K45I, and A71V) and wild-type Gag or Gag with five mutations, while other clones, $rHIV_{30/46/77pro}{}^{WTgag}$ and $rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$, were generated with three protease mutations (D30N, M46I, and V77I) and wild-type Gag or Gag with five mutations. Both sets of protease mutations were seen when HIV-1 was propagated in the presence of NFV. The IC_{50}s were determined by employing MT-4 cells exposed to each recombinant HIV-1 clone (50 $TCID_{50}$) in the presence of each PI, with the inhibition of p24 Gag protein production as an end point. All values were determined in triplicate, and the data are shown as mean values ±1 standard deviation of results from two or three independent experiments. The numbers in parentheses are changes (*n*-fold) compared to the IC_{50} of each PI for HIV_{WT}.

NFV-related resistance mutation, N88S, renders HIV-1 susceptible to APV (33, 49). Since the acquisition of viral resistance to PIs such as NFV was significantly delayed when HIV-1 had the Gag mutations seen in HIV_{APVp33}, we asked if another NFV-related resistance mutation (D30N) would render HIV-1 more susceptible to APV. We also asked whether the presence of multiple NFV resistance-associated mutations (D30N, M46I, and V77I) would make HIV-1 susceptible to APV. Moreover, we examined the effects of the Gag mutations seen in HIV_{APVp33} on HIV-1 susceptibilities to APV and NFV.

As shown in Table 2, the N88S mutant clone $rHIV_{N88Spro}{}^{WTgag}$ was more susceptible to APV than HIV_{WT} by a factor of 20, in agreement with the reports by Ziermann et al. and Resch et al. (33, 49). We found that the D30N mutation in $rHIV_{D30Npro}{}^{WTgag}$ also made HIV-1 more susceptible to APV, by a factor of 10. Interestingly, $rHIV_{10/30/45/71pro}{}^{WTgag}$, with the four mutations L10F, D30N, K45I, and A71V, was more resistant to NFV than HIV_{WT} by a factor of 9; however, the recombinant virus remained more susceptible to APV than HIV_{WT} (Table 2). The introduction of the five Gag mutations (E12K, L75R, H219Q, V390D, and R409K) into $rHIV_{10/30/45/71pro}{}^{WTgag}$, generating $rHIV_{10/30/45/71pro}{}^{12/75/219/390/409gag}$, did not change the susceptibility profile (Table 2). Another recombinant HIV-1 clone with three protease mutations (D30N, M46I, and V77I), $rHIV_{30/46/77pro}{}^{WTgag}$, was also more resistant to NFV (by a factor of 9) and more susceptible to APV than HIV_{WT}. The introduction of the five Gag mutations, generating $rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$, did not affect the susceptibility of $rHIV_{30/46/77pro}{}^{WTgag}$ to APV or NFV (Table 2).

Taken together, the data suggest that, as seen in the case of the lamivudine (3TC) resistance-associated mutation M184V that restores zidovudine (ZDV) sensitivity (37), NFV resistance-associated mutations paradoxically render HIV-1 more susceptible to APV.

DISCUSSION

Certain amino acid substitutions in Gag are known to occur in common with resistance to PIs (11, 15, 32, 36); however, no salient features such as patterns and orders of the occurrence have been identified for a number of amino acid substitutions seen in Gag in PI-resistant HIV-1 variants. The roles and impact of such amino acid substitutions in Gag for the replication of HIV-1 have not been delineated, either. These limitations have been worsened since the functions and tertiary structures of entire HIV-1 Gag proteins remain to be determined, although some structures of certain parts of Gag proteins have been lately elucidated (13, 34, 41).

In the present study, we attempted to determine the effects of non-cleavage site mutations in Gag which emerged during the in vitro selection of HIV-1 in the presence of APV on the viral acquisition of resistance to APV and other currently existing PIs. When we selected HIV-1 in vitro in the presence of increasing concentrations of APV, six amino acid substitutions apparently critical for the development of APV resistance emerged. Such substitutions included five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K) and one cleavage site mutation, L449F (Fig. 1B).

HIV-1 variants containing PI resistance-conferring amino acid substitutions in protease plus wild-type Gag often have highly limited replicative abilities (7, 31). Indeed, in the present study, the recombinant HIV-1 clone containing the protease of HIV_{APVp33} plus a wild-type Gag ($rHIV_{APVp33pro}{}^{WTgag}$) or the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}{}^{449gag}$) failed to replicate in MT-2 cells (Fig. 2A), indicating that neither of the two Gag species supported the growth of HIV_{APVp33}. However, a recombinant HIV-1 clone containing the protease of HIV_{APVp33} and the five Gag non-cleavage site mutations, $rHIV_{APVp33pro}{}^{12/75/219/390/409gag}$, replicated moderately under the same conditions (Fig. 2A), an observation in agreement with reports by others that some PI resistance-associated mutations compromise the catalytic activity of protease and/or alter polyprotein processing, often leading to slower viral replication (29, 36, 43). Since some of the five non-cleavage site mutations emerged before mutations in protease developed, we examined the effects of three sets of non-cleavage site amino acid mutations upon the emergence of APV resistance. Interestingly, HIV-1 with either of two sets of Gag mutations ($rHIV_{WTpro}{}^{219/409gag}$ and $rHIV_{WTpro}{}^{12/75/219/390/409gag}$) acquired APV resistance significantly faster than HIV_{WT} (Fig. 3), while such mutations alone did not alter the susceptibilities of HIV to the PIs examined (Table 1), a finding providing the first report that Gag mutations expedite the emergence of PI-resistant HIV-1 variants. At this time, it is apparently unknown whether certain Gag mutations associated with viral resistance to PIs persist when highly active antiretroviral therapy (HAART) regimens including a PI(s) are interrupted or changed to regimens containing no PIs. However, the non-cleavage site mutations in Gag examined in this study did not reduce the viral fitness (Fig. 2 and 6), suggesting that Gag mutations may persist longer in circulation and/or in the HIV-1 reservoir in the body than mutations in protease when antiretroviral therapy including a PI(s) is interrupted. Such persisting Gag mutations may enable HIV-1 to rapidly acquire resistance

to that very PI when treatment with the PI is resumed. It is of note that on the other hand, two sets of Gag non-cleavage site mutations seen in HIV_{APVp33} (H219Q and R409K and E12K, L75R, H219Q, V390D, and R409K) significantly delayed the emergence of resistance to other PIs such as RTV and NFV (Fig. 5). These data suggest that if a HAART regimen including APV is changed to an alternative regimen, the inclusion of a different PI in the alternative regimen is likely to delay the emergence of resistance to the different PI.

It is known that the L449F cleavage site mutation renders recombinant HIV-1 carrying a protease mutation (I50V) more resistant to APV (25). In the present study, a recombinant HIV-1 clone containing the protease of HIV_{APVp33} plus the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}{}^{449gag}$) failed to replicate (Fig. 2A). These data strongly suggest that the L449F mutation alone prevents HIV_{APVp33} from replicating, although HIV_{APVp33} did not contain the I50V mutation. The observation in the present study that the addition of five non-cleavage site mutations to $rHIV_{APVp33pro}{}^{449gag}$, generating $rHIV_{APVp33pro}{}^{12/75/219/390/409/449gag}$, restored the replicative ability of the virus indicates that the presence of non-cleavage site Gag mutations plays an important role in the replication of APV-resistant HIV-1 variants.

Since $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ acquired resistance to APV more rapidly than HIV_{WT} (Fig. 3), while $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ significantly delayed the acquisition of resistance to other PIs (Fig. 5), we examined whether the replication rates of $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ and HIV_{WT} were associated with the observed contrasting resistance acquisition patterns by using the CHRA (21). We found that $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ outgrew HIV_{WT} regardless of the presence or absence of PIs (Fig. 6), suggesting that the difference in the replication rates of $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ and HIV_{WT} was not the cause for the contrasting resistance acquisition patterns. As for the reason why $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ outgrew HIV_{WT}, it is well explained by the presence of the H219Q mutation. His-219 is located within the cyclophilin A (CypA) binding loop of p24 Gag protein. It is thought that CypA plays an essential role in the HIV-1 replication cycle (4, 35) by destabilizing the capsid (p24 Gag protein) shell during viral entry and uncoating (12) and/or by performing an additional chaperone function, thus facilitating correct capsid condensation during viral maturation (17, 39). CypA is also known to support the replication of HIV-1 by binding to the Ref-1 restriction factor and/or TRIM5α, the human cellular inhibitors that impart resistance to retroviral infection (18, 38). It has also been demonstrated previously that the effect of CypA on HIV-1 replicative ability is bimodal: both high and low CypA contents limit HIV-1 replication (14). We have demonstrated previously that certain human cells, such as MT-2 and H9 cells, contain large amounts of CypA (14). We have determined more recently that MT-2 cells contain more CypA by about fivefold and that MT-4 cells contain about three times more than peripheral blood mononuclear cells (PBMCs) (unpublished data). In fact, HIV-1 produced in MT-4 cells contains large amounts of CypA, presumably resulting in compromised replication of the HIV-1. However, the H219Q mutation apparently reduces the incorporation of CypA into the virions through significantly distorting the CypA binding loop and restores the replicative ability of virions produced in MT-4 cells (14). Therefore, H219Q should contribute at least in part to the replication advantage of $rHIV_{WTpro}{}^{12/75/219/390/409gag}$. It is noteworthy that of 156 different HIV-1 strains whose sequences were compiled in the *HIV Sequence Compendium 2008* (22), 95 and 45 strains had histidine and glutamine, respectively, at position 219. Hence, position 219 is a polymorphic amino acid site, and it is thought that this polymorphic position is associated with the acquisition of resistance to certain PIs. Indeed, we have observed that $rHIV_{WTpro}{}^{219gag}$ overgrew $rHIV_{WTpro}{}^{WTgag}$ in the CHRA using fresh phytohemagglutinin-stimulated PBMCs (14). Since H219Q confers a replication advantage on HIV-1 in PBMCs, it is likely that HIV-1 with H219Q may acquire resistance more rapidly than HIV-1 without H219Q.

