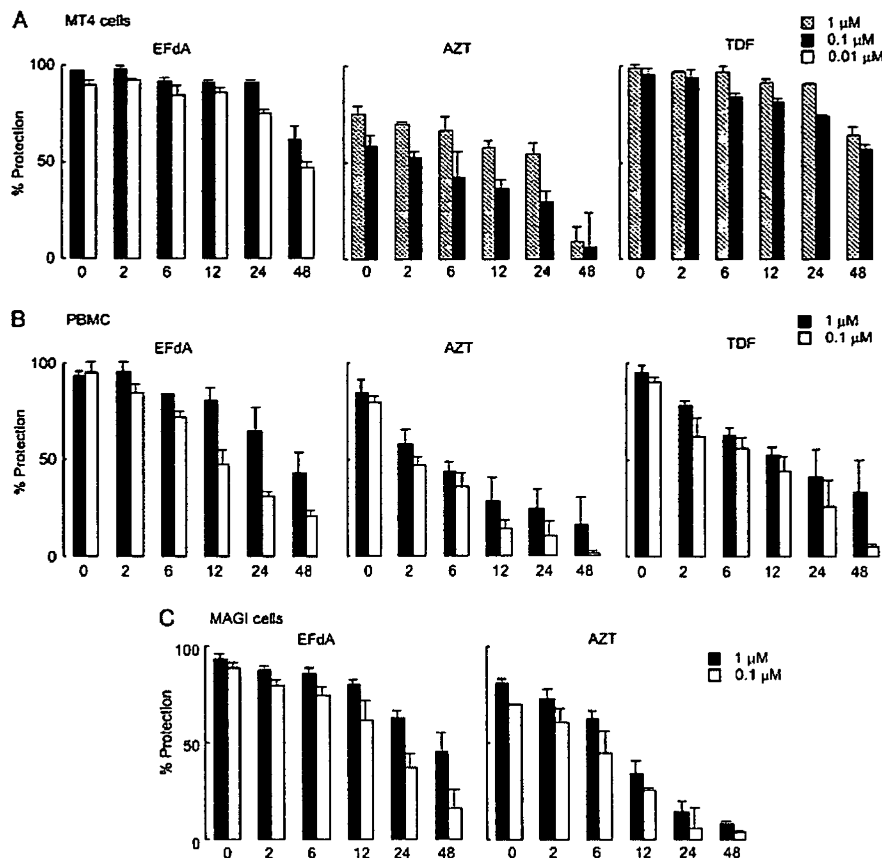

FIG. 4. Persistence of anti-HIV-1 activity after removal of EFdA, AZT, and TDF from culture media. MT-4 (A), PBMC (B), or CCR5$^+$-MAGI cells (C) were exposed to 0.01, 0.1, or 1 μM EFdA (or AZT) for 24 h, thoroughly washed to deplete extracellular drugs, cultured for various periods of time (0, 2, 6, 12, 24, and 48 h), exposed to HIV-1, and further cultured for an additional 5 days with MT-4 cells and PBMC or for an additional 48 h with CCR5$^+$-MAGI cells. Anti-HIV-1 activity was monitored using p24 production or with an MTT assay or a MAGI assay.

daily or twice-daily regimen has produced an improved prognosis (11, 13, 26), we examined whether the pharmacodynamics of EFdA potentially supported a once- or twice-a-day regimen by determining the profiles of anabolic phosphorylation of EFdA in human CD4$^+$ T cells. With regard to the use of these CD4$^+$ human T-cell lines, the intracellular metabolism of certain nucleosides is known to be considerably affected by the status of cells, depending upon proliferation rates, activation states, donors, and other factors (9, 10). It should be noted that the EC_{50} value of EFdA against HIV-1_{Ba-L} determined with PBMC was 0.0004 μM, while that against HIV-1_{NL4-3} determined with MT-4 cells was 0.001 μM. The difference between the values was only a factor of ~3. Thus, we assumed that the phosphorylation pattern and the ratios of EFdA-TP over its possible competitive counterpart, dATP, should be comparable, and we employed two human CD4$^+$ T-cell lines, CEM and MT-4 cells. The present data from these cell lines showed that EFdA efficiently underwent cellular uptake into the cytoplasm and was readily phosphorylated to EFdA-MP, -DP, and -TP (Fig. 2A and B). However, all EFdA phosphates persisted significantly longer than AZT phosphates. Indeed, in both CEM and MT-4 cells exposed to AZT,

TABLE 2. Inhibitory effects of EFdA against DNA polymerase α, β, and γ[a]

Primer/template	IC_{50} value (μM ± SD)					K_i value (μM ± SD) of polymerase γ (D_{21}/D_{36})
	Polymerase α (calf thymus DNA)	Polymerase β		Polymerase γ		
		D_{21}/D_{36}	Calf thymus DNA	D_{21}/D_{36}	Calf thymus DNA	
EFdA-TP	>100	>100	>100	>100	10 ± 2	24.4 ± 7.9
ddA-TP	>100	3 ± 0.3	0.2 ± 0.07	2 ± 0.3	0.2 ± 0.02	4.6 ± 1.7

[a] In steady-state kinetic assays, DNA primer/template of 21 and 36 nucleotides (D_{21}/D_{36}) or activated calf thymus DNA was employed. The IC_{50} values were determined in the presence of 0.3 mM dATP. The IC_{50} values and K_i values represent means ± standard deviations (SD) from two independent experiments. Values of K_m for dATP and K_{cat} were 0.55 ± 0.13 and 0.4 ± 0.03, respectively.

