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A Novel Bis-Tetrahydrofuranylurethane-Containing Nonpeptidic Protease Inhibitor (PI), GRL-98065, Is Potent against Multiple-PI-Resistant Human Immunodeficiency Virus In Vitro▽

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We designed, synthesized, and identified GRL-98065, a novel nonpeptidic human immunodeficiency virus type 1 (HIV-1) protease inhibitor (PI) containing the structure-based designed privileged cyclic ether-derived nonpeptide P2 ligand, 3(*R*),3a(*S*),6a(*R*)-bis-tetrahydrofuranylurethane (bis-THF), and a sulfonamide isostere, which is highly potent against laboratory HIV-1 strains and primary clinical isolates (50% effective concentration [EC_{50}], 0.0002 to 0.0005 μM) with minimal cytotoxicity (50% cytotoxicity, 35.7 μM in $CD4^+$ MT-2 cells). GRL-98065 blocked the infectivity and replication of each of the HIV-1_{NL4-3} variants exposed to and selected by up to a 5 μM concentration of saquinavir, indinavir, nelfinavir, or ritonavir and a 1 μM concentration of lopinavir or atazanavir (EC_{50}, 0.0015 to 0.0075 μM), although it was less active against HIV-1_{NL4-3} selected by amprenavir (EC_{50}, 0.032 μM). GRL-98065 was also potent against multiple-PI-resistant clinical HIV-1 variants isolated from patients who had no response to existing antiviral regimens after having received a variety of antiviral agents, HIV-1 isolates of various subtypes, and HIV-2 isolates examined. Structural analyses revealed that the close contact of GRL-98065 with the main chain of the protease active-site amino acids (Asp29 and Asp30) is important for its potency and wide-spectrum activity against multiple-PI-resistant HIV-1 variants. The present data demonstrate that the privileged nonpeptide P2 ligand, bis-THF, is critical for the binding of GRL-98065 to the HIV protease substrate binding site and that this scaffold can confer highly potent antiviral activity against a wide spectrum of HIV isolates.

Highly active antiretroviral therapy (HAART) has had a major impact on the AIDS epidemic in industrially advanced nations; however, no eradication of human immunodeficiency virus type 1 (HIV-1) appears to be currently possible, in part due to the viral reservoirs remaining in blood and infected tissues. Moreover, we have encountered a number of challenges in bringing the optimal benefits of the currently available therapeutics of AIDS and HIV-1 infection to individuals receiving HAART (2, 29, 30). They include (i) drug-related toxicities; (ii) partial restoration of immunologic functions once individuals have developed AIDS; (iii) development of various cancers as a consequence of survival prolongation; (iv) flameup of inflammation in individuals receiving HAART or immune reconstruction syndrome; and (v) increased cost of antiviral therapy. Such limitations and flaws of HAART are exacerbated by the development of drug-resistant HIV-1 variants (1, 5, 12, 13, 20).

Successful antiviral drugs, in theory, exert their virus-specific effects by interacting with viral receptors, virally encoded enzymes, viral structural components, viral genes, or their transcripts without disturbing the cellular metabolism or function. However, at present, no antiretroviral drugs or agents are likely to be completely specific for HIV-1 or to be devoid of toxicity or side effects in the therapy of AIDS, which has been a critical issue because patients with AIDS and its related diseases will have to receive antiretroviral therapy for a long period of time, perhaps for the rest of their lives. Thus, the identification of new class of antiretroviral drugs which have a unique mechanism(s) of action and produce no or minimal side effects remains an important therapeutic objective.

We have been focusing on the design and synthesis of nonpeptidyl protease inhibitors (PIs) that are potent against HIV-1 variants resistant to the currently approved PIs. One such anti-HIV-1 agent, darunavir (DRV)/TMC114, contains a structure-based designed privileged nonpeptidic P2 ligand, 3(*R*),3a(*S*),6a(*R*)-bis-tetrahydrofuranylurethane (bis-THF) (6, 7, 18). DRV has recently been approved as a therapeutic agent for the treatment of individuals who harbor multidrug-resistant HIV-1 variants and do not respond to previously existing HAART regimens. Incorporation of bis-THF also conferred on other PIs, including brecanavir/GW640385, potent antiviral

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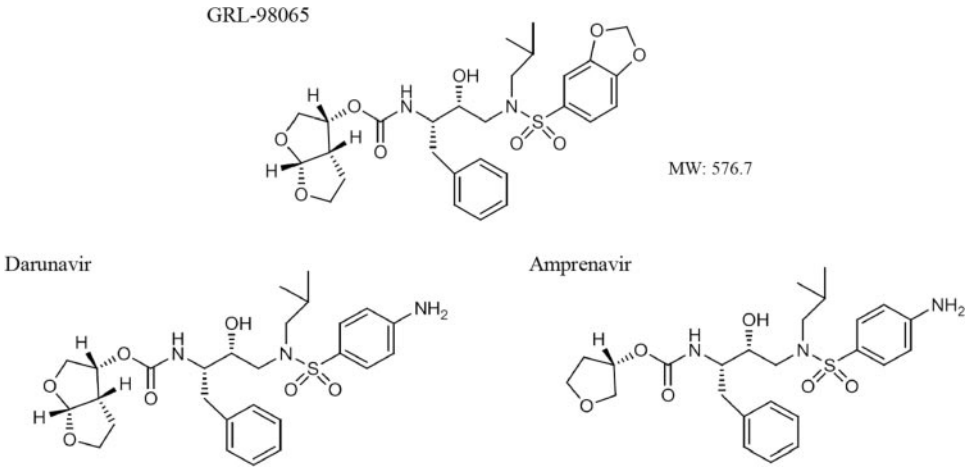

FIG. 1. Structures of GRL-98065, darunavir, and amprenavir.

activity against a wide spectrum of PI-resistant HIV-1 variants (9, 10, 23), although clinical development of brecanavir has been discontinued due to its inherent formulation difficulty.

In the present work, we report the synthesis and biological properties of a potent nonpeptidic HIV-1 protease inhibitor, GRL-98065, which also contains bis-THF and a sulfonamide isostere. GRL-98065 exerts highly potent activity against a wide spectrum of laboratory HIV-1 strains and primary clinical isolates, including multiple-PI-resistant variants, with minimal cytotoxicity. GRL-98065 was also active against HIV-1 isolates of various subtypes, as well as the HIV-1 isolates examined. Structural analyses revealed that the close contact (backbone hydrogen bonding) of GRL-98065 with the main chain of the protease active-site amino acids (Asp29 and Asp30) is critical for its potency and wide-spectrum activity against multiple-PI-resistant HIV-1 variants.

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 (PAA Laboratories GmbH, Linz, Austria) plus 50 U of penicillin and 100 μg of kanamycin per ml. The following HIV strains were used for the drug susceptibility assay: HIV-1_{LAI}, HIV-1_{NL4-3}, HIV-2_{EHO}, HIV-2_{ROD}, clinical HIV-1 strains from drug-naive patients with AIDS (HIV-$1_{ERS104pre}$) (28), and six HIV-1 clinical isolates that were originally isolated from patients with AIDS who had received anti-HIV-1 therapy heavily (for 32 to 83 months) and that were genotypically and phenotypically characterized as multiple-PI-resistant HIV-1 variants. We also used five HIV-1 isolates of different subtypes: HIV-$1_{92UG029}$ (subtype A; X4), HIV-$1_{92UG037}$ (subtype A; R5), HIV-1_{Ba-L} (subtype B; R5), HIV-$1_{97ZA003}$ (subtype C; R5), and HIV-$1_{92TH019}$ (subtype E; R5). These five HIV-1 isolates were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH. To determine whether each clinical HIV-1 isolate used in the present study was a syncytium-inducing (X4 virus) or non-syncytium-inducing (R5 virus) strain, MT-2 cells (10^5) were exposed to an aliquot of viral stock supernatant containing 100 50% tissue culture infectious doses ($TCID_{50}$s) of the virus and cultured in 12-well culture plates. Cultures were maintained for 4 weeks and were examined under an inverted microscope to determine the syncytium-inducing or non-syncytium-inducing nature of the virus, as described previously (33).

Antiviral agents. GRL-98065 (Fig. 1), a novel nonpeptidic PI containing bis-THF, was designed and synthesized by Ghosh and coworkers as described below. Saquinavir (SQV) and ritonavir (RTV) were kindly provided by Roche Products, Ltd. (Welwyn Garden City, United Kingdom) and Abbott Laboratories (Abbott Park, IL), respectively. Amprenavir (APV) was a kind gift from GlaxoSmithKline (Research Triangle Park, NC). Nelfinavir (NFV), indinavir (IDV), and lopinavir (LPV) were kindly provided by Japan Energy, Inc., Tokyo, Japan. Atazanavir (ATV) was a kind gift from Bristol Myers Squibb (New York, NY).