Two groups, Ziermann et al. and Resch et al., have reported that an NFV-related resistance mutation, N88S, renders HIV-1 susceptible to APV (33, 49), and indeed, Zachary et al. have reported an anecdotal finding that the infection of an individual with HIV-1 containing N88S was successfully managed with an ensuing APV-based regimen (46). Therefore, we examined the effect of another NFV resistance-associated mutation, D30N, in addition to that of the N88S mutation on HIV-1 susceptibility to APV. It was found that the mutations (D30N and N88S) clearly increased the susceptibility of HIV-1 to APV by 10- and 20-fold, respectively. These data are reminiscent of the observation that the 3TC resistance-associated mutation M184V in a background of mutations conferring resistance to ZDV restores ZDV sensitivity (37) and that ZDV-3TC combination therapy has proven to be more beneficial than ZDV monotherapy in patients harboring HIV-1 with the M184V mutation (9, 23), although the structural mechanism of the restoration of ZDV sensitivity by M184V is not clear. When a set of four protease mutations (L10F, D30N, K45I, and A71V), which had emerged by passage 10 when HIV_{WT} was selected with NFV, were introduced into HIV_{WT}, generating $rHIV_{10/30/45/71pro}{}^{WTgag}$, the recombinant HIV-1 clone was more resistant to NFV than HIV_{WT} by a factor of 9 while the clone was slightly more sensitive to APV (Table 2). When we introduced $mGag^{12/75/219/390/409gag}$ into HIV-1 containing a set of three NFV resistance-associated protease mutations (D30N, M46I, and V77I), generating $rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$, the recombinant clone was more resistant to NFV by a factor of 8 but more sensitive to APV by a factor of 6.7 (Table 2).

There has been a report that dual PI therapy with APV plus NFV is generally safe and well tolerated and that the combination of APV with NFV may have the most beneficial pharmacokinetic interactions, based on the results of a phase II clinical trial of dual PI therapies, APV in combination with IDV, NFV, or SQV, although this phase II trial was handicapped by the presence of substantial PI resistance at the baseline and the small number of patients in the study, precluding conclusions about the relative activities or toxicities of the dual PI combinations (10). The hypothesis that a HAART regimen combining APV with NFV may bring about more

favorable antiviral efficacy for HIV-1-infected individuals should merit further study.

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GRL-02031, a Novel Nonpeptidic Protease Inhibitor (PI) Containing a Stereochemically Defined Fused Cyclopentanyltetrahydrofuran Potent against Multi-PI-Resistant Human Immunodeficiency Virus Type 1 In Vitro▽

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We generated a novel nonpeptidic protease inhibitor (PI), GRL-02031, by incorporating a stereochemically defined fused cyclopentanyltetrahydrofuran (Cp-THF) which exerted potent activity against a wide spectrum of human immunodeficiency virus type 1 (HIV-1) isolates, including multidrug-resistant HIV-1 variants. GRL-02031 was highly potent against laboratory HIV-1 strains and primary clinical isolates, including subtypes A, B, C, and E (50% effective concentration [EC_{50}] range, 0.015 to 0.038 μM), with minimal cytotoxicity (50% cytotoxic concentration, >100 μM in $CD4^+$ MT-2 cells), although it was less active against two HIV-2 strains ($HIV\text{-}2_{EHO}$ and $HIV\text{-}2_{ROD}$) (EC_{50}, ~0.60 μM) than against HIV-1 strains. GRL-02031 at relatively low concentrations blocked the infection and replication of each of the $HIV\text{-}1_{NL4\text{-}3}$ variants exposed to and selected by up to 5 μM of saquinavir, amprenavir, indinavir, nelfinavir, or ritonavir and 1 μM of lopinavir or atazanavir (EC_{50} range, 0.036 to 0.14 μM). GRL-02031 was also potent against multi-PI-resistant clinical HIV-1 variants isolated from patients who had no response to the conventional antiretroviral regimens that then existed, with EC_{50}s ranging from 0.014 to 0.042 μM (changes in the EC_{50}s were less than twofold the EC_{50} for wild-type HIV-1). Upon selection of $HIV\text{-}1_{NL4\text{-}3}$ in the presence of GRL-02031, mutants carrying L10F, L33F, M46I, I47V, Q58E, V82I, I84V, and I85V in the protease-encoding region and G62R (within p17), L363M (p24-p2 cleavage site), R409K (within p7), and I437T (p7-p1 cleavage site) in the *gag*-encoding region emerged. GRL-02031 was potent against a variety of $HIV\text{-}1_{NL4\text{-}3}$-based molecular infectious clones containing a single primary mutation reported previously or a combination of such mutations, although it was slightly less active against HIV-1 variants containing consecutive amino acid substitutions: M46I and I47V or I84V and I85V. Structural modeling analysis demonstrated a distinct bimodal binding of GRL-02031 to protease, which may provide advantages to GRL-02031 in blocking the replication of a wide spectrum of HIV-1 variants resistant to PIs and in delaying the development of resistance of HIV-1 to GRL-02031. The present data warrant the further development of GRL-02031 as a potential therapeutic agent for the treatment of infections with primary and multidrug-resistant HIV-1 variants.

The currently available combination therapy or highly active antiretroviral therapy (HAART) with two or more reverse transcriptase inhibitors and protease inhibitors (PIs) for human immunodeficiency virus (HIV) type 1 (HIV-1) infection and AIDS has been shown to suppress the replication of HIV-1 and extend the life expectancy of HIV-1-infected individuals (35, 38). However, the ability to provide effective long-term antiretroviral therapy for HIV-1 infection has become a complex issue, since those who initially achieved favorable viral suppression to undetectable levels have experienced treatment failure (11, 18, 28). In addition, it is evident that with these anti-HIV drugs, only partial immunologic reconstitution is attained in patients with advanced HIV-1 infection.

Nevertheless, recent analyses have revealed that the life expectancy of HIV-infected patients treated with HAART increased between 1996 and 2005, that the mortality rates for HIV-infected persons have become much closer to general mortality rates since the introduction of HAART, and that first-line HAART with boosted PI-based regimens results in less resistance within and across drug classes (2, 3, 18, 46).

In the development of new anti-HIV-1 therapeutics, we have faced a variety of challenges different from those faced during the design of the first-line drugs (7, 10, 39). The issue of the emergence of drug-resistant HIV-1 variants is one of the most formidable challenges in the era of HAART. Indeed, it is of note that the very features that contribute to the specificities and the efficacies of reverse transcriptase inhibitors and PIs provide the virus with a strategy to develop resistance (15, 19, 35), and it seems inevitable that this resistance issue will remain problematic for many years to come, although a few recently developed drugs, such as darunavir (DRV) and tipranavir, have been relatively successful as treatments for individuals carrying multidrug-resistant HIV-1 variants (5, 20).

In particular, a number of studies indicate that cross-resis-

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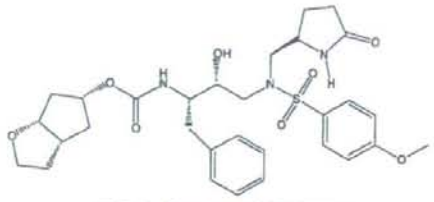

FIG. 1. Structure of GRL-02031.

tance is a major obstacle to antiviral therapy with PIs (19, 24). Obviously, the emergence of viral resistance, difficulties with compliance with the complicated treatment protocols, and adverse side effects urge the development of new classes of PIs (i) that have potent activities against existing resistant HIV-1 variants and that do not allow or delay the emergence of resistance, (ii) that have improved pharmacokinetics parameters in humans, and (iii) that have less severe side effects (43).

The present paper represents the first demonstration of the results of antiviral analyses of a novel PI which contains cyclopentanyltetrahydrofuran (Cp-THF) and which is highly potent against a wide spectrum of HIV isolates, including a variety of multi-PI-resistant clinical strains, in vitro. In addition, we selected GRL-02031-resistant HIV-1 variants in vitro and characterized their virological properties and susceptibilities to other PIs. We also demonstrated that the emergence of HIV-1 variants resistant to GRL-02031 requires multiply accumulated amino acid substitutions in the protease-encoding region. Moreover, in an attempt to explain why GRL-02031 can exert potent activity against a wide spectrum of HIV-1 variants resistant to multiple PIs, we performed structural modeling and molecular docking and examined the interactions of GRL-02031 with HIV-1 protease.

MATERIALS AND METHODS

Cells and viruses. MT-2 and MT-4 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, MD), 50 U/ml penicillin, and 50 μg/ml of streptomycin. The following HIV-1 strains were employed for the drug susceptibility assay (see below): HIV-1_{LAI}, HIV-$1_{Bs\text{-}L}$, HIV-1_{JRFL}, HIV-$1_{NL4\text{-}3}$, HIV-2_{EHO}, and HIV-2_{ROD}; two clinical HIV-1 strains isolated from drug-naive patients with AIDS, HIV-$1_{ERS104pre}$ and HIV-1_{MOKW} (30, 45); and seven HIV-1 clinical isolates which were originally isolated from patients with AIDS who had received 9 to 11 anti-HIV-1 drugs over the past 32 to 83 months and which were genotypically and phenotypically characterized as multi-PI-resistant HIV-1 variants (47, 48). HIV-$1_{92UG037}$, HIV-$1_{97ZA003}$, and HIV-$1_{92TH019}$ were obtained from the NIH AIDS Reagent Program. All primary HIV-1 strains were passaged once or twice in 3-day-old phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBMs), and the culture supernatants were stored at −80°C until use.

Antiviral agents. GRL-02031 (Fig. 1), a novel nonpeptidic PI containing Cp-THF, was designed and synthesized. Detailed methods for the synthesis of GRL-02031 will be described elsewhere by A. K. Ghosh et al. 3′-Azido-2′,3′-dideoxythymidine (AZT; zidovudine) was purchased from Sigma (St. Louis, MO). Saquinavir (SQV) and ritonavir (RTV) were kindly provided by Roche Products Ltd. (Welwyn Garden City, United Kingdom) and Abbott Laboratories (Abbott Park, IL), respectively. Amprenavir (APV) was a kind gift from Glaxo-Wellcome, Research Triangle Park, NC. Nelfinavir (NFV) and indinavir (IDV) were kindly provided by Japan Energy Inc, Tokyo, Japan. Lopinavir (LPV) was synthesized by previously published methods (48). Atazanavir (ATV) was a kind gift from Bristol-Myers Squibb (New York, NY).