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not only the intracellular levels of AZT-DP and AZT-TP but also that of the accumulated AZT-MP rapidly declined in comparison to EFdA phosphates (Fig. 3A and B). These data suggest that AZT phosphates are more vulnerable to intracellular catalysis than EFdA phosphates. The data also suggest that both AZT-MP and -DP get catalyzed without undergoing further phosphorylation. Indeed, the intracellular $t_{1/2}$ of EFdA-TP, an active metabolite of EFdA, was much greater, at 17.2 h, than that of AZT-TP (at 2.8 h) (Fig. 3). It is noteworthy that the intracellular $t_{1/2}$ of the triphosphate forms of d4T, ddC, 3TC, ddI, ABC, and TDF (PMPApp) were reportedly 3.5, 2.6, 10.5 to 15.5, 25 to 40, 3.3, and 15.4 h, respectively (22). Compared with the half-lives of these FDA-approved drugs, EFdA-TP's intracellular half-life (17.2 h) was relatively long, and these results suggest its favorable intracellular pharmacokinetics. We therefore asked whether the longer intracellular persistence of EFdA-TP resulted in more persistent anti-HIV-1 activity of EFdA as EFdA was removed from the culture medium. It was noted that when MT-4 cells were incubated with EFdA (0.1 μM) for 24 h, thoroughly washed to remove EFdA from the culture medium, cultured for various periods of time without adding EFdA, exposed to HIV-1, and further cultured for 5 days, substantial levels of antiviral activity (at post-24- and -48 h, protection values were 91 and 61%, respectively) were seen. The post-24 and -48 h protection values of TDF (0.1 μM), an FDA-approved once-daily anti-HIV-1 drug, were 74 and 57%, respectively (Fig. 4A). In contrast, substantially lower levels of antiviral activity were observed for AZT than for EFdA. When the cells were preincubated with 0.1 μM AZT, only 6% protection was seen with MT-4 cells (Fig. 4). This relatively poor protective activity of AZT should stem from the relatively short $t_{1/2}$ of AZT-TP (8). Thus, the present data that indicate EFdA-TP has a substantially long $t_{1/2}$ of 17 h, in addition to the observed in vitro persistence of antiviral activity, suggest that a once-daily or twice-daily regimen of EFdA is possible.

In regard to the in vitro selection of HIV-1 variants resistant to EFdA, we previously reported that the 3TC resistance-conferring M184V substitution in reverse transcriptase is the major substitution that reduces anti-HIV-1 activity of 4′-ethynyl analogs, although the EC_{50} value change with the M184V substitution was only approximately sixfold (14). In the present work, when we examined MDR HIV-1 variants containing a number of mutations including M184V, the level of resistance was similarly moderate, with changes in their EC_{50} values ranging from 4- to 21-fold (Table 1). However, against such MDR HIV-1 variants, the absolute EC_{50} values remained lowest for EFdA compared to those of four representative FDA-approved antiviral agents (AZT, TDF, APV, and SQV) (Table 1). Thus, it is possible that the "genetic barrier" to HIV-1 acquisition of EFdA resistance can be substantially higher than at least the agents examined in the present study.

It was noted that when cells were exposed to high concentrations (1 and 10 μM) of [^{3}H]EFdA, the amounts of EFdA phosphates increased proportionately (Fig. 2B). This profile of EFdA phosphates contrasted with those of AZT phosphates, which showed that levels of AZT-TP increased only slightly when the cells were exposed to higher concentrations of AZT (Fig. 2D). This phosphorylation profile of AZT stems from the fact that thymidylate kinase has a good affinity for AZT-MP (K_m of ~8 μM), comparable to that of dT-MP (K_m of ~4 μM), while AZT-MP has an extremely low V_{max} value (only 0.3% relative to the V_{max} of dT-MP) (8), resulting in the accumulation of AZT-MP and low levels of AZT-TP. These data suggest that the intracellular anabolic phosphorylation of EFdA to EFdA-TP is substantially efficient, which explains the reason that EFdA exerts such a potent and persistent anti-HIV-1 activity.