Synthesis of GRL-98065. The synthesis of GRL-98065 is summarized in Fig. 2. In brief, to a stirred solution of secondary amine 1 (8) (83 mg, 0.25 mmol), *N*,*N*-diisopropylethylamine (65 μl, 0.37 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (3 mg, 0.03 mmol) in THF (2 ml) at room temperature, sulfonyl chloride 2 (21) (60 mg, 0.27 mmol) in THF (1 ml) was added. The mixture was stirred at room temperature for 4 h and then concentrated in vacuum, and the residue was chromatographed with 25% ethyl acetate in hexanes to give sulfonamide 3 (105 mg, 81%) as a white amorphous solid. ^{1}H nuclear magnetic resonance (NMR) ($CDCl_3$, 500 MHz): δ, 7.33 to 7.17 (m, 7H), 6.86 (d, 1H, J = 8 Hz), 6.06 (s, 2H), 4.67 (d, 1H, 8 Hz), 3.78 (d, 2H, J = 24 Hz), 3.08 to 3.06 (m, 2H), 3.02 to 2.98 (m, 1H), 2.96 to 2.86 (m, 2H), 2.84 to 2.80 (m, 1H), 1.88 to 1.82 (m, 1H), 1.34 (s, 9H), 0.88 (dd, 6H, J = 6.5 Hz, 15.5 Hz). ^{13}C NMR ($CDCl_3$, 500 MHz): δ, 189.4, 151.3, 148.1, 137.8, 131.6, 129.4, 128.4, 126.3, 108.2, 107.5, 102.2, 79.6, 72.7, 58.6, 54.6, 53.6, 35.4, 28.1, 27.1, 20.0, 19.8. To a stirred solution of sulfonamide 3 (57 mg, 0.11 mmol) in CH_2Cl_2 (3 ml), trifluoroacetic acid (1 ml) was added. The resulting solution was stirred at room temperature for 1 h, and then the solvent was removed in a vacuum. The residue was dissolved in acetonitrile (2 ml), and

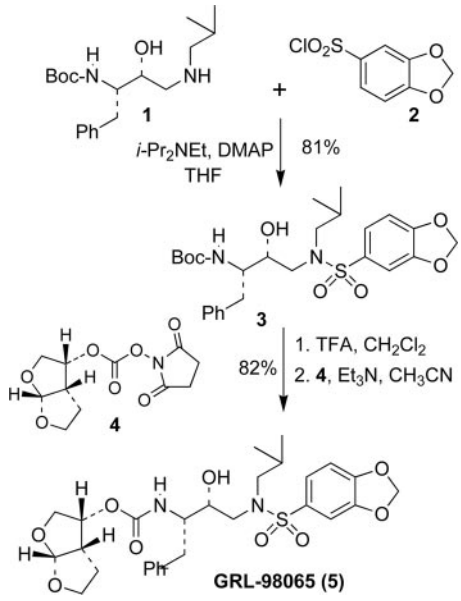

FIG. 2. Synthesis of GRL-98065.

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TABLE 1. Antiviral activities of GRL-98065 against HIV-1_{LAI}, HIV-2_{EHO}, and HIV-2_{ROD} and cytotoxicities[a]

Drug	EC_{50} (μM) for:			CC_{50} (μM)	Selectivity index[b]
	HIV-1_{LAI}	HIV-2_{EHO}	HIV-2_{ROD}		
GRL-98065	0.0005 ± 0.0001	0.0032 ± 0.0007	0.0045 ± 0.0004	35.7	71,400
SQV	0.008 ± 0.001	0.0030 ± 0.0004	0.0043 ± 0.0002	16.4	2,050
RTV	0.054 ± 0.001	0.21 ± 0.05	0.26 ± 0.01	31.1	580
IDV	0.048 ± 0.007	0.024 ± 0.005	0.054 ± 0.003	69.8	1,450
NFV	0.032 ± 0.004	0.030 ± 0.006	0.240 ± 0.009	8.1	250
APV	0.036 ± 0.002	0.25 ± 0.08	0.57 ± 0.01	>100	>2,780
LPV	0.007 ± 0.001	0.0026 ± 0.0008	0.0049 ± 0.0008	>100	>14,300
ATV	0.0048 ± 0.0001	0.005 ± 0.002	0.013 ± 0.006	27.6	5,750
DRV	0.0039 ± 0.0009	0.0080 ± 0.0009	0.0068 ± 0.0004	83.1	21,310

[a] MT-2 cells (2×10^3) were exposed to 100 $TCID_{50}$s of HIV-1_{LAI} or each HIV-2 isolate and cultured in the presence of various concentrations of each PI, and EC_{50}s were determined by using the MTT assay. All assays were conducted in duplicate, and data shown represent mean values (±1 standard deviation) derived from results of three independent experiments.

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

triethylamine (45 μl, 0.32 mmol) and mixed carbonate 4 (8) (30 mg) were added. The mixture was stirred at room temperature for 4 h and then concentrated in vacuum. Column chromatography over silica gel with 30% and then 50% of ethyl acetate in hexanes gave the inhibitor GRL-98065 (5, 51 mg, 82%) as a white amorphous solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ, 7.35 to 7.17 (m, 7H), 6.89 (d, 1H, J = 8.5 Hz), 6.09 (s, 2H), 5.64 (d, 1H, J = 5.5 Hz), 5.05 to 4.97 (m, 2H), 3.97 to 3.94 (m, 1H), 3.89 to 3.83 (m, 3H), 3.72 to 3.67 (m, 2H), 3.16 to 2.95 (m, 4H), 2.93 to 2.87 (m, 1H), 2.83 to 2.79 (m, 2H), 1.86 to 1.81 (m, 1H), 1.68 to 1.55 (m, 1H), 1.50 to 1.42 (m, 1H), 0.94 to 0.88 (dd, 6H, J = 6.5 Hz, 21 Hz). ^{13}C NMR ($CDCl_3$, 500 MHz): δ, 155.4, 151.5, 148.3, 137.5, 131.2, 129.3, 129.2, 128.6, 126.5, 123.0, 109.2, 108.3, 107.3, 102.3, 73.0, 72.8, 70.6, 69.6, 58.8, 55.0, 53.7, 45.2, 35.5, 27.1, 25.7, 19.2.

Drug susceptibility assay. The susceptibilities of HIV-1_{LAI}, HIV-2_{EHO}, and HIV-2_{ROD} to various drugs and the cytotoxicities of those drugs were determined by using the MTT assay. Briefly, MT-2 cells (2×10^4/ml) were exposed to 100 $TCID_{50}$s of HIV-1_{LAI}, HIV-2_{EHO}, or HIV-2_{ROD} in the presence or absence of various concentrations of drugs in 96-well microculture plates and 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 4 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. In some experiments, MT-2 cells were chosen as target cells in the MTT assay, since these cells undergo greater HIV-1-elicited cytopathic effects than MT-4 cells. To determine the sensitivities of HIV-$1_{Ba\text{-}L}$, HIV-$1_{ERS104pre}$, clinical multidrug-resistant HIV-1 isolates, and different subtypes of HIV-1 isolates to drugs, phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBMs) (10^6/ml) were exposed to 50 $TCID_{50}$s of each HIV-1 isolate and cultured in the presence or absence of various concentrations of drugs in 10-fold serial dilutions in 96-well microtiter culture plates. To determine the drug susceptibilities of certain laboratory HIV-1 strains (HIV-$1_{NL4\text{-}3}$), MT-4 cells were used as target cells. MT-4 cells (10^5/ml) were exposed to 100 $TCID_{50}$s of wild-type HIV-$1_{NL4\text{-}3}$ and PI-resistant HIV-$1_{NL4\text{-}3}$ in the presence or absence of various concentrations of drugs and were incubated at 37°C. On day 7 of culture, the supernatant was harvested and the amount of p24 Gag protein was determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio, Inc., Tokyo, Japan) (22). The drug concentrations that suppressed the production of the p24 Gag protein by 50% (50% effective concentrations [EC_{50}s]) were determined by comparison with the level of p24 production in drug-free control cell cultures. All assays were performed in duplicate or triplicate. PHA-PBMs were derived from a single donor in each independent experiment. Thus, to obtain the data, three different donors were recruited.

Generation of PI-resistant HIV-1 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, each at an initial concentration of its EC_{50}. 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 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 1 or 5 μM. In the experiments for selecting drug-resistant variants, MT-4 cells were exploited as target cells, since HIV-1 in general replicates at greater levels in MT-4 cells than in MT-2 cells.

Determination of nucleotide sequences. Molecular cloning and determination of the nucleotide sequences of HIV-1 strains passaged in the presence of anti-HIV-1 agents were performed as described previously (18). 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 with 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, 10 μl of Premix *Taq* (Ex *Taq* version; Takara Bio, Inc., Otsu, Japan), and 10 pmol of each of the first PCR primers in a total volume of 20 μl. The PCR conditions used were an initial 3 min at 95°C, followed by 30 cycles of 40 s at 95°C, 20 s at 55°C, and 2 min at 72°C, with a final 10 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 PCR conditions of an initial 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 20 s at 55°C, and 2 min at 72°C, with a final 10 min of extension at 72°C. 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 a model 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Determination of replication kinetics of GRL-98065-resistant HIV-$1_{NL4\text{-}3}$ variant and wild-type HIV-$1_{NL4\text{-}3}$. MT-4 cells (2.4×10^5) were exposed to the GRL-98065-selected HIV-1 variant at passage 40 (HIV-$1_{GRL98065p40}$) or wild-type HIV-$1_{NL4\text{-}3}$ preparation containing 30 ng p24 in six-well culture plates for 3 h, and the MT-4 cells were divided into three fractions, each cultured with or without GRL-98065 (final concentration of MT-4 cells, 10^4/ml; drug concentrations, 0, 0.01, and 0.1 μM). Amounts of p24 were measured every 2 days for up to 9 days.