Drug susceptibility assay. The susceptibilities of HIV-1_{LAI}, HIV-$1_{Bs\text{-}L}$, HIV-2_{EHO}, HIV-2_{ROD}, and the primary HIV-1 isolates to various drugs were determined as described previously (26), with minor modifications. Briefly, MT-2 cells (2×10^4/ml) were exposed to 100 50% tissue culture infectious dose ($TCID_{50}$s) of HIV-1_{LAI}, HIV-$1_{Bs\text{-}L}$, HIV-2_{EHO}, or HIV-2_{ROD} in the presence or the absence of various concentrations of drugs in 96-well microculture plates; and the plates were incubated at 37°C for 7 days. After 100 μl of the medium was removed from each well, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10 μl, 7.5 mg/ml in phosphate-buffered saline) was added to each well in the plate, followed by incubation at 37°C for 2 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 in a kinetic microplate reader (Vmax; Molecular Devices, Sunnyvale, CA). All assays were performed in duplicate or triplicate.

To determine the sensitivities of the primary HIV-1 isolates to drugs, PHA-PBMs (10^6/ml) were exposed to 50 $TCID_{50}$s of each primary HIV-1 isolate and cultured in the presence or the absence of various concentrations of drugs in 10-fold serial dilutions in 96-well microculture plates. To determine the drug susceptibilities of certain laboratory HIV-1 strains, MT-4 cells were employed as target cells, as described previously (26), with minor modifications. In brief, MT-4 cells (10^5/ml) were exposed to 100 $TCID_{50}$s of drug-resistant HIV-1 strains in the presence or the absence of various concentrations of drugs and were incubated at 37°C. On day 7 of culture, the supernatants were harvested and the amounts of the p24 Gag protein were determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo, Japan) (29). The drug concentrations that suppressed the production of p24 Gag protein by 50% (EC_{50}) were determined by comparison of the amount of p24 Gag protein produced in drug-treated cell cultures with the level of p24 Gag protein produced in a drug-free control cell culture. All assays were performed in triplicate.

Generation of PI-resistant HIV-1 variants in vitro. MT-4 cells (10^5/ml) were exposed to HIV-$1_{NL4\text{-}3}$ (500 $TCID_{50}$s) and cultured in the presence of various PIs at an initial concentration of 0.01 to 0.03 μM. Viral replication was monitored by determination of the amount of p24 Gag produced by MT-4 cells. The culture supernatants were harvested on day 7 and were used to infect fresh MT-4 cells for the next round of culture in the presence of increasing concentrations of each drug. When the virus began to propagate in the presence of the drug, the drug concentration was generally increased two- to threefold. Proviral DNA samples obtained from the lysates of infected cells were subjected to nucleotide sequencing. This drug selection procedure was carried out until the drug concentration reached 5 μM.

Determination of nucleotide sequences. Molecular cloning and determination of the nucleotide sequences of HIV-1 isolates passaged in the presence of anti-HIV-1 agents were performed as described previously (26, 47). 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 of PCR 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 5 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 employed were as follows: an initial 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 3 min at 72°C, with a final 8-min 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 columns; Amersham Biosciences Corp., Piscataway, NJ), cloned directly, and subjected to sequencing with an ABI model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The viral RNA in the selection culture should contain a number of noninfectious (or dead) virions due to randomly occurring amino acid substitutions, which could provide misleading results if the sequences of such noninfectious or dead virions were erroneously taken into account. The viral DNA extracted from the newly infected cells in the present cell-free transmission system represents the infectious virions in the previous culture.

Generation of recombinant HIV-1 clones. The PCR products obtained as described above were digested with two enzymes, ApaI and SmaI; and the fragments obtained were introduced into pHIV-1_{NL3ma}, designed to have a SmaI site by changing two nucleotides (2590 and 2593) of pHIV-$1_{NL4\text{-}3}$, as described previously (14, 25). To generate HIV-1 clones carrying the desired mutations, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the mutation-containing genomic fragments were introduced into pHIV-1_{NL3ma}. Determination of the nucleotide

TABLE 1. Antiviral activity of GRL-02031 against HIV-1_{LAI}[a]

Compound	EC_{50} (μM)	CC_{50}(μM)	Selectivity index
GRL-02031	0.028 ± 0.003	>100	>3,600
SQV	0.014 ± 0.005	9.9 ± 3.6	710
APV	0.033 ± 0.012	>100	>3,000
IDV	0.044 ± 0.007	69.8 ± 3.1	1,600
RTV	0.038 ± 0.004	21.3 ± 0.9	560
NFV	0.023 ± 0.006	ND	ND
LPV	0.032 ± 0.007	ND	ND

[a] MT-2 cells (2×10^4/ml) were exposed to 100 $TCID_{50}$s of HIV-1_{LAI} and were cultured in the presence of various concentrations of PIs, and the EC_{50}s were determined by using the MTT assay on day 7 of culture. All assays were conducted in duplicate. The data shown represent mean values (±1 standard deviation) derived from the results of three independent experiments. ND, not determined. Selectivity index, CC_{50}/EC_{50}.

sequences of the plasmids confirmed that each clone had the desired mutations but no unintended mutations. Each recombinant plasmid was transfected into 293T cells with Lipofectoamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), and the infectious virions thus generated were harvested for 48 h after transfection and stored at −80°C until use.

Structural analysis of GRL-02031 interactions with wild-type HIV-1 protease. The interactions of GRL-02031 with wild-type HIV-1 protease were examined by computational structural modeling and molecular docking on the basis of the published crystallographic data for protease complexed with PIs. Besides accounting for the conformational flexibility of the inhibitor, the polarization induced in the inhibitor by the protease was taken into consideration by employing polarizable quantum charges in the docking computations. The use of polarizable quantum charges has recently been shown to substantially improve the prediction of protein-ligand complex structures (4). The quantum mechanical polarized ligand docking protocol provided with the Glide (version 4.5), QSite (version 4.5), Jaguar (version 7.0), and Maestro (version 8.5) software (Schrödinger, LLC, New York, NY) was used as described below. The crystal structures 2FDE (protease-brecanavir complex) and 2IEN (protease-DRV complex) were used as templates in separate docking calculations to determine the binding mode of GRL-02031 with wild-type protease. The crystal coordinates were obtained from the Protein Data Bank (http://www.rcsb.org/). Hydrogens were optimized by placing constraints on the heavy atoms. The crystal water that mediates the interaction between PIs and the protease flap was retained, and all other crystal waters were deleted. Close interaction in the protease was annealed, and the docking grid was set up. Polarizable ligand charges were determined at the B3LYP/6-31G* level. The extraprecision mode of the Glide program (12, 13), which has a higher penalty for unphysical interactions, was used.

RESULTS

In vitro activity of GRL-02031 against laboratory and primary HIV strains and cytotoxicity of GRL-02031. We designed and synthesized ~80 different novel nonpeptidyl PIs containing a Cp-THF moiety and examined them for their anti-HIV activities and cytotoxicities in vitro. Among them, we found that GRL-02031 (Fig. 1) was the most potent against a laboratory HIV-1 strain, HIV-1_{LAI}, and had a favorable cytotoxicity profile, as examined with target MT-2 cells. As shown in Table 1, GRL-02031 showed an anti-HIV-1 activity profile comparable to that of most of the Food and Drug Administration (FDA)-approved PIs examined in the present study, although its toxicity profile was apparently more favorable, with a 50% cytotoxic concentration (CC_{50}) of >100 μM and a selectivity index (CC_{50}/EC_{50}) of >3,600.

GRL-02031 was further tested against two R5 laboratory HIV-1 strains (HIV-1_{Ba-L} and HIV-1_{JRFL}), three different subtypes of primary HIV-1 strains (HIV-$1_{92UG037}$ [subtype A], HIV-$1_{97ZA003}$ [subtype C], and HIV-$1_{92TH019}$ [subtype E]), and two HIV-2 strains (HIV-2_{ROD} and HIV-2_{EHO}). GRL-02031 was found to be potent against all these HIV-1 strains and had EC_{50}s that ranged from 0.015 to 0.038 μM, as tested by the use of target PHA-PBMs, while GRL-02031 was moderately active against two HIV-2 strains (EC_{50}, ~0.60 μM), as tested by the use of MT-2 cells (data not shown).

GRL-02031 exerts potent activity against a wide spectrum of primary HIV-1 variants resistant to multiple PIs. We next examined the activity of GRL-02031 against a variety of primary HIV-1 strains which were isolated from those with AIDS who had failed a number of anti-HIV therapeutic regimens after they had received 9 to 11 anti-HIV-1 drugs over the previous 32 to 83 months and who proved to be highly resistant to multiple PIs (47, 48). These primary strains contained 9 to 14 amino acid substitutions in the protease-encoding region of the HIV-1 genome which have been reported to be associated with HIV-1 resistance to various PIs (RTV, IDV, NFV, SQV, APV, and LPV) (8). The substitutions identified included Leu-10 → Ile (L10I; seven of seven isolates), M46I/L (six of seven isolates), I54V (five of seven isolates), L63P (seven of seven isolates), A71V/T (six of seven isolates), V82A or V82T (seven of seven isolates), and L90M (five of seven isolates) (see footnote *a* of Table 2).

All drugs examined showed potent activity against two reference wild-type primary strains (X4 HIV-$1_{ERS104pre}$ [45] and R5 HIV-1_{MOKW} [30]), with the EC_{50}s ranging 0.004 to 0.036 μM (Table 2). However, all the primary strains examined were highly resistant to AZT, with the EC_{50}s being from 24- to >200-fold greater than the EC_{50} against HIV-$1_{ERS104pre}$. It was noted that SQV and LPV were still active against one or two of the seven strains and had EC_{50}s that differed 3- to 4-fold from those for HIV-$1_{ERS104pre}$; however, all the other FDA-approved PIs examined in this study except DRV failed to exert activity and had EC_{50}s 6- to >63-fold greater than the EC_{50} for HIV-$1_{ERS104pre}$. In contrast, GRL-02031, like DRV, potently blocked all seven primary strains and had EC_{50}s that ranged from 0.014 to 0.043 μM. It should be noted that the change in the EC_{50} of GRL-02031 for all seven multi-PI-resistant isolates tested was less than twofold compared with the EC_{50} for a wild-type primary strain, HIV-$1_{ERS104pre}$.