As noted above, EFdA was efficiently converted to its active form, EFdA-TP, whose intracellular $t_{1/2}$ was substantially longer (as long as ~17 h) than that of AZT-TP (Fig. 3). However, there was a concern that the long intracellular persistence of EFdA-TP might cause cellular DNA damages, particularly since EFdA retains a 3′-OH group, which may get incorporated into the growing cellular DNA chain, resulting in human DNA chain termination. All the currently available nucleoside reverse transcriptase inhibitors (NRTI) are not devoid of adverse effects such as lactic acidosis and peripheral neuropathy, which are thought to be associated with the interactions of NRTI-TP and cellular DNA polymerases. Therefore, we examined the effects of EFdA-TP on DNA polymerases α, β, and γ, using ddA-TP, the active form of ddI, as a control. EFdA-TP had virtually no significant inhibition against DNA polymerases α and β, although it had moderate inhibitory effects against DNA polymerase γ, with an IC_{50} value of 10 μM when calf thymus DNA was used as a template/primer. The K_i value of EFdA-TP, determined using D_{21}/D_{36} as the template/primer, was 24.4 μM, while that of ddA-TP was 4.6 μM. The anti-HIV-1 drug ddI is known to cause damages in DNA polymerase γ-mediated mitochondrial DNA synthesis, and one can be concerned about the possibility that EFdA may also cause mitochondrial DNA damages since the K_i value (24.4 μM) of EFdA-TP with DNA polymerase γ was only 5.3-fold less than that of ddA-TP (4.6 μM). However, EFdA is much more potent, with an EC_{50} value (~0.0004 μM with PHA-PBMC exposed to HIV-1_{Ba-L} [Table 1]) higher than that of ddI (EC_{50}, ~1.5 μM in PHA-PBMC exposed to HIV-1_{Ba-L}) (28), and indeed, the ratio of the K_i value to the IC_{50} value for EFdA is as great as 61,000. Thus, EFdA could produce more potent antiviral effects with fewer adverse effects when used as a therapeutic agent for HIV-1 infection and AIDS, although it is important that the antiviral effects and safety of experimental agents be determined only through rigorously controlled preclinical and clinical trials.

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Emergence of human immunodeficiency virus type 1 variants containing the Q151M complex in children receiving long-term antiretroviral chemotherapy

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Abstract

We examined 28 children with HIV-1 infection who were not responding to existing antiviral regimens and were enrolled into clinical trials conducted at the National Cancer Institute to receive salvage therapy. In 3 of the 28 patients (10.7%), the Q151M complex amino acid substitutions were identified. The three patients had received nucleoside reverse transcriptase inhibitor (NRTI) monotherapy and/or combination regimens with multiple NRTIs for 4.3–8.6 years prior to the study. Recombinant infectious clones generated by incorporating the RT-encoding region of HIV-1 isolated from patients' plasma samples were highly resistant to zidovudine, didanosine and stavudine, while they were moderately resistant to lamivudine and tenofovir disoproxil fumarate (TDF). TDF-containing regimens reduced HIV-1 viremia in two of the three children carrying the Q151M complex. These data suggest that the Q151M could be prevalent in pediatric patients with long-term NRTI monotherapy and/or dual NRTI regimens and that HAART regimens containing TDF may be meritorious in such patients.
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Keywords: Multi-dideoxynucleoside resistance; Reverse transcriptase; HIV-1; Q151M; Pediatric AIDS

1. Introduction

Highly active antiretroviral therapy (HAART) has dramatically improved the quality of life and survival of individuals infected with human immunodeficiency virus type 1 (HIV-1) (Pillay et al., 2000; Lee and Henderson, 2001; Yeni et al., 2002). However, drug-resistant HIV-1 variants often emerge, resulting in treatment failure, which has been a major obstacle in achieving optimal therapeutic efficacy of HAART (Larder and Kemp, 1989; Shirasaka et al., 1993; Shafer et al., 1999; Grabar et al., 2000; Wainberg and Turner, 2004). A set or subset of five substitutions in the viral reverse transcriptase (RT), including Ala62 → Val (A62V), V75I, F77L, F116Y, and Q151M, confers multi-dideoxynucleoside resistance (MDR) on HIV-1 and has been referred to as the 'Q151 complex' (Shirasaka et al., 1993, 1995; Shafer et al., 1994; Schmit et al., 1996; Kavlick et al., 1998; Shulman and Winters, 2003; Zaccarelli et al., 2004). The frequency of the emergence of the Q151M complex-associated amino acid substitutions appears to be relatively low, ranging from 1.9% to 6.25% depending on the cohorts examined (Lawrence et al., 1999; Rousseau et al., 2001; Englund et al., 2004; Zaccarelli et al., 2004), although a few studies have shown a relatively higher frequency ranging from 8.8% to 17% (Kavlick et al., 1998; Re et al., 2003). The reason for the high frequency observed in such cohorts appears to be that those individuals received nucleoside reverse transcriptase inhibitor (NRTI) monotherapy and/or dual NRTI combination therapy for substantially longer periods of time in

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the pre-HAART era (Kavlick and Mitsuya, 2001; Yin et al., 2006).

To our knowledge, there are only two works reporting the emergence of HIV-1 containing the Q151M complex in children with HIV-1 infection (Englund et al., 2004; Hazra et al., 2005), but no detailed study of such Q151M-carrying HIV-1 variants has been conducted. A previous study by Palumbo et al. (2001) has shown no significant association between perinatal transmission and the presence of zidovudine (ZDV) or NRTI resistance-associated mutations. They also showed that the mutation patterns identified in children were not identical to those of their mothers. Thus, it is possible that HIV-1 variants carrying the Q151M complex are not readily transmitted. It is also possible that the number of pediatric cases examined for the presence of drug-resistant HIV-1 variants is