Crystallographic analysis. Recombinant HIV-1 protease was expressed and purified as described previously (31). GRL-98065 was dissolved in dimethyl sulfoxide. Crystals were grown by the hanging-drop vapor diffusion method from 4.9 mg/ml protease solution buffered at pH 4.8 with 25 mM sodium acetate in the presence of 10% (wt/vol) sodium chloride, 6% dioxane, and 10% (vol/vol) dimethyl sulfoxide. The crystal was mounted in a fiber loop with 20 to 30% (vol/vol) glycerol as a cryoprotectant. Diffraction data were collected at the National Synchrotron Light Source, beamline X-26C. The data were processed in the space group $P2_12_12$ with unit cell parameters of a = 58.25 Å, b = 85.83 Å, and c = 45.97 Å by using the HKL2000 program (25). The structure was solved by molecular replacement with AMoRe (24) using 1FG6, from the Protein Data Bank, as the starting model. Refinement was carried out using SHELX-97 (27) and manual adjustment with O (16). Alternate conformations for protease residues, inhibitor, water, and other solvent molecules were modeled when observed, as described previously (31). Anisotropic B factors were applied, and

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hydrogen atoms were calculated in the last round of crystallographic refinement by using SHELXL.

Analysis of GRL-98065 interactions with mutant proteases with molecular docking. A model was generated from the crystal structure. Hydrogens were added and optimized, with constraints on heavy atom positions, using the OPLS2005 force field as implemented in MacroModel, version 9.1. Structural figures were generated using Maestro, version 7.5. The interactions of GRL-98065 with six mutant HIV-1 proteases were elucidated with molecular docking using Glide version 4.0 (Schrödinger, LLC, New York, NY). The crystal structures of these mutant proteases were accessed from the Protein Data Bank (PDB), and the native ligand was removed. Close interaction in the protease was annealed, and the docking grid was set up. The conformation of GRL-98065 in its complex with wild-type protease was taken as the starting ligand conformation. The conformational flexibility of GRL-98065 when it binds to protease was taken into account during the docking calculations. The extra-precision mode of Glide, which has a higher penalty for unphysical interactions, was used (4).

RESULTS

Antiviral activity of GRL-98065 against HIV-1_{LAI} and HIV-2. We designed and synthesized GRL-98065 and examined its antiviral activity against a variety of HIV-1 isolates. We found that GRL-98065 was highly potent in vitro against a laboratory wild-type HIV-1 strain, HIV-1_{LAI}, compared to clinically available Food and Drug Administration (FDA)-approved PIs, with EC_{50}s of ~0.0005 μM, as examined with the MTT assay using MT-2 target cells, while its cytotoxicity was seen only at high concentrations, with 50% cytotoxicities (CC_{50}s) of 35.7 μM and a selectivity index of 71,400 (Table 1). In contrast, FDA-approved PIs had EC_{50}s ranging from 0.0039 to 0.054 μM. The selectivity index of GRL-98065 hence proved to be very high at 71,400. GRL-98065 was also examined in comparison with two different strains of HIV-2, HIV-2_{EHO} and HIV-2_{ROD}. The potency of GRL-98065 against the HIV-2 strains examined was less than that against HIV-1_{LAI} by factors of 6 to 9; however, its absolute EC_{50}s were comparable to those of four FDA-approved PIs (SQV, LPV, ATV, and DRV) which showed similar antiviral potencies against HIV-1_{LAI} and HIV-2 strains (Table 1).

GRL-98065 is potent against PI-selected laboratory HIV-1 variants. We also examined GRL-98065 against a variety of HIV-1 variants in vitro selected with each of seven FDA-approved PIs (SQV, RTV, IDV, NFV, APV, LPV, and ATV). These variants were selected by propagating HIV-1_{NL4-3} in the presence of increasing concentrations of each of these PIs (up to 1 or 5 μM) in MT-4 cells (18), and such variants proved to have acquired various PI resistance-associated amino acid substitutions in the protease-encoding region of the viral genome (Table 2). Each of the variants (HIV-$1_{SQV5\mu M}$, HIV-$1_{RTV5\mu M}$, HIV-$1_{IDV5\mu M}$, HIV-$1_{NFV5\mu M}$, and HIV$_{APV5\mu M}$), except HIV-$1_{LPV1\mu M}$ and HIV-$1_{ATV1\mu M}$, was highly resistant to the PI with which the variant was selected and showed significant resistance, with an EC_{50} of >1 μM (*n*-fold differences of 29 to 143). Interestingly, HIV-$1_{LPV1\mu M}$, which was only moderately resistant to LPV, with an EC_{50} of 0.31 μM, was highly resistant to both RTV and IDV, with an EC_{50} of >1 μM. HIV-$1_{ATV1\mu M}$ was resistant to ATV, with an EC_{50} of 0.33 μM (79-fold difference). The activities of GRL-98065 against all of the variants except HIV-$1_{APV5\mu M}$ were found to be relatively well maintained, with *n*-fold differences of 5 to 25. It was of note that even with the 5- to 25-fold differences in the EC_{50}s from those against wild-type HIV-1_{NL4-3}, EC_{50}s were all <0.0075 μM except against HIV-$1_{APV5\mu M}$. GRL-98065 was relatively

TABLE 2. Antiviral activities of GRL-98065 against laboratory PI-resistant HIV-1 variants[a]

Virus	Amino acid substitutions in protease-encoding region	EC_{50} (μM) of drug								
		SQV	RTV	IDV	NFV	APV	LPV	ATV	DRV	GRL-98065
HIV-1_{NL4-3}	None (wild type)	0.007 ± 0.002	0.033 ± 0.002	0.034 ± 0.004	0.033 ± 0.007	0.026 ± 0.007	0.031 ± 0.009	0.0042 ± 0.0004	0.0030 ± 0.0001	0.0003 ± 0.0002
HIV-$1_{SQV5\mu M}$	L10I/G48V/I54V/L63P/A71V/G73S/V82T	>1 (>143)	>1 (>30)	>1 (>29)	0.48 ± 0.04 (15)	0.33 ± 0.03 (13)	0.27 ± 0.09 (9)	0.326 ± 0.001 (78)	0.0058 ± 0.0003 (2)	0.006 ± 0.003 (20)
HIV-$1_{RTV5\mu M}$	M46I/V82F/I84V	0.010 ± 0.008 (1)	>1 (>30)	0.25 ± 0.01 (7)	0.21 ± 0.05 (6)	0.28 ± 0.02 (11)	0.16 ± 0.02 (5)	0.018 ± 0.008 (4)	0.018 ± 0.004 (6)	0.0025 ± 0.0003 (8)
HIV-$1_{IDV5\mu M}$	L10F/L24I/M46I/I54V/L63P/A71V/G73S/V82T	0.059 ± 0.004 (8)	>1 (>30)	>1 (>29)	0.47 ± 0.07 (14)	0.17 ± 0.01 (7)	0.26 ± 0.01 (8)	0.06 ± 0.02 (14)	0.015 ± 0.007 (5)	0.0037 ± 0.0007 (12)
HIV-$1_{NFV5\mu M}$	L10F/K20T/D30N/K45I/A71V/V77I	0.024 ± 0.008 (3)	0.051 ± 0.005 (2)	0.27 ± 0.05 (8)	>1 (>30)	0.060 ± 0.004 (2)	0.024 ± 0.001 (1)	0.021 ± 0.006 (5)	0.0033 ± 0.0001 (1)	0.0024 ± 0.0008 (8)
HIV-$1_{APV5\mu M}$	L10F/M46I/I50V/A71V/I84V/L90M	0.031 ± 0.004 (4)	0.29 ± 0.02 (9)	0.200 ± 0.007 (6)	0.27 ± 0.05 (8)	>1 (>38)	0.23 ± 0.02 (7)	0.003 ± 0.001 (1)	0.33 ± 0.03 (110)	0.032 ± 0.004 (107)
HIV-$1_{LPV1\mu M}$	L10F/M46I/I54V/V82A	0.032 ± 0.002 (5)	>1 (>30)	>1 (>29)	0.49 ± 0.04 (15)	0.31 ± 0.02 (12)	0.31 ± 0.02 (10)	0.040 ± 0.002 (10)	ND	0.0075 ± 0.0003 (25)
HIV-$1_{ATV1\mu M}$	L23I/K43I/M46I/I50L/G51A/A71V	0.037 ± 0.004 (5)	0.12 ± 0.06 (4)	0.388 ± 0.001 (11)	0.22 ± 0.04 (7)	0.20 ± 0.07 (8)	0.033 ± 0.006 (1)	0.33 ± 0.06 (79)	0.0034 ± 0.0001 (1)	0.0015 ± 0.0009 (5)
HIV-$1_{GRL98065p20}$	A28S/K43I/C67Y/V82I/I85V/L89M	0.012 ± 0.005 (2)	0.13 ± 0.05 (4)	0.04 ± 0.02 (1)	0.08 ± 0.02 (2)	0.058 ± 0.009 (2)	0.034 ± 0.007 (1)	0.006 ± 0.002 (2)	0.021 ± 0.009 (7)	0.0038 ± 0.0006 (13)
HIV-$1_{GRL98065p30}$	T12I/E21K/A28S/E34K/K43I/M46I/V82I/I85V/L89M	0.022 ± 0.009 (3)	0.30 ± 0.03 (9)	0.059 ± 0.009 (2)	0.27 ± 0.07 (8)	0.33 ± 0.09 (13)	0.06 ± 0.003 (2)	0.008 ± 0.001 (2)	0.036 ± 0.001 (12)	0.008 ± 0.004 (27)
HIV-$1_{GRL98065p40}$	E21K/A28S/K43I/M46I/I50V/D60N/A71V/V82I/I85V/L89M	0.032 ± 0.002 (5)	0.38 ± 0.09 (12)	0.28 ± 0.02 (8)	0.34 ± 0.01 (10)	>1 (>38)	0.19 ± 0.07 (6)	0.011 ± 0.007 (3)	0.21 ± 0.03 (70)	0.18 ± 0.03 (600)