Selection of HIV-1_{NL4-3} with GRL-02031. We then attempted to select a laboratory X4 HIV-1 strain (HIV-1_{NL4-3}) by propagating it in MT-4 cells in the presence of increasing concentrations of APV, IDV, or GRL-02031, as described previously (47). The virus was initially exposed to 0.03 μM APV, 0.02 μM IDV, or 0.02 μM GRL-02031. At passages 21 and 27, HIV-1_{NL4-3} was capable of propagating in the presence of 167- and 250-fold greater concentrations of APV and IDV, respectively. At passage 26, HIV-1_{NL4-3} was capable of propagating in the presence of a 250-fold greater concentration of IDV; however, 37 passages were required until the virus became similarly resistant to GRL-02031 and capable of propagating in the presence of 5 μM (Fig. 2).

We also determined the nucleic acid sequences of the protease-encoding region of the proviral DNA isolated from the cells exposed to GRL-02031 at passages 5, 15, 22, 30, and 37 (Fig. 3). At passage 5, no significant amino acid substitutions were identified; however, by passage 15, the virus had acquired the L10F substitution, which has been reported to be associated with PI resistance (6, 32). By passage 22, all eight clones of the virus examined had additionally acquired a flap muta-

TABLE 2. Antiviral activity of GRL-02031 against clinical HIV-1 isolates in PHA-PBMs[a]

Virus	Phenotype	EC_{50} (μM)								
		AZT	SQV	APV	IDV	NFV	RTV	LPV	DRV	GRL-02031
HIV-$1_{ERS104pre}$ (wild type)	X4	0.005 ± 0.002	0.009 ± 0.005	0.025 ± 0.007	0.021 ± 0.008	0.016 ± 0.004	0.036 ± 0.008	0.032 ± 0.002	0.0035 ± 0.0003	0.027 ± 0.002
HIV-1_{MOKW} (wild type)	R5	0.016 ± 0.011	0.004 ± 0.002	0.015 ± 0.004	0.019 ± 0.005	0.023 ± 0.008	0.036 ± 0.008	0.029 ± 0.0005	0.003 ± 0.0005	0.026 ± 0.007
HIV-1_{TM}(MDR)	X4	0.89 ± 0.11 (178)	0.27 ± 0.08 (30)	0.35 ± 0.11 (14)	>1 (>48)	>1 (>63)	>1 (>30)	0.23 ± 0.18 (7)	0.004 ± 0.001 (1)	0.026 ± 0.004 (1)
HIV-1_{MM}(MDR)	R5	0.45 ± 0.08 (90)	0.27 ± 0.06 (30)	0.40 ± 0.10 (16)	>1 (>48)	>1 (>63)	>1 (>30)	0.62 ± 0.28 (19)	0.017 ± 0.008 (5)	0.041 ± 0.004 (2)
HIV-1_{JSL} (MDR)	R5	0.18 ± 0.11 (36)	0.32 ± 0.13 (36)	0.66 ± 0.17 (26)	>1 (>48)	>1 (>63)	>1 (>30)	0.74 ± 0.32 (23)	0.026 ± 0.005 (7)	0.043 ± 0.003 (2)
HIV-1_A(MDR)	X4	0.12 ± 0.11 (24)	0.10 ± 0.07 (11)	0.16 ± 0.08 (6)	>1 (>48)	0.33 ± 0.07 (21)	>1 (>30)	0.32 ± 0.003 (10)	0.003 ± 0.0003 (1)	0.014 ± 0.007 (1)
HIV-1_B(MDR)	X4	>1 (>200)	0.30 ± 0.02 (33)	0.31 ± 0.04 (12)	>1 (>48)	>1 (>63)	>1 (>30)	0.25 ± 0.007 (8)	0.026 ± 0.017 (7)	0.029 ± 0.007 (1)
HIV-1_C(MDR)	X4	0.28 ± 0.20 (56)	0.033 ± 0.010 (4)	0.22 ± 0.13 (9)	>1 (>48)	0.36 ± 0.12 (23)	>1 (>30)	0.46 ± 0.24 (14)	0.007 ± 0.005 (2)	0.027 ± 0.009 (1)
HIV-1_G(MDR)	X4	0.60 ± 0.56 (120)	0.027 ± 0.006 (3)	0.23 ± 0.13 (9)	0.34 ± 0.07 (16)	0.23 ± 0.11 (14)	>1 (>30)	0.13 ± 0.04 (4)	0.007 ± 0.004 (2)	0.028 ± 0.001 (1)

[a] The amino acid substitutions identified in the protease-encoding region of HIV-$1_{ERS104pre}$, HIV-1_{TM}, HIV-1_{MM}, HIV-1_{JSL}, HIV-1_A, HIV-1_B, HIV-1_C, and HIV-1_G, compared to the consensus B sequence cited from the Los Alamos National Laboratory database include L63P, L10I/K14R/R41K/M46L/I54V/L63P/A71V/V82A/L90M/I93L, L10I/K43T/M46L/I54V/L63P/A71V/V82A/L90M/Q92K, L10I/L24I/L33F/E35D/M36I/N37S/M46L/I54V/R57K/I62V/L63P/A71V/G73S/V82A, L10I/I15V/E35D/N37E/R45S/I54V/L63P/A71V/V82T/L90M/I93L/C95F, L10F/K14R/L33I/M36I/M46I/P53L/K55R/I62V/L63P/A71V/G73S/V82A/L90M/I93L, L10I/I15V/K20R/L24I/M36I/M46L/I54V/I62V/L63P/K70Q/V82A/L89M, and L10I/V11I/T12E/I15V/L19I/R41K/M46L/L63P/A71T/V82A/L90M, respectively. HIV-1_{MOKW} was confirmed to lack known drug resistance-associated amino acid substitutions. The EC_{50}s were determined by employing PHA-PBMs as target cells and the inhibition of p24 Gag protein production as the endpoint. All values were determined in triplicate, and those shown were derived from the results of three independent experiments. Numbers in parentheses represent the fold changes in the EC_{50} for each isolate compared to the EC_{50} for HIV-$1_{ERS104pre}$. MDR, multidrug resistant.

FIG. 2. In vitro selection of HIV-1 variants resistant to GRL-02031. HIV-1_{NL4-3} was passaged in the presence of increasing concentrations of APV (open circles), IDV (solid circles), or GRL-02031 (open triangles) in MT-4 cells. The selection was carried out in a cell-free manner for a total of 21 to 37 passages with drug concentrations escalating from 0.02 to 5 μM. The nucleotide sequences of the proviral DNAs were determined by using cell lysates of HIV-1-infected MT-4 cells at the termination of each indicated passage.

tion (I47V) and the primary substitution (V82I). By passage 30, in addition to L10F, I47V, and V82I, the virus had acquired L33F, M46I, and I85V. By passage 37, the virus had gained I84V and another Q58E substitution, an unusual mutation not associated with resistance to other HIV PIs. Genetic characterization of the protease-encoding gene of the HIV-1 variants selected with APV and IDV revealed that those variants had acquired previously reported mutations, such as L10F, V32I, M46I, and I84V in APV-selected HIV-1 (HIV-1_{APV^r}) and L10F, V32I, K43T, M46I, L63P, G73C, and V82A in HIV-1_{IDV^r}.

Amino acid substitutions in Gag protein of HIV-1 exposed to GRL-02031. We also determined the amino acid sequences of the Gag proteins of HIV-1_{NL4-3} exposed to GRL-02031. By passage 15, an unusual amino acid substitution, L363M, located at the p24-p2 cleavage site, had emerged. By passage 22, the virus had acquired another amino acid substitution, I437T, located close to the p7-p1 cleavage site. By passage 30 and beyond, G62R (within p17) and R409K (within p7) were seen in addition to L363M and I437T (data not shown).

Anti-HIV-1 profile of a GRL-02031-resistant HIV-1 variant. We then examined the anti-HIV-1 profile of a GRL-02031-resistant HIV-1 variant. In this experiment, we employed eight HIV-1 variants which were selected in the presence of up to 1 or 5 μM of each of seven FDA-approved PIs and GRL-02031. Each of these eight variants contained a variety of previously known PI resistance-associated amino acid substitutions (see footnote *a* of Table 3). As shown in Table 3, when each HIV-1 variant was examined in target MT-4 cells, each HIV-1 variant except the variant selected with 1 μM LPV in vitro (HIV-$1_{LPV\text{-}1\ \mu M}$) and the variant selected with 1 μM ATV in vitro (HIV-$1_{ATV\text{-}1\ \mu M}$), proved to be highly resistant to the PI with which the virus was selected, with EC_{50}s exceeding 1 μM. However, GRL-02031 was potent against all the PI-resistant variants, although the compound was relatively less potent against the variant selected with 5 μM SQV in vitro (HIV-

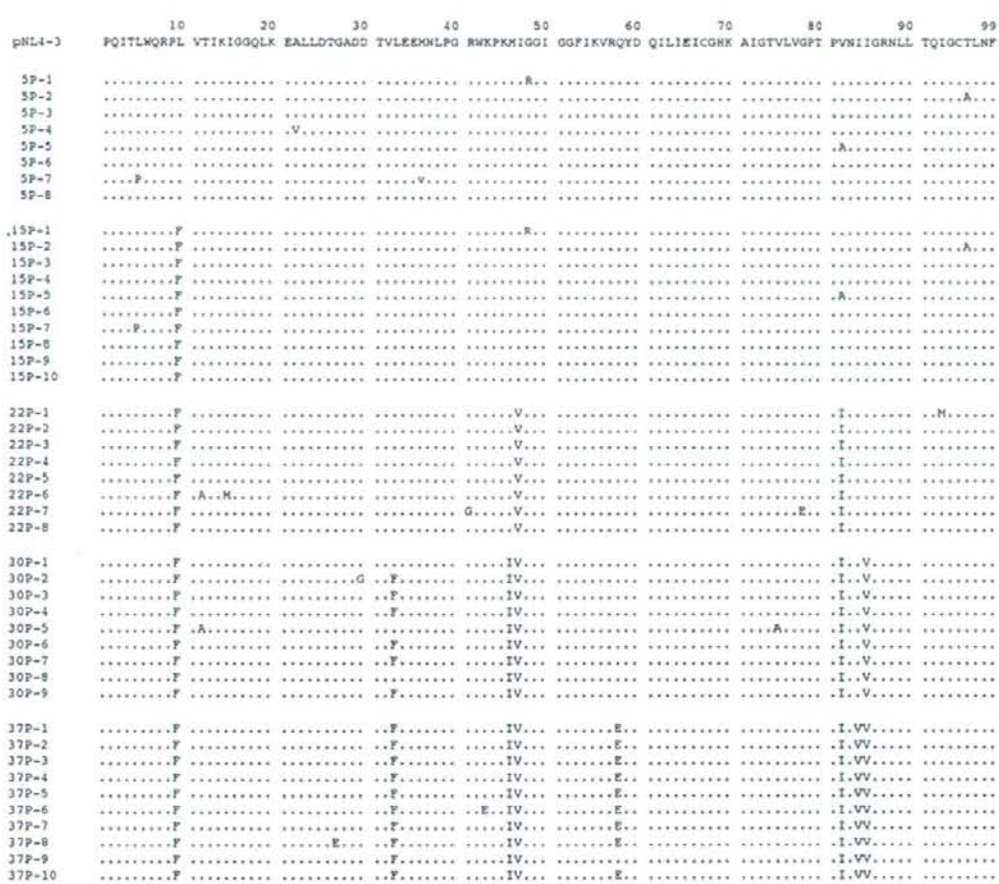

FIG. 3. Sequence analysis of the protease-encoding region of HIV passaged in the presence of GRL-02031. The amino acid sequences of the proteases deduced from the nucleotide sequences of the protease-encoding region of HIV clones determined at five different passages are illustrated. The identity of each amino acid with that from pNL4-3 (top row) at each individual amino acid position is indicated by a dot.

$1_{SQV\text{-}5\ \mu M}$) (a sixfold increase in the EC_{50} of SQV compared with that of GRL-02031).

When the virus was selected with up to 5 μM GRL-02031 (HIV-$1_{GRL\text{-}02031\text{-}5\ \mu M}$) was examined in MT-4 cells, the EC_{50} of GRL-02031 turned out to be >1 μM although HIV-$1_{GRL\text{-}02031\text{-}5\ \mu M}$ remained susceptible to other PIs, in particular, SQV, IDV, and NFV. The HIV-$1_{LPV\text{-}1\ \mu M}$ variant was substantially resistant to APV, IDV, NFV, RTV, LPV, and ATV; however, this variant was highly susceptible to GRL-02031 and had an EC_{50} of 0.038 μM (Table 3). HIV-$1_{ATV\text{-}1\ \mu M}$ variant was also substantially resistant to IDV, NFV, and ATV; however, this variant was susceptible to LPV and GRL-02031. Of note, LPV, which has currently been widely used as a first-line therapeutic among HAART regimens, was not active against three HIV-1 variants (HIV-$1_{SQV\text{-}5\ \mu M}$, a variant selected with 5 μM IDV [HIV-$1_{IDV\text{-}5\ \mu M}$], and a variant selected with 5 μM NFV [HIV-$1_{NFV\text{-}5\ \mu M}$]), with the differences in the EC_{50}s being more than 16-fold compared to the value for wild-type strain HIV-$1_{NL4\text{-}3}$. This anti-HIV-1 profile of LPV greatly contrasted with that of GRL-02031. GRL-02031 was highly potent against all the variants examined except HIV-$1_{SQV\text{-}5\ \mu M}$ (sixfold change in the EC_{50} compared to that for HIV-$1_{NL4\text{-}3}$). It is also noteworthy that SQV, IDV, and NFV remained potent against HIV-$1_{GRL\text{-}02031\text{-}5\ \mu M}$, suggesting that the combination of GRL-02031 and SQV, IDV, or NFV could exert complementarily augmented activity against multi-PI-resistant HIV-1 variants.

Sensitivities of infectious molecular HIV-1 clones carrying various amino acid substitutions to GRL-02031. Finally, we attempted to determine the profile of the activity of GRL-02031 against a variety of HIV-$1_{NL4\text{-}3}$-based molecular infectious clones containing a single primary mutation previously reported or a combination of such mutations (Table 4) (21, 31, 40–42). Interestingly, no significant changes in EC_{50}s were observed when HIV-1 clones containing only one of the amino acid substitutions (L10F, L33F, M46I, I47V, Q58E, V82I,

TABLE 3. Antiviral activities of GRL-02031 against laboratory PI-resistant HIV-1 variants[a]

Virus	EC_{50} (μM)							
	SQV	APV	IDV	NFV	RTV	LPV	ATV	GRL-02031
$HIV\text{-}1_{NL4\text{-}3}$	0.008 ± 0.004	0.028 ± 0.009	0.014 ± 0.004	0.018 ± 0.009	0.021 ± 0.009	0.018 ± 0.001	0.0043 ± 0.0004	0.023 ± 0.008
$HIV\text{-}1_{SQV\text{-}5\ \mu M}$	>1 (>125)	0.21 ± 0.09 (8)	>1 (>71)	0.32 ± 0.04 (18)	>1 (>48)	0.70 ± 0.22 (39)	0.32 ± 0.02 (74)	0.14 ± 0.06 (6)
$HIV\text{-}1_{APV\text{-}5\ \mu M}$	0.016 ± 0.010 (2)	>1 (>36)	0.22 ± 0.15 (16)	0.17 ± 0.10 (10)	>1 (>48)	0.14 ± 0.05 (8)	0.0032 ± 0.012 (1)	0.037 ± 0.004 (2)
$HIV\text{-}1_{IDV\text{-}5\ \mu M}$	0.022 ± 0.009 (3)	0.26 ± 0.14 (9)	>1 (>71)	0.65 ± 0.16 (36)	>1 (>48)	>1 (>56)	0.063 ± 0.022 (15)	0.047 ± 0.002 (2)
$HIV\text{-}1_{NFV\text{-}5\ \mu M}$	0.028 ± 0.009 (4)	0.078 ± 0.026 (3)	0.27 ± 0.06 (19)	>1 (>56)	0.08 ± 0.05 (4)	0.29 ± 0.03 (16)	0.024 ± 0.005 (6)	0.036 ± 0.002 (2)
$HIV\text{-}1_{RTV\text{-}5\ \mu M}$	0.010 ± 0.008 (1)	0.43 ± 0.27 (15)	0.30 ± 0.07 (21)	0.24 ± 0.10 (13)	>1 (>48)	0.11 ± 0.08 (6)	0.021 ± 0.007 (5)	0.077 ± 0.031 (3)
$HIV\text{-}1_{LPV\text{-}1\ \mu M}$	0.033 ± 0.003 (4)	0.32 ± 0.02 (11)	>1 (>71)	0.51 ± 0.06 (28)	>1 (>48)	0.30 ± 0.04 (17)	0.041 ± 0.002 (10)	0.038 ± 0.004 (2)
$HIV\text{-}1_{ATV\text{-}1\ \mu M}$	0.034 ± 0.006 (4)	0.18 ± 0.06 (6)	0.33 ± 0.10 (24)	0.21 ± 0.03 (12)	0.14 ± 0.02 (7)	0.025 ± 0.011 (1)	0.31 ± 0.05 (72)	0.033 ± 0.002 (1)
$HIV\text{-}1_{GRL\text{-}02031\text{-}5\ \mu M}$	0.008 ± 0.001 (1)	0.21 ± 0.02 (8)	0.044 ± 0.015 (3)	0.011 ± 0.004 (1)	0.26 ± 0.10 (12)	0.14 ± 0.09 (8)	0.036 ± 0.005 (8)	>1 (>43)

[a] The amino acid substitutions identified in the protease-encoding region of $HIV\text{-}1_{SQV\text{-}5\ \mu M}$, $HIV\text{-}1_{APV\text{-}5\ \mu M}$, $HIV\text{-}1_{IDV\text{-}5\ \mu M}$, $HIV\text{-}1_{NFV\text{-}5\ \mu M}$, $HIV\text{-}1_{RTV\text{-}5\ \mu M}$, $HIV\text{-}1_{LPV\text{-}1\ \mu M}$, $HIV\text{-}1_{ATV\text{-}1\ \mu M}$, and $HIV\text{-}1_{GRL\text{-}02031\text{-}5\ \mu M}$ compared to the consensus B sequence cited from the Los Alamos National Laboratory database include L10I/G48V/I54V/L90M, L10F/V32I/M46I/I54M//A71V/I84V, L10F/L24I/M46I/L63P/A71V/G73S/V82T, L10F/D30N/K45I/A71V/T74S, M46I/V82F/I84V, L10F/M46I/I54V/V82A, L23I/K43I/M46I/I50L/G51A/A71V, and L10F/L33F/M46I/I47V/Q58E/V82I/I84V/I85V, respectively. MT-4 cells (1×10^4) were exposed to each HIV-1 isolate (100 $TCID_{50}$), and the inhibition of p24 Gag protein production by the drug was used as the endpoint. The numbers in parentheses represent the fold changes in the EC_{50} for each isolate compared to the EC_{50} for $HIV\text{-}1_{NL4\text{-}3}$. The data shown are mean values (±1 standard deviation) derived from the results of three independent experiments conducted in triplicate.

I84V, or I85V) which emerged in the selection process in the present work (Fig. 3) were tested with GRL-02031. We tested each of these one-mutation-containing infectious clones against a selected PI, and again, no significant changes in EC_{50}s were seen (Table 4).

It was noted that increases in the EC_{50}s of GRL-02031 were seen only when more than two amino acid substitutions were introduced into $HIV\text{-}1_{NL4\text{-}3}$. A substantial reduction in susceptibility (differences in EC_{50}s of more than threefold) was seen when the virus had two mutations (M46I/I47V or I84V/I85V). Further increases in the EC_{50}s were seen when four substitutions (L10F/I47V/V82I/I85V) or five substitutions (L10F/M46I/I47V/V82I/I85V) were introduced. Moreover, we generated molecular clones containing a primary mutation with which HIV-1 is known to acquire substantial resistance to a PI(s) (such as D30N, G48V, I50V, and L90M) and determined the EC_{50}s of GRL-02031 (Table 4). We found that GRL-02031 was potent against all such molecular clones with a primary mutation, with the differences in the EC_{50}s being 0.7- to 1.7-fold in comparison with the EC_{50} for $HIV\text{-}1_{NL4\text{-}3}$, although $HIV\text{-}1_{D30N}$ and $HIV\text{-}1_{G48V}$ showed moderate to substantial levels of resistance to NFV (5.6-fold change) and SQV (5.1-fold change) (Table 4). These data suggest that HIV-1 can acquire substantial resistance to GRL-02031 only when it gains multiple mutations in the protease, a potentially advantageous property of GRL-02031.