[a] MT-4 cells (10^4) were exposed to 100 $TCID_{50}$s of each HIV-1, and inhibition of p24 Gag protein production by each drug was used as an end point. Numbers in parentheses represent *n*-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 data shown represent mean values (±1 standard deviation) derived from results of three independent experiments. ND, not determined.

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less potent against HIV-$1_{APV5\mu M}$, with an EC_{50} of 0.032 μM (107-fold difference), presumably due to the structural resemblance between GRL-98065 and APV, both of which contain a sulfonamide isostere (Fig. 1).

GRL-98065 exerts potent activities against highly PI-resistant clinical HIV-1 strains. In our previous work, we isolated highly multiple-PI-resistant primary HIV-1 strains, HIV-$1_{MDR/TM}$, HIV-$1_{MDR/MM}$, HIV-$1_{MDR/JSL}$, HIV-$1_{MDR/B}$, HIV-$1_{MDR/C}$, and HIV-$1_{MDR/G}$, from patients with AIDS who had failed then-existing anti-HIV regimens after receiving 9 to 11 anti-HIV-1 drugs over 32 to 83 months (32). These primary strains contained 9 to 14 amino acid substitutions in the protease-encoding region which have reportedly been associated with HIV-1 resistance to various PIs (see footnote *a* of Table 3). The EC_{50}s of RTV, IDV, and NFV against clinical multidrug-resistant HIV-1 strains were mostly >1 μM, and the activities of the other four PIs (SQV, APV, LPV, and ATV) were also significantly compromised, as examined in PHA-PBMs with target cells using p24 production inhibition as an end point. However, GRL-98065 exerted quite potent antiviral activity, and its EC_{50}s against those clinical variants were as low as 0.006 μM or less (Table 3). The potency of GRL-98065 proved that the compound was most potent against the six representative multidrug-resistant clinical HIV-1 variants compared to the currently available approved PIs, including the two recently approved PIs LPV and ATV. Generally, GRL-98065 exerted more-potent antiviral activities against various wild-type HIV-1 strains, drug-resistance variants, and HIV-2 strains than DRV by 2- to 10-fold.

GRL-98065 is potent against HIV-1 strains of diverse subtypes. GRL-98065 was further examined as to whether the compound exerted antiviral activity against HIV-1 strains of diverse subtypes in vitro. It was found that GRL-98065 exerted highly potent activity against HIV-1 isolates of all subtypes (subtypes A, B, C, and E) examined (Table 3), with EC_{50}s from 0.0002 to 0.0005 μM. It is noteworthy that GRL-98065 was significantly more potent than SQV, LPV, and ATV, whose EC_{50}s were fairly low compared with other FDA-approved PIs (RTV, IDV, NFV, and APV) by factors of 8 to 41.5, 11 to 26.5, and 6.5 to 12, respectively.

In vitro selection of HIV-1 variants resistant to GRL-98065. We attempted to select HIV-1 variants with GRL-98065 by propagating a laboratory HIV-1 strain, HIV-1_{NL4-3}, in MT-4 cells in the presence of increasing concentrations of GRL-98065 as described previously (32). HIV-1_{NL4-3} was initially exposed to 0.0005 μM GRL-98065 and underwent 40 passages to be capable of propagating at a 1,000-fold greater concentration (0.5 μM). Judging by the amounts of p24 Gag protein secreted in the culture medium, the replicative capacity of HIV-1_{NL4-3} at passage 40 was generally well maintained (~900 ng/ml). We compared whether the emergence of GRL-98065-resistant HIV-1 is delayed in comparison with the emergence of resistant HIV-1 upon APV, LPV, or ATV selection (Fig. 3). HIV-1 variants resistant to APV, LPV, or ATV, which replicated at >1 μM, emerged by passages 25, 30, and 39, respectively, while resistance to GRL-98065 emerged by passage 42, strongly suggesting that the emergence of GRL-98065-resistant HIV-1 variants was substantially delayed compared to that for the three PIs tested. Genetic characterization of the protease-encoding region disclosed that those variants resistant to

TABLE 3. Antiviral activities of GRL-98065 against multidrug-resistant clinical isolates and various subtypes in PHA-PBMs

Virus[a]	EC_{50} (μM)								
	SQV	RTV	IDV	NFV	APV	LPV	ATV	DRV	GRL-98065
HIV-$1_{ERS104pre}$ (wild type: X4)	0.008 ± 0.003	0.025 ± 0.005	0.024 ± 0.008	0.015 ± 0.004	0.029 ± 0.005	0.007 ± 0.001	0.0038 ± 0.0004	0.0038 ± 0.0007	0.0005 ± 0.0002
HIV-$1_{MDR/TM}$ (X4)	0.18 ± 0.05 (23)	>1 (>40)	>1 (>42)	>1 (>67)	0.30 ± 0.04 (10)	0.36 ± 0.09 (51)	0.038 ± 0.009 (10)	0.0043 ± 0.0007 (1)	0.0032 ± 0.0006 (6)
HIV-$1_{MDR/MM}$ (R5)	0.14 ± 0.04 (18)	>1 (>40)	>1 (>42)	>1 (>67)	0.48 ± 0.09 (17)	0.38 ± 0.08 (54)	0.045 ± 0.0001 (12)	0.016 ± 0.007 (4)	0.0038 ± 0.0006 (8)
HIV-$1_{MDR/JSL}$ (R5)	0.29 ± 0.05 (36)	>1 (>40)	>1 (>42)	>1 (>67)	0.43 ± 0.05 (15)	0.70 ± 0.19 (100)	0.54 ± 0.20 (142)	0.027 ± 0.009 (7)	0.006 ± 0.002 (12)
HIV-$1_{MDR/B}$ (X4)	0.27 ± 0.06 (34)	>1 (>40)	>1 (>42)	>1 (>67)	0.36 ± 0.09 (12)	0.30 ± 0.03 (43)	0.25 ± 0.003 (66)	0.04 ± 0.01 (11)	0.0039 ± 0.0005 (8)
HIV-$1_{MDR/C}$ (X4)	0.035 ± 0.004 (4)	>1 (>40)	>1 (>42)	0.42 ± 0.06 (28)	0.25 ± 0.05 (9)	0.31 ± 0.05 (44)	0.021 ± 0.006 (6)	0.009 ± 0.005 (2)	0.0027 ± 0.0003 (5)
HIV-$1_{MDR/G}$ (X4)	0.033 ± 0.005 (4)	>1 (>40)	0.64 ± 0.11 (27)	0.37 ± 0.05 (25)	0.32 ± 0.02 (11)	0.16 ± 0.04 (23)	0.032 ± 0.002 (8)	0.007 ± 0.005 (2)	0.0034 ± 0.0003 (7)
HIV-$1_{92UG029}$ (subtype A: X4)	0.0048 ± 0.0005	0.071 ± 0.011	0.044 ± 0.009	0.043 ± 0.006	0.046 ± 0.006	0.007 ± 0.001	0.006 ± 0.002	ND	0.0005 ± 0.0002
HIV-$1_{92UG037}$ (subtype A: R5)	0.0032 ± 0.0003	0.041 ± 0.008	0.034 ± 0.003	0.056 ± 0.014	0.027 ± 0.005	0.005 ± 0.001	0.0025 ± 0.0002	ND	0.0004 ± 0.0001
HIV-1_{Ba-L} (subtype B: R5)	0.0083 ± 0.0005	0.023 ± 0.006	0.022 ± 0.005	0.018 ± 0.004	0.025 ± 0.006	0.0053 ± 0.0004	0.0013 ± 0.0004	ND	0.0002 ± 0.0001
HIV-$1_{97ZA003}$ (subtype C: R5)	0.0067 ± 0.0008	0.039 ± 0.004	0.037 ± 0.006	0.037 ± 0.007	0.033 ± 0.005	0.0073 ± 0.0006	0.0034 ± 0.0001	ND	0.0005 ± 0.0001
HIV-$1_{92TH019}$ (subtype E: R5)	0.0030 ± 0.0001	0.030 ± 0.009	0.021 ± 0.001	0.029 ± 0.004	0.021 ± 0.006	0.0033 ± 0.0005	0.0027 ± 0.0001	ND	0.0003 ± 0.0001