Structural analysis of GRL-02031 interactions with wild-type protease. Finally, we conducted molecular and structural analyses of the interactions of GRL-02031 with protease (Fig. 4). By refined structural modeling based on the previously published crystal structures 2FDE (protease-brecanavir complex) and 2IEN (protease-DRV complex), we found that the oxygen atom of Cp-THF has a hydrogen bond interaction with Asp29 in the S-2 pocket of the protease. The hydrogen bond interactions of GRL-02031 with Asp25 and Gly27, which have been observed for various PIs, were also predicted to be present. In addition, GRL-02031 has hydrogen bond interactions mediated through a water molecule with flap residues Ile50 and Ile50′. Of note, GRL-02031 has an *R* configuration at the pyrrolidone stereocenter. Interestingly, the structural models demonstrated that for the *R*-stereochemical configuration, two distinct binding modes of GRL-02031 were found in the S-2′ pocket. The 2-pyrrolidone group and the methoxybenzene moiety can orient toward Asp29′ and Asp30′ for configuration 1 and configuration 2, respectively (Fig. 4A and B). In configuration 1, the 2-pyrrolidone oxygen has hydrogen bond interactions with Asp29′ in the S-2′ pocket. In configuration 2, the methoxybenzene orients toward the S-2′ pocket and forms tight hydrogen bonds with Asp30′. The interactions of PIs (48) with Asp29′ and/or Asp30′ have been reported to be mediated by water molecules. It is likely that the presence of water molecules may influence the relative abundance of configurations 1 and 2. The alternate bimodal binding feature observed in this molecular analysis should provide advantages to the PI in maintaining its antiviral potency when the HIV-1 protease either has a polymorphism or develops amino acid substitutions under drug pressure.

We also examined the lipophilic potential of the computationally defined cavity for the binding of GRL-02031 within the HIV protease (Fig. 4C). It was revealed that GRL-02031 fits

TABLE 4. Sensitivities of infectious molecular HIV clones carrying various amino acid substitutions to GRL-02031[a]

Recombinant HIV-1 clone	EC_{50} (μM) of GRL-02031	Fold change in EC_{50}	Fold change in EC_{50} compared with that of other PIs	Fold change in EC_{50} of other PIs reported previously (reference)
pNL4-3 (wild-type)	0.023 ± 0.008	1.0		
L10F	0.037 ± 0.001	1.6	1.4 (APV)	1.5 (IDV) (42)
L33F	0.028 ± 0.005	1.2	1.0 (RTV)	1.4 (APV) (31)
M46I	0.028 ± 0.009	1.2	1.2 (RTV)	1.0 (APV) (40)
I47V	0.037 ± 0.006	1.6	1.2 (APV)	2.2 (APV) (31)
Q58E	0.033 ± 0.007	1.4	1.0 (APV)	Not previously reported
V82I	0.035 ± 0.001	1.5	1.5 (RTV)	1.9 (APV) (31)
I84V	0.030 ± 0.0001	1.3	2.2 (IDV)	10.6 (IDV) (41)
I85V	0.024 ± 0.011	1.0	2.1 (RTV)	Not previously reported
M46I/I47V	0.073 ± 0.009	3.2	1.3 (APV)	1.0 (APV) (40)
V82I/I85V	0.035 ± 0.002	1.5	1.6 (RTV)	Not previously reported
I84V/I85V	0.097 ± 0.010	4.2	14.8 (RTV)	Not previously reported
L10F/I47V/V82I/I85V	0.43 ± 0.06	18.7	1.9 (RTV)	Not previously reported
L10F/M46I/I47V/V82I/I85V	>1	>43	10.0 (APV)	Not previously reported
D30N	0.020 ± 0.009	0.9	5.6 (NFV)	6.0 (NFV) (41)
G48V	0.040 ± 0.0008	1.7	5.1 (SQV)	7.0 (SQV) (21)
I50V	0.015 ± 0.008	0.7	1.2 (APV)	3.5 (APV) (31)
L90M	0.032 ± 0.001	1.4	1.0 (SQV)	3.0 (SQV) (21)

[a] MT-4 cells (1×10^4/ml) were exposed to 100 $TCID_{50}$s of each infectious molecular HIV clone, and the inhibition of p24 Gag protein production by the drug was used as the endpoint on day 7 in culture. The fold change represents the ratio of the EC_{50} for each mutant clone to the EC_{50} for wild-type $HIV\text{-}1_{NL4\text{-}3}$. All assays were performed in triplicate, and the values shown are mean values (±1 standard deviation) derived from the results of three independent experiments.

tightly in the binding cavity and has favorable polar and nonpolar interactions with the active-site residues of the HIV-1 protease. The van der Waals surfaces of Ile47 and Ile47′ and of Ile84′ demonstrate that they form tight nonpolar interactions with GRL-02031. Our antiviral data showing that the I47V substitution is associated with HIV-1 resistance to GRL-02031 (Tables 3 and 4) are in agreement with this structural finding, in that the substitution should reduce GRL-02031's interaction with protease and helps develop HIV-1 resistance to the inhibitor.

DISCUSSION

In the present work, we demonstrated that GRL-02031 suppresses the replication of a wide spectrum of HIV-1 isolates and is potent against a variety of HIV-1 variants highly resistant to multiple PIs, with the differences in the EC_{50}s being less than twofold in comparison with the EC_{50} for wild-type strain $HIV\text{-}1_{ERS104pre}$ (Table 2). Additionally, when $HIV\text{-}1_{NL4\text{-}3}$ was propagated in the presence of increasing concentrations of IDV, APV, or GRL-02031, the time of emergence of HIV-1 variants highly resistant to GRL-02031 was substantially delayed compared to that of IDV- or APV-resistant HIV-1 variants (Fig. 2). Indeed, 21, 27, and 37 passages were required for HIV-1 to acquire the ability to propagate in the presence of APV, IDV, and GRL-02031 at 5 μM, respectively. In this regard, when we generated a variety of PI-resistant HIV-1 variants by propagating laboratory strain $HIV\text{-}1_{NL4\text{-}3}$ in the presence of increasing concentrations of a PI in MT-4 cells using the same procedure as that used in the present study, it required 27, 23, 22, 21, and 14 passages for the virus to propagate in the presence of 5 μM of SQV, APV, IDV, NFV, and RTV, respectively (26). However, it should be noted that the population size of HIV-1 in a culture is relatively small and that the viral acquisition of mutations can be affected by stochastic phenomena. For example, mutations take place at random and the rates of mutations in the HIV-1 genome may not be reproducible, although certain mutations that severely compromise viral replication would not remain in culture.

During the selection of $HIV\text{-}1_{NL4\text{-}3}$ with GRL-02031, the L10F substitution, one of the secondary substitutions, first appeared. The L10F mutation occurs distal to the active site of the enzyme and is thought to act in concert with active-site mutations and compensate for a possible functional deficit caused by the latter (6, 32). Mutations at Leu-10 reportedly occur in 5 to 10% of HIV-1 isolates recovered from untreated HIV-1-infected individuals but increase in prevalence by 60 to 80% in heavily treated patients (19, 22). However, the virological and structural significance of the L10F substitution in HIV-1 resistance to GRL-02031 is presently unknown.

By passage 37, two active-site mutations (V82I and I84V) emerged. These V82 and I84 residues represent active-site residues whose side chains are involved in the formation of the protease substrate cleft and that make direct contact with certain PIs (48), and the V82I substitution has been shown to be effective in conferring resistance when it is combined with a second active-site mutation, such as V32I (23). Another active-site mutation (I85V) and two flap mutations (M46I and I47V) also emerged by passage 30. Both Met46 and Ile47 are located in the flap region of the enzyme; the I47V substitution is reported to be associated with viral resistance to APV and JE-2147 (40, 48). The lipophilic potential of the computationally defined cavity for the binding of GRL-02031 within the HIV protease seems to be related to a finding that the van der Waals surfaces of Ile47 and Ile47′ and of Ile84′ form tight nonpolar interactions with GRL-02031 (Fig. 4C). Our antiviral data showing that the I47V substitution is associated with HIV-1 resistance to GRL-02031 (Table 3) are in agreement with this structural finding. However, it is also of note that HIV-1 acquires substantial resistance to GRL-02031 when the virus gains multiple mutations in the protease (Table 4), as

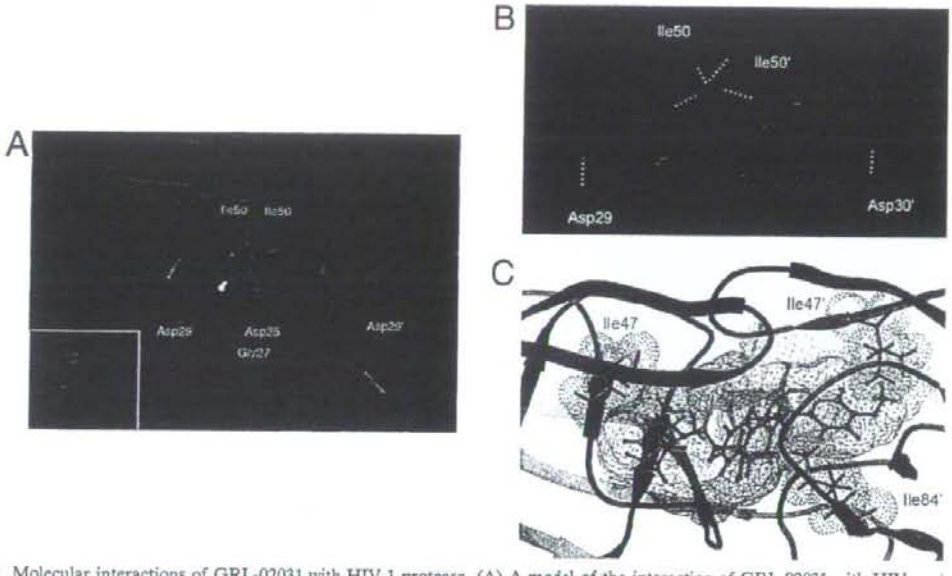

FIG. 4. Molecular interactions of GRL-02031 with HIV-1 protease. (A) A model of the interaction of GRL-02031 with HIV protease. The bird's-eye view of the docked pose (inset) is presented along with a blown-up figure highlighting the important hydrogen bond interactions. The inhibitor is predicted to have hydrogen bond interactions with Asp25, Gly27, Asp29, Ile50, Asp29', and Ile50'. Note that the pyrrolidone oxygen (red stick) interacts with the S-2' subpocket and forms a hydrogen bond interaction with Asp29'. (B) Superimposed binding configurations of GRL-02031 with the HIV-1 protease. The carbons are shown in gray in configuration 1 and in green in configuration 2. Selected hydrogen bond interactions of configuration 2 are shown. In configuration 2, the methoxybenzene interacts with the S-2' site and forms a hydrogen bond interaction with Asp30'. The interaction of the P-2 ligand Cp-THF is the same in both configurations. (C) The binding cavity of HIV protease with lipophilic potential is shown. GRL-02031 fits tightly in the binding cavity and has favorable polar and nonpolar interactions with the active-site residues of the HIV-1 protease. The van der Waals surfaces of Ile47 and Ile47' (both in magenta) and of Ile84' (in purple) demonstrate that they form tight nonpolar interactions with GRL-02031. The protease residues are shown in stick representation. The following atoms are indicated by designated colors: C, gray; O, red; N, blue; S, yellow; H, cyan. Both protease chains are shown in green. The figure was generated with the MOLCAD program (Sybyl, version 8.0; Tripos, L.P, St. Louis, MO).

seen in the case of DRV (9). This resistance profile (i.e., the requirement of multiple mutations) of GRL-02031 may also confer certain advantage in the resistance profile of GRL-02031.

Two mutations at conserved residues, L33F and Q58E, also emerged by passage 37 and were present in 10 and 9 of 10 clones, respectively. L33F has primarily been reported in patients treated with RTV or APV (37). The L33F substitution alone did not change the susceptibility of HIV-1 to GRL-02031 (Table 4), although it has recently gained attention because of its association with resistance to the FDA-approved PI, tipranavir (33).

In the HIV-1 variants selected with GRL-02031, four amino acid substitutions in the Gag proteins (G62R, R409K, L363M, and I437T) were seen by passage 37. R409K within the p7 Gag seems to be associated with viral resistance to APV (14), although the significance of G62R within p17 is as yet unknown. The p7-p1 cleavage-site mutation I437T has been reported to be associated with ATV resistance (17). It is of note that by passage 15, an unusual amino acid substitution, L363M, emerged; this substitution has not previously been reported in relation to PI resistance. This L363M is located at the p24-p2 cleavage site, which represents the C terminus of the capsid (CA) p24 protein that is highly conserved and that is involved in virion assembly. The deletion of this cluster or the introduction of mutations such as L363A is known to cause significant impairment of particle formation and infectivity (34). It is noteworthy that L363M appears in HIV-1 variants resistant to a maturation inhibitor, PA-457 [3-*O*-(3',3'-dimethylsuccinyl) betulinic acid], which binds to the CA-p2 cleavage site or its proximity, blocks the cleavage by protease during virion maturation, and exerts activity against HIV-1 (27, 44, 49).

It was noted that GRL-02031 and SQV remained active against most of the PI-selected HIV-1 variants and that SQV, IDV, and NFV remained potent against HIV-$1_{GRL\text{-}02031\text{-}5\ \mu M}$ (Table 3), suggesting that the combination of GRL-02031, SQV, IDV, and NFV can exert complementarily augmented activity against multi-PI-resistant HIV-1 variants. Such a difference in the resistance profile of GRL-02031 when it is used with SQV and NFV may be due to the differences in binding and antiviral potency associated with the D30N and G48V mutations (Table 4).

In an attempt to explain why GRL-02031 can exert potent activity against a wide spectrum of HIV-1 variants resistant to multiple PIs, we performed structural modeling and molecular docking of the interactions of GRL-02031 with protease (Fig. 4). Interestingly, our structural modeling analysis demonstrated that there are two distinct binding modes of GRL-

02031 in the S-2′ pocket of the protease. Either the 2-pyrrolidone group or the methoxybenzene moiety can orient toward Asp29′ and Asp30′ (configuration 1 and configuration 2, respectively) (Fig. 4B). It is presumed that such alternate binding modes provide distinct advantages to GRL-02031 in maintaining its antiviral activity against a wide spectrum of HIV-1 variants resistant to other currently available PIs. The alternate binding modes could explain the reason why the development of resistance to GRL-02031 is substantially delayed compared to the time to the development of resistance to APV or IDV (Fig. 2). In addition, the models of GRL-02031 indicated that it is capable of forming hydrogen bond interactions with the backbone atoms of Asp29, Asp29′, and/or Asp30′. Such backbone interactions have been shown to be important in maintaining potency not only against wild-type protease but also against drug-resistant mutant proteases (1, 15, 16, 36). This may also explain why GRL-02031 maintains its potency against a wide variety of drug-resistant mutant proteases.

It is of note that the difference seen with GRL-02031 (one- to twofold) seems substantially less than that seen with DRV (one- to sevenfold) (Table 2). Although this difference may not be translated into an actual difference in the clinical setting, it is worth noting that GRL-02031 may have certain advantages in its activity against highly drug-resistant HIV-1 variants. Considering that the acquisition of multiple amino acid substitutions is required for the emergence of HIV-1 resistance to GRL-02031, the profile of HIV-1 resistance to GRL-02031, which is apparently different from the profiles for the other PIs, might result in an advantage for GRL-02031, although further evaluations, including testing of the compound in the clinical setting, are required.

Taken together, GRL-02031 exerts potent activity against a wide spectrum of laboratory and clinical wild-type and multidrug-resistant HIV-1 strains without significant cytotoxicity in vitro and substantially delays the emergence of HIV-1 variants resistant to GRL-02031. These data warrant further consideration of GRL-02031 as a candidate as a novel PI for the treatment of AIDS.

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Letter

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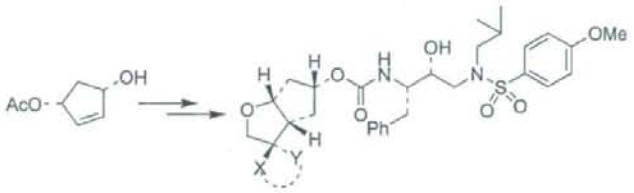

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Design and Synthesis of Stereochemically Defined Novel Spirocyclic P2-Ligands for HIV-1 Protease Inhibitors

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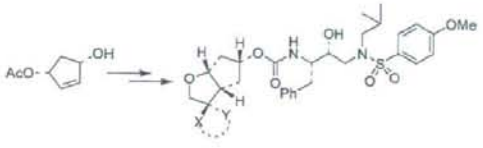
ABSTRACT



The synthesis of a series of stereochemically defined spirocyclic compounds and their use as novel P2-ligands for HIV-1 protease inhibitors are described. The bicyclic core of the ligands was synthesized by an efficient nBu$_3$SnH-promoted radical cyclization of a 1,6-enyne followed by oxidative cleavage. Structure-based design, synthesis of ligands, and biological evaluations of the resulting inhibitors are reported.

The introduction of highly active antiretroviral therapy (HAART) in 1996, in combination with HIV-1 protease inhibitors and reverse transcriptase inhibitors, has dramatically changed the management of HIV/AIDS.[1] The advent of HAART has significantly reduced morbidity and mortality and has improved the quality of life for HIV-infected patients, particularly in developed nations.[2] Despite this important breakthrough, current and future management of HIV/AIDS is being challenged by the rapid emergence of multi-drug-resistant HIV-1 strains and drug-related side effects.[3] Consequently, development of novel and effective treatment regimens are critically important.

In our continuing effort to design a new generation of HIV-1 protease inhibitors (PIs) that combat drug resistance, we developed a series of exceedingly potent PIs. A number of these nonpeptidyl PIs have shown superb antiviral activity and drug-resistance profiles. In our structure-based design strategies, we introduced the "backbone binding concept" with the presumption that an inhibitor that makes maximum interactions in the protease active site, particularly hydrogen bonding with the backbone atoms, may retain its potency against mutant strains.[4] Darunavir (**1**, Figure 1), which has been approved by the FDA for the treatment of patients harboring multi-drug-resistant HIV-1 strains, has emerged from this approach.[5,6] Our detailed X-ray structural analysis of protein–ligand complexes revealed an extensive hydrogen

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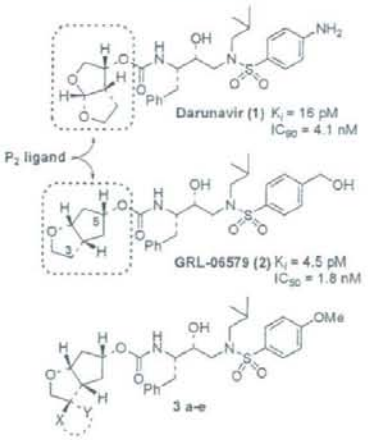

Figure 1. Structure of HIV protease inhibitors.

bonding network with HIV-1 protease backbone atoms and most notably with the designed bis-THF P2-ligand.[7]

More recently, we reported another novel PI, GRL-06579 (**2**), which features a stereochemically defined bicyclic hexahydrocyclopentylfuran (Cp-THF) P2-ligand in the hydroxyethylsulfonamide isostere core.[8] The X-ray crystallographic analysis of **2**-bound HIV-1 protease documented extensive hydrogen bonding interactions including the Cp-THF oxygen with the backbone atoms in the S2-subsite.[8]

The favorable drug-resistance profile of this PI containing the Cp-THF ligand logically prompted us to design several structural analogs. We set out to introduce new functionalities on this bicyclic core that could create additional interactions within the enzyme catalytic site. The 3-position of the Cp-THF ligand appeared particularly suitable for this purpose, because of its proximity to the flap region and the S2-subsite of the protease. Based upon our analysis of the X-ray crystal structure of **2**-bound protease, we planned to investigate the effect of a structurally constrained spirocyclic motif at the 3-position of the Cp-THF ring. We speculated that a cyclic ether oxygen or an oxazolidinone carbonyl oxygen may be positioned in this cyclic motif to accept a hydrogen bond from the enzyme active site residues or a backbone NH. Such a functionality would fill in the hydrophobic pocket in the S2-subsite as well. Furthermore, this structural feature may improve the pharmacological profile of these inhibitors.[9,10]

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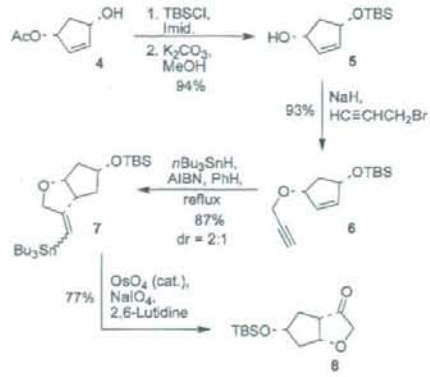
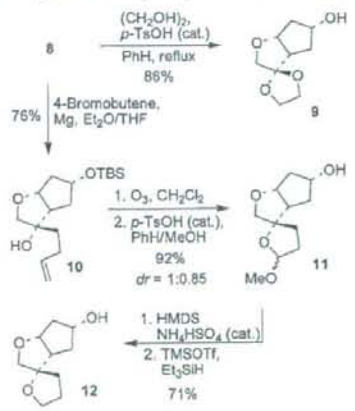
Scheme 1. Synthesis of Bicyclic Ketone **8**



We initially set out to synthesize a series of spirocyclic Cp-THF-derived P2-ligands and their corresponding HIV-protease inhibitors (**3a**−**e**). A synthetic strategy was devised so that all analogs could be synthesized from a common precursor that gives rapid access to new polycyclic molecular probes. The general synthesis of the bicyclic core of our new P2-ligands was accomplished in enantiomerically pure form as shown in Scheme 1. Optically active monoacetate **4** was obtained in 95% ee by desymmetrization of the corresponding *meso*-diacetate with acetyl cholinesterase.[11] Protection of alcohol **4** as a TBS ether followed by methanolysis of the acetyl group furnished compound **5**. Propargylation of **5** using propargyl bromide in the presence of NaH provided alkyne **6** in excellent yield.