[a] Amino acid substitutions identified in the protease-encoding region compared to the consensus type B sequence cited from the Los Alamos database include L63P in HIV-$1_{ERS104pre}$; L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90M, I93L in HIV-$1_{MDR/TM}$; L10I, K43T, M46L, I54V, L63P, A71V, V82A, L90M, and Q92K in HIV-$1_{MDR/MM}$; L10I, L24I, I33F, E35D, M36I, N37S, M46L, I54V, R57K, I62V, L63P, A71V, G73S, and V82A in HIV-$1_{MDR/JSL}$; L10I, K14R, L33I, M36I, M46I, F53L, K55R, I62V, L63P, A71V, G73S, V82A, L90M, and I93L in HIV-$1_{MDR/B}$; L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, and L89M in HIV-$1_{MDR/C}$; and L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, and L90M in HIV-$1_{MDR/G}$. HIV-$1_{ERS104pre}$ served as a source of wild-type HIV-1. EC_{50}s were determined by using PHA-PBMs as target cells, and inhibition of p24 Gag protein production by each drug was used as an end point. Numbers in parentheses represent *n*-fold changes of EC_{50}s for each isolate compared to EC_{50}s for wild-type HIV-$1_{ERS104pre}$. All assays were conducted in duplicate or triplicate, and data shown represent mean values (±1 standard deviation) derived from results of three independent experiments. PHA-PBMs were derived from a single donor in each independent experiment. ND, not determined.

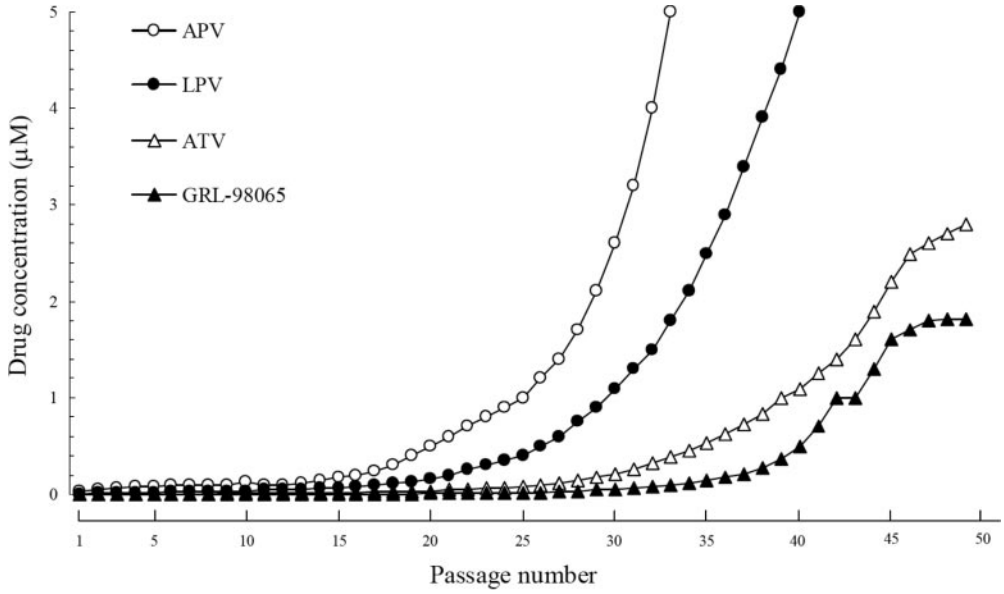

FIG. 3. In vitro selection of PI-resistant HIV-1 variants. HIV-1_{NL4-3} was propagated in MT-4 cells in the presence of increasing concentrations of amprenavir (○), lopinavir (●), atazanavir (△), or GRL-98065 (▲). Each passage of virus was done in a cell-free fashion.

each of the three PIs had acquired previously reported mutations (Table 3). The protease-encoding region of the proviral DNA isolated from infected MT-4 cells was cloned and sequenced at passages 5, 10, 15, 20, 25, 30, 33, and 40 upon GRL-98065 selection. Individual protease sequences and their frequency at each passage are depicted in Fig. 4. By passage 10 (HIV-$1_{GRL98065p10}$), the wild-type protease gene sequence was seen in 8 of 13 clones, although one or two sporadic amino acid substitutions were noted in 5 of the 13 clones. However, by passage 15 and beyond, the virus acquired the K43I substitution. As the passage proceeded, more amino acid substitutions emerged. In HIV-$1_{GRL98065p25}$, K43I, M46I, V82I, I85V, and L89M were seen, along with A28S (9 of 20 clones). Val82 is an active-site amino acid residue whose side chain has direct contacts with inhibitor atoms (33), and the V82I substitution has been shown to be effective in conferring resistance when combined with a second active-site mutation, such as V32I (17). By passage 30, more amino acid substitutions, such as E21K and E34K, were seen, while the latter was not seen in HIV-$1_{GRL98065p33}$. The A28S substitution, which was first seen in HIV-$1_{GRL98065p20}$, never became predominant in the later passages, and the percentage of HIV-1 carrying A28S remained around 50% (45% in HIV-$1_{GRL98065p25}$, 60% in HIV-$1_{GRL98065p30}$, 36% in HIV-$1_{GRL98065p33}$, and 64% in HIV-$1_{GRL98065p40}$). It should be noted that as we described previously (32), the A28S substitution, located at the active site of the enzyme, was seen early (at passage 15) in HIV-1 selected in the presence of TMC126, the prototype of GRL-98065, and this particular mutation never disappeared but was consistently seen at frequencies of ~50%, suggesting that the A28S substitution was critical in conferring resistance to TMC126 (32). E21K coexisted with A28S by passage 30 and beyond, being seen in four of six clones at passage 30. The substitution I50V, seen in HIV-1 resistant to APV, did not coexist with A28S throughout the passage. This profile was previously seen in the case of TMC126-selected HIV-1 variants, as described previously (32). The M46I substitution first emerged at passage 25 and was present in 4 of 10 clones at passage 30 (Fig. 4). Met46 is located on the flap region of the enzyme. The I47V substitution reportedly emerges with viral resistance to APV but was not seen in GRL-98065-resistant variants. We examined whether the virus acquired mutations in the Gag region at passages 5, 10, 15, 20, 25, 30, 33, and 40 of GRL-98065 selection. It was found that by passage 25, the virus had acquired the R275K substitution. By passage 33 and beyond, the G412D substitution emerged and persisted. By passage 40, the p7/p1 cleavage site substitution, I437T, was seen in four of nine clones (Fig. 5).

GRL-98065-resistant HIV-1_{NL4-3} variant maintained robust replicative activity. We determined replication kinetics of HIV-$1_{GRL98065p40}$ and wild-type HIV-1_{NL4-3}. HIV-$1_{GRL98065p40}$ generally propagated well regardless of the presence of GRL-98065 in culture medium. As shown in Fig. 6, when HIV-$1_{GRL98065p40}$ was propagated in MT-4 cells in the presence or absence of 0.01 or 0.1 μM GRL-98065, there was no marked difference observed in the replication kinetics of HIV-$1_{GRL98065p40}$ compared to that of HIV-1_{NL4-3} without GRL-98065.

Reduced sensitivities of GRL-98065-selected HIV-1 variants to various PIs. We also examined the susceptibilities of HIV-$1_{GRL98065p40}$ to eight FDA-approved PIs with MT-4 cells (Table 2). HIV-$1_{GRL98065p40}$ was highly resistant to GRL-98065, with a 600-fold-greater EC_{50} (0.18 μM) relative to the EC_{50} of GRL-98065 against HIV-1_{NL4-3}. However, HIV-$1_{GRL98065p40}$ was still susceptible to SQV and ATV, with relatively low EC_{50}s of 0.032 μM (fivefold difference relative to that for HIV-1_{NL4-3}) and 0.011 μM (threefold difference), respectively. However, APV was no longer active against HIV-$1_{GRL98065p40}$, with an EC_{50} of >1 μM. The loss of APV's activity against