The construction of the bicyclic core was accomplished by an intramolecular radical cyclization of alkyne **6** using nBu$_3$SnH and AIBN in benzene at reflux. This provided vinyl stannane **7** as a mixture of *cis*/*trans* diastereoisomers (2:1),

Scheme 2. Synthesis of Spirocyclic Ketal and Ether Ligands



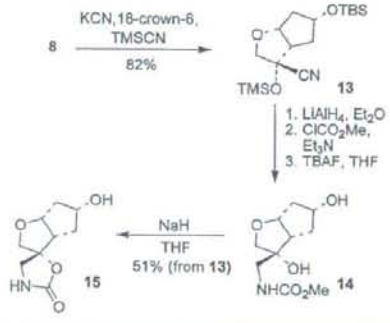
along with trace amounts of the olefin, which presumably formed during purification on silica gel. The mixture of isomers was directly oxidized with a catalytic amount of OsO_4 in the presence of $NaIO_4$ and 2,6-lutidine to afford the key intermediate, ketone **8** in 77% yield.

We first turned our attention to the synthesis of spirocyclic ketal **9** and ether **12**. Molecular modeling of the corresponding inhibitors suggested that the ligand oxygens could be within hydrogen bonding distance to the NH amide bonds of both Asp30 and Asp29 residues.

Spirocyclic dioxolane ligand **9** was obtained in 86% yield by treatment of ketone **8** with ethylene glycol in benzene with a catalytic amount of *p*-TsOH. Synthesis of ether **12** was achieved in four consecutive steps starting from ketone **8**. Reaction of **8** with homoallyl magnesium bromide furnished compound **10** in 76% yield. Ozonolysis of the terminal alkene and refluxing the resulting crude aldehyde in benzene/methanol followed by azeotropic distillation of the excess methanol afforded methyl acetal **11** as a mixture (1:0.85) of diastereoisomers. Reduction of this acetal intermediate **11** furnished the desired alcohol **12** by applying a one-pot procedure involving (1) TMS-protection of the alcohol with hexamethyldisilazane and (2) subsequent reduction of the acetal with triethylsilane.[12]

We have designed spirocyclic oxazolidinone ligands that could potentially exploit polar interactions with the backbone atoms and residues in the HIV-1 protease active site. Their respective syntheses are highlighted in Scheme 3 and 4.

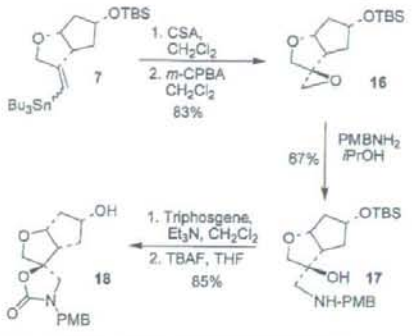
Scheme 3. Synthesis of Spirooxazolidinone Ligand **15**



Cyanohydrin **13** was synthesized in 82% yield from ketone **8**. $LiAlH_4$-reduction of the cyanide provided the corresponding amine, which exhibited partial TMS-deprotection. Therefore, the crude mixture was directly submitted to the next steps with (1) formation of the methyl carbamate derivative and (2) removal of the silyl ethers with TBAF in THF. The resulting diol **14** was then treated with NaH in THF to give oxazolidinone ligand **15** in 51% yield over four steps (from **13**).

Synthesis of oxazolidinone **18** started with vinylstannane **7** (Scheme 4). Proto-destannylation of **7** was carried out with CSA in CH_2Cl_2. Epoxidation of the resulting olefin with *m*-CPBA gave epoxide **16** as a major diastereomer (93:7 ratio). Opening of the epoxide with *p*-methoxybenzylamine

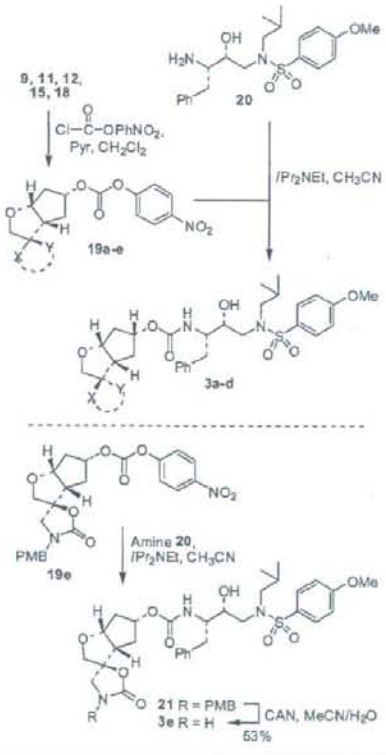
Scheme 4. Synthesis of Spirooxazolidinone Ligand **18**



gave amino alcohol **17** in 67% yield. The carbonyl was installed using triphosgene and Et_3N in CH_2Cl_2. Deprotection of the TBS-group provided the desired oxazolidinone **18**.

The synthesis of polycyclic PIs is shown in Scheme 5. Various synthetic ligands were reacted with 4-nitrophenylchloroformate and pyridine to form the corresponding activated carbonates, **19a–e**. Reaction of the respective

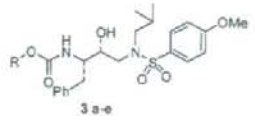
Scheme 5. Synthesis of Inhibitors **3a–e**



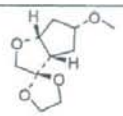
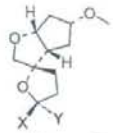
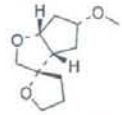
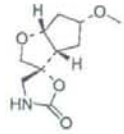
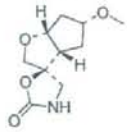
active carbonate with known[8] amine **20** in the presence of diisopropylethylamine afforded PIs **3a**−**d**. For the synthesis of inhibitor **3e**, amine **20** was reacted with active carbonate **19e** to provide urethane **21**. Removal of the PMB group from **21** by exposure to ceric ammonium nitrate (CAN) afforded inhibitor **3e**.

We examined all inhibitors for their enzymatic potency as well as their cellular activity, and the results are displayed in Table 1. As shown, most inhibitors exhibited excellent enzymatic potency. Dioxolane-based analogue **3a** displayed a K_i value of 0.16 nM. Inhibitor **3b** contains the spirocyclic methyl acetal as a mixture (1:0.85 ratio) of diastereomers. These diastereomers were separated by HPLC, and the stereochemical identity of each diastereomer was determined by extensive NOESY experiments. Diastereomer **3b**-(*S*) showed an enzymatic K_i of 0.81 nM. The **3b**-(*R*) isomer is slightly more potent (K_i = 0.38 nM). The removal of the methoxy group from **3b** resulted in inhibitor **3c**, which showed a loss of enzyme inhibitory activity. Both inhibitors **3b** and **3c** have shown comparable antiviral activity. We have examined stereochemically defined oxazolidinone derivatives as P2-ligands. Inhibitor **3d** displayed a K_i of 0.29 nM. Diastereomeric inhibitor **3e** is slightly more potent than **3d** in both enzyme inhibitory as well as in antiviral assays (IC_{50} = 21 nM in MT-2 cells). The inhibitors in Table 1 in general are significantly less potent than UIC-PI (TMC-126),[14] the corresponding methoxysulfonamide derivative of darunavir or Cp-THF-containing inhibitor **2**.[8]

Table 1. Enzymatic and Antiviral Activity of PIs



3 a-e

Inhibitor	R	K_i (nM)[a]	IC_{50} (μM)[b]
3a		0.16	0.28
3b		**3b**-(*S*) isomer (X=OMe,Y=H): 0.81 **3b**-(*R*) isomer (X=H,Y=OMe): 0.38	0.23[c]
3c		2.22	0.17
3d		0.29	0.093
3e		0.17	0.021

[a] K_i determined following protocol as described by Toth and Marshall, mean values of at least four determinations.[13] [b] MT-2 cells (2×10^4/mL) were exposed to 100 $TCID_{50}$ of HIV-1_{LAI} and cultured in the presence of various concentrations of PIs, and the IC_{50}'s were determined by using the MTT assay on day 7 of culture.[6] [c] Tested as a 1:0.85 mixture.

In conclusion we have designed and synthesized a series of inhibitors containing stereochemically defined novel spirocyclic P2-ligands. The syntheses of these ligands were carried out from the key intermediate **8**, which was efficiently prepared in optically active form by using a radical cyclization as the key step. The spirooxazolidinone-derived inhibitor **3e** is the most potent inhibitor in this series. While these inhibitors contain novel P2-ligands, it appears that the spirocyclic motif at the 3-position of the Cp-THF ring resulted in a significant reduction in potency. Further design and optimization of the ligand binding site interactions are in progress.

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Supporting Information Available: Experimental procedures, spectral data, and ^1H NMR and ^{13}C NMR spectra for compounds **5**−**21** and **3a**−**e**. This material is available free of charge via the Internet at http://pubs.acs.org.

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