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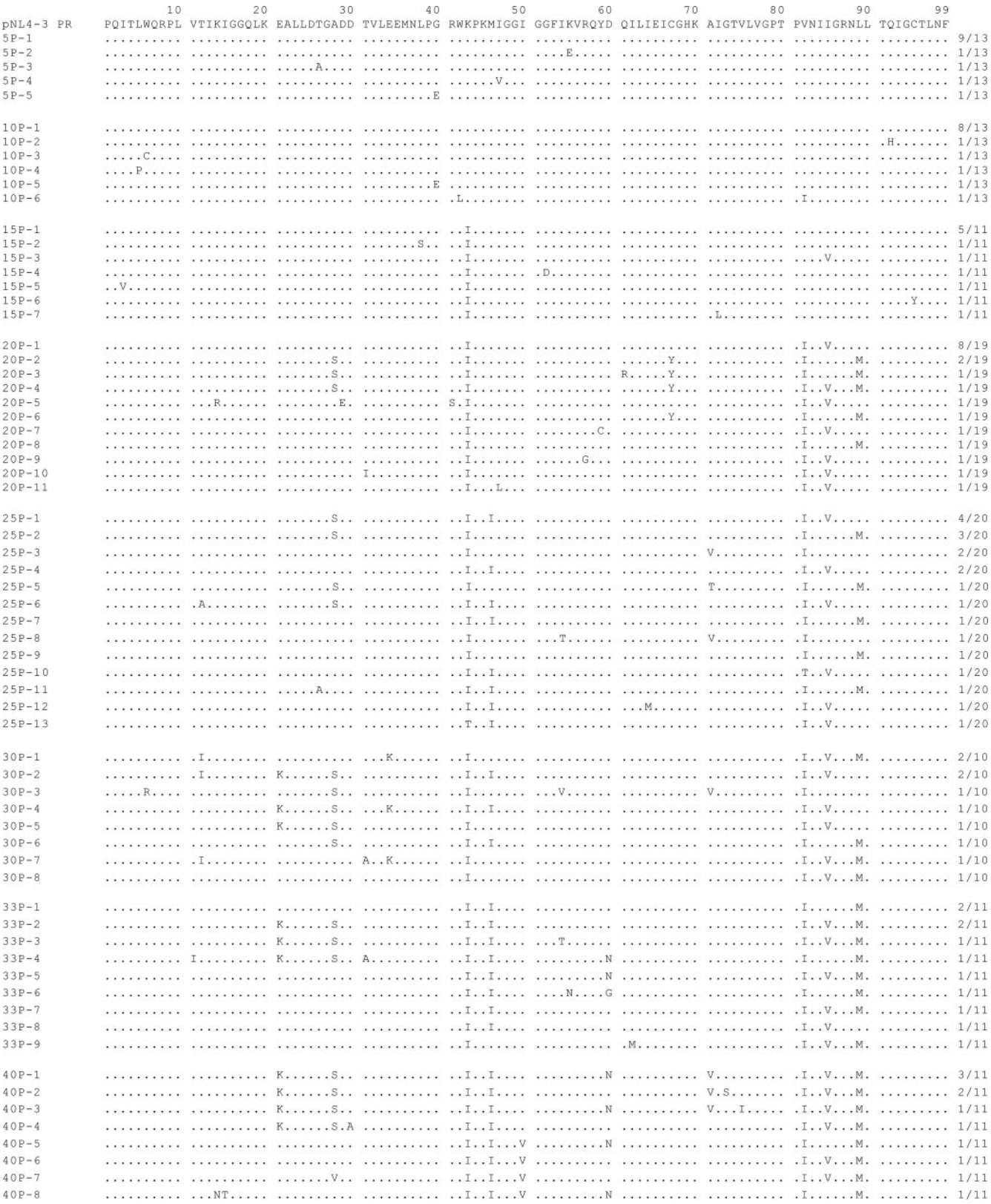

FIG. 4. Amino acid sequences of protease-encoding regions of HIV-1_{NL4-3} variants selected in the presence of GRL-98065. The amino acid sequence of protease, deduced from the nucleotide sequence of the protease-encoding region of each proviral DNA isolated at each indicated time, is shown. The amino acid sequence of wild-type HIV-1_{NL4-3} protease is illustrated at the top as a reference.

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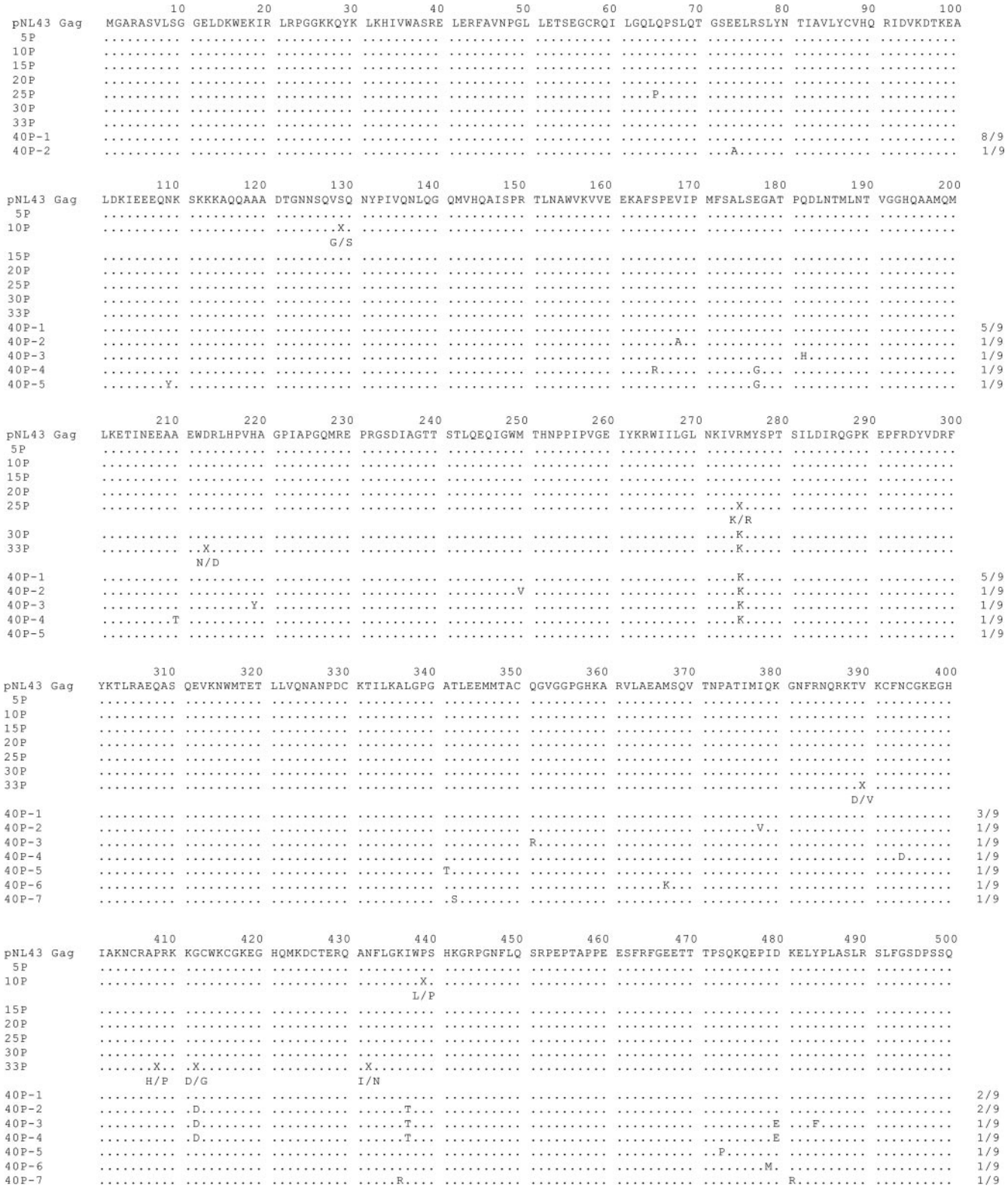

FIG. 5. Amino acid sequences of Gag-encoding regions of HIV-1 variants selected in the presence of GRL-98065. The amino acid sequence of Gag, deduced from the nucleotide sequence of the Gag-encoding region of each proviral DNA isolated at each indicated time, is shown. The amino acid sequence of wild-type HIV-1_{NL4-3} Gag is illustrated at the top as a reference.

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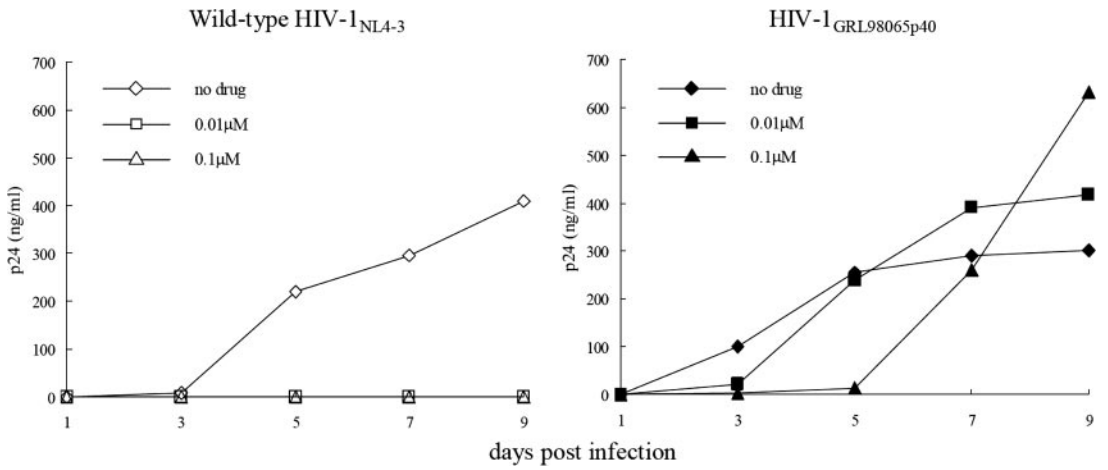

FIG. 6. Replication kinetics of GRL-98065-resistant HIV-1 variant and HIV-1_{NL4-3}. MT-4 cells (2.4×10^5) were exposed to an HIV-$1_{GRL98065p40}$ or wild-type HIV-1_{NL4-3} preparation containing 30 ng p24 in six-well culture plates for 3 h, and these MT-4 cells were divided into three fractions, each cultured with or without GRL-98065 (final concentration of MT-4 cells, 10^4/ml; drug concentrations, 0, 0.01, and 0.1 μM). Amounts of p24 were measured every 2 days for up to 9 days.

HIV-$1_{GRL98065p40}$ was thought to be due to APV's structural relatedness to GRL-98065.

Crystal structure analysis of HIV-1 protease with GRL-98065. The crystal structure of HIV-1 protease complexed with GRL-98065 was refined to a residual factor of 0.147 at a 1.6-Å resolution in order to determine the molecular basis for the inhibitor potency. The crystallographic statistics are listed in Table 4. The inhibitor was found to bind in two overlapping conformations with equivalent interactions with protease, as observed for DRV (18, 31). GRL-98065 has hydrogen bond interactions with the backbone atoms of Asp29, Asp30, Gly27, and Asp30′ and with the side chain atoms of Asp25 and Asp25′ (Fig. 7A). The protease formed very similar hydrogen bond interactions with GRL-98065 and DRV, with a few exceptions. The equivalent atoms of GRL-98065 and DRV superimpose with a root mean square deviation of 0.04 Å, excluding the aniline group of DRV and the 1,3-benzodioxole group of GRL-98065. The 1,3-benzodioxole group of GRL-98065 and the aniline group of DRV each formed a hydrogen bond with Asp30′—however, they interact with different atoms of Asp30′. GRL-98065 interacts with the Asp30′ amide, while the aniline of DRV interacts with the carbonyl oxygen of Asp30′ (Fig. 7B). More significantly, the other oxygen of the 1,3-benzodioxole group of GRL-98065 formed a water-mediated interaction with the amide of the flap residue, Gly48′, while DRV had no equivalent interaction with Gly48′. These additional interactions of GRL-98065 with Gly48′ in the flexible flap region should stabilize its binding to protease and mimic the interactions of the peptide substrates more closely than does DRV.

TABLE 4. Crystallographic data collection and refinement statistics

Parameter[a]	Value for wild-type protease
Space group	$P2_12_12$
Unit cell dimensions (Å)	
a	58.25
b	85.83
c	45.97
Resolution range (Å)	50–1.60
Unique reflections	31,128
R_{merge} (%) overall (final shell)	7.1 (38.8)
$<I>/<\sigma(I)>$ overall (final shell)	11.0 (4.7)
Completeness (%) overall (final shell)	99.7 (99.4)
Data range for refinement (Å)	10–1.60
R_{factor} (%)	14.7
R_{free} (%)	20.8
No. of solvent atoms (total occupancies)	207.7
Root-mean-square deviation from ideal	
Bonds (Å)	0.009
Angle distance (Å)	0.030
Average *B* factors (Å^2)	
Main chain	15.5
Side chain	19.0
Inhibitor	10.7
Solvent	28.7
Residual density (max/min) (eÅ^{-3})	0.38/−0.40

[a] R_{merge}, $\Sigma|I - <I>|/\Sigma(I)$; R_{factor}, $\Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$; R_{free}, R_{factor} calculated for 5% reference set; max/min, maximum/minimum.

Structural analysis of interaction with mutant protease. We finally attempted to predict the molecular interactions of GRL-98065 with a variety of mutant proteases by molecular docking. We first analyzed the hydrogen bond interactions of various PIs (including DRV) with wild-type protease, employing previously published coordinates for each PI complexed with wild-type protease (PDB identifiers, 1S6G, 1HXB, 1HXW, 1SDT, 1OHR, 1HPV, 1MUI, and 2AQU) (Table 5). It was noted that GRL-98065 and DRV have four hydrogen bond interactions with backbone atoms of Asp29, Asp30, and Asp30′. None of the other PIs examined have more than two hydrogen bond interactions with these residues. These results should corroborate that GRL-98065 and DRV exert highly potent antiviral activities against wild-type HIV-1 strains.

We next examined six mutant proteases with 2 to 11 amino acid substitutions (PDB identifiers, 2FDD, 1SGU, 1HSH, 2AZC, 1B6K, and 2AVV). Even though the mutations in each of these proteases do not exactly match the mutations shown in Table 2 and Table 3, they cover the range of amino acid substitutions observed in multidrug-resistant protease. The

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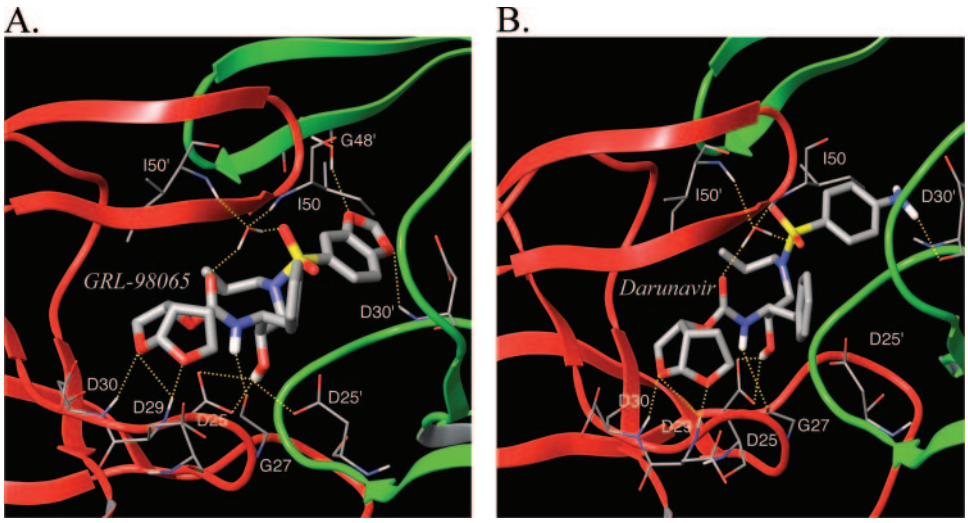

FIG. 7. Selected hydrogen bond interactions of GRL-98065 with wild-type HIV-1 protease. (A) The bis-THF group forms hydrogen bond interactions with backbone atoms of Asp29 and Asp30. There is a hydrogen bond with the backbone atom of Gly27. The hydroxyl group forms hydrogen bonds with the side chains of the catalytic aspartates. One oxygen of the benzodioxole group forms a hydrogen bond interaction with Asp30′, and the other oxygen of the benzodioxole group forms a water-mediated hydrogen bond interaction with Gly48′. (B) Hydrogen bond interactions between DRV and protease (PDB identifier, 1S6G) are shown. Most interactions between GRL-98065 and DRV are similar, except for interactions with Asp30′ and Gly48′. GRL-98065 interacts with the Asp30′ amide, while DRV interacts with the Asp30′ carbonyl oxygen. The benzodioxole oxygen of GRL-98065 has a water-mediated interaction with Gly48′ in the flap. This interaction appears to stabilize the binding site more for GRL-98065 and may be partly responsible for its greater antiviral potency than that of DRV.

docking calculations show that for these mutant proteases, GRL-98065 is able to maintain most of the four hydrogen bond interactions observed for the wild-type protease. In particular, we observed that the hydrogen bond interaction with Asp29 is maintained in four out of six mutant proteases. Taken together, these results are likely to explain why GRL-98065 is able to show greater potency than other clinically approved PIs against a wide spectrum of multiple-PI-resistant HIV-1 variants.

TABLE 5. Hydrogen bond distances of protease inhibitors with selected active-site residues[a]

Inhibitor	PDB ID	Hydrogen bond distance(s) (Å)		
		Asp29	Asp30	Asp30′
GRL-98065		1.9/2.4	2.4	2.5
DRV	1S6G	2.3/2.4	2.4	2.5
SQV	1HXB	1.9	2.2	NP
RTV	1HXW	2.1	NP	2.2
IDV	1SDT	2.1	NP	NP
NFV	1OHR	NP	NP	NP
APV	1HPV	2.8	2.6	NP
LPV	1MUI	1.7	NP	NP
ATV	2AQU	1.9	NP	NP

[a] Hydrogen atoms were added and optimized with constraints on heavy atoms using the OPLS2005 force field (MacroModel, version 9.1; Schrödinger, LLC). Hydrogen bond tolerances used were as follows: 3.0 Å for H—A distance; D—H—A angle greater than 90°; and H—A—B angle greater than 60°, where H is the hydrogen atom, A is the acceptor, D is the donor, and B is a neighbor atom bonded to the acceptor. Values for separate interactions are separated by a shill. NP (not present) denotes that a hydrogen bond is not present between the inhibitor and the particular residue. ID, identifier.

DISCUSSION

GRL-98065, which contains a unique component, bis-THF, and a sulfonamide isostere, suppressed a wide spectrum of HIV-1, HIV-2, and primary HIV-1 strains of different subtypes over a very narrow spread of EC_{50}s ranging from 0.0002 to 0.0045 μM (Tables 1 and 3). GRL-98065 was highly potent against a variety of multidrug-resistant clinical HIV-1 isolates, with EC_{50}s of 0.003 to 0.006 μM, while the existing FDA-approved PIs examined either failed to suppress the replication of those isolates or required much higher concentrations for viral inhibition (Table 3). When examined against laboratory PI-selected HIV-1 variants (except against HIV-$1_{APV5\mu M}$), GRL-98065 also exerted potent activity, with EC_{50}s ranging 0.0015 to 0.0075 μM (Table 2). It is of note that GRL-98065 was less potent against APV-resistant HIV-$1_{APV5\mu M}$; however, it is thought that this relative cross-resistance is due to the structural similarities of GRL-98065 with APV. It is intriguing that the activity of SQV against laboratory PI-selected variants, except for the SQV-selected variant, was fairly well maintained, a profile generally consistent with our results and those of other groups (11, 32, 33). However, when SQV was examined against multidrug-resistant primary HIV-1 strains, high concentrations of SQV were required to suppress the replication of four of six strains tested (EC_{50}s ranging from 0.14 to 0.29 μM) (Table 3). In contrast, GRL-98065 exerted highly potent activity against all the six primary strains examined. As shown in Table 3, even the replication of the most PI-resistant primary strain, HIV-1_{JSL}, against which EC_{50}s of the eight PIs, including ATV and DRV, were 0.027 to >1 μM, was effectively suppressed by GRL-98065 at a fairly low concentration, with an EC_{50} of 0.006 μM.

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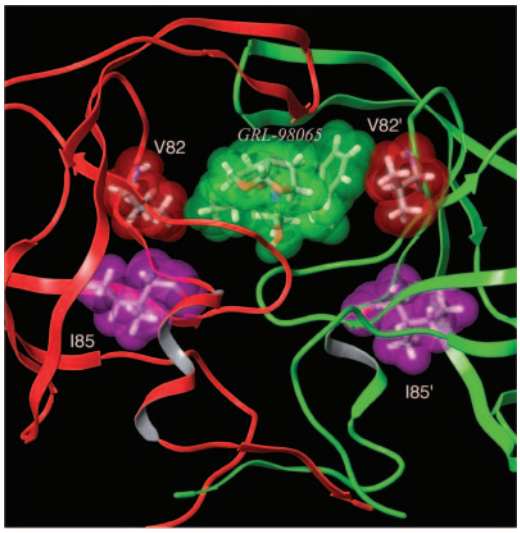

FIG. 8. Interactions between GRL-98065 and wild-type protease. van der Waals surfaces of GRL-98065 (green), Val82 (red), and Ile85 (magenta) are shown. There are strong van der Waals interactions of GRL-98065 with Val82 and Val82′. Note that Val82 was replaced with isoleucine as a primary resistance mutation during in vitro passage of HIV-1 in the presence of GRL-98065. However, Ile85 does not have van der Waals contact with the inhibitor, suggesting that I85V emerged as a secondary mutation during in vitro selection with the inhibitor.

The observed greater potency of GRL-98065 than those of the existing FDA-approved PIs examined in the present study appears to stem, at least in part, from the ability of the two conformationally constrained ring oxygen atoms in its bis-THF group to form hydrogen bonds with the main chain amide hydrogen atoms of Asp29 and Asp30 in the S2 subsite (Fig. 7). Since the main chain atoms cannot be changed by viral amino acid substitutions, the interactions of GRL-98065 and the two catalytic site amino acids are unlikely to be substantially affected, perhaps resulting in GRL-98065's broad spectrum of activity against multidrug-resistant variants.

It is noteworthy that Asp30 can be mutated to asparagine when HIV-1 is exposed to NFV (26). This mutation, D30N, is a primary resistance mutation for NFV that results in formation of a hydrogen bond with the side chain of Asp30 (26). GRL-98065 does not have direct interaction with the side chain of Asp30 (Fig. 7). Consistent with this observation, exposure of HIV-1 to GRL-98065 did not select mutations at codon 30, and GRL-98065 was active against D30N-carrying HIV-$1_{NFV5\mu M}$, which was highly resistant to NFV, with an EC_{50} value of >1 μM (Table 2).

In the present HIV-1 selection experiment with GRL-98065, by passage 30 and beyond, 10 major amino acid substitutions (E21K, A28S, K43I, M46I, I50V, D60N, A71V, V82I, I85V, and L89M) were identified. It is noteworthy that mutation of Val82, whose side chain makes direct contacts with a number of PIs (3), was not seen in HIV-1 selected with TMC126 that has bis-THF and exerts potent activity against a wide spectrum of HIV-1 strains (32). Presumably, V82I arises due to the fact that GRL-98065 has a tight and direct contact with Val82 (Fig. 8), while Ile85 does not have van der Waals contact with the inhibitor, suggesting that I85V emerged as a secondary mutation during the in vitro selection with GRL-98065 (Fig. 4). In GRL-98065-selected HIV-1, neither of the active-site amino acid substitutions, I84V or V32I, emerged. These two substitutions are known to confer high levels of PI resistance on HIV-1, in particular when combined with V82I (17). The absence of these mutations may contribute to the observed delayed acquisition and relatively low level of resistance to GRL-98065.

It is also of note that during selection with GRL-98065, the unique A28S mutation in the active site of the enzyme emerged. The A28S mutation was seen during the selection of HIV-1_{NL4-3} with TMC126, where the mutation never became predominant but persisted within TMC126-selected HIV-1 variants at frequencies of ~50%. In a previous biochemical study conducted by Hong et al. (14), the A28S mutation in HIV protease caused a more than 1,500-fold decrease in k_{cat}/K_m values for peptide substrates. These results suggest that A28S represents a critical mutation for GRL-98065 resistance but also confers a severe replication disadvantage on the virus. It should be noted, however, that the population size of HIV-1 in a culture is relatively small and the appearance of mutations can be affected by stochastic phenomena, i.e., rates and orders of appearance of mutations. In order to address the issue of mutation appearance, clinical studies on GRL-98065 are ultimately needed.

The crystal structure reveals that GRL-98065 has a series of hydrogen bond interactions with backbone atoms of Asp29, Asp30, Asp30′, and Gly27 of the protease (Fig. 7). Like other protease inhibitors, GRL98065 also has hydrogen bond interactions with the side chain atoms of Asp25 and Asp25′. Besides the water-mediated hydrogen bond interactions with Ile50 and Ile50′, there is a water-mediated hydrogen bond interaction with the flap residue Gly48′. Thus, GRL-98065 makes favorable polar interactions with Asp29 and Asp30 as well as with the flap residues. These hydrogen bond interactions, besides various favorable van der Waals contacts, are likely to be responsible for the strong binding of the inhibitor and its potent antiviral activity observed in the present work. Comparison of the crystal structure of HIV-1 protease with that of GRL-98065 and the crystal structure of the complex with the recently approved inhibitor DRV shows that the interactions with the S2 site of the protease are shared by the two PIs, but the nature of the hydrogen bonds with residues in the S2′ site differs (Fig. 7). The water-mediated interaction of GRL-98065 with flap residue 48′ is not observed for DRV. These differences in interactions might be partly responsible for the low EC_{50} of GRL-98065 compared to that of DRV (Tables 1 to 3).

We also attempted to gain a structural understanding of why GRL-98065 is able to maintain a highly favorable potency against a variety of laboratory PI-resistant HIV-1 variants and multidrug-resistant clinical isolates. The resistance of PIs due to mutations arises because of possible loss of direct hydrogen bond interactions with specific residues (e.g., D30N for NFV and G48V for SQV) or loss of van der Waals contact (e.g., with V82A and I84V for first-generation PIs). Analysis of mutant protease crystal structures in comparison with that of wild-type protease showed that the backbone atoms of mutant protease undergo minimal conformational changes on mutation (9, 15, 19). The loss of binding in many cases seems to be due to loss of weaker van der Waals contacts between the inhibitor and the protease. We hypothesize

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that if an inhibitor maintains strong hydrogen bond interactions with the wild-type protease, particularly with backbone atoms of multiple residues that are conserved (e.g., Asp29 and Gly27), then the loss of van der Waals contacts due to mutations may not result in a drastic loss of binding affinity. Thus, inhibitors without multiple strong hydrogen bond interactions with wild-type protease would be more susceptible to loss of binding due to loss of weaker van der Waals contacts than inhibitors with multiple hydrogen bond interactions. In this respect, we analyzed the hydrogen bond interactions of several PIs with wild-type protease (Table 5). It is noteworthy that only GRL-98065 and DRV have four hydrogen bond interactions with backbone atoms of Asp29 and Asp30 and of Asp30′. None of the other clinically approved PIs studied here have more than two hydrogen bond interactions with these residues. Thus, GRL-98065 is likely to preserve the hydrogen bond interactions and bind tightly with mutant protease.

The present data suggest that GRL-98065 has several advantages: (i) it exerts potent activity against a wide spectrum of drug-resistant HIV-1 variants, presumably due to its interactions with the main chains of the active-site amino acids Asp29 and Asp30; (ii) its unique contact with HIV-1 protease differs from that of other PIs; (iii) the viral acquisition of resistance is substantially delayed; and (iv) at least several PIs, including SQV and ATV, remain active in vitro against the virus selected in vitro with GRL-98065. It is of note that GRL-98065 possesses substantially favorable features as a potential therapeutic for AIDS, as described above; however, its oral bioavailability, pharmacokinetics/pharmacodynamics, biodistribution, etc., are yet to be determined in further rigorous preclinical and clinical testing.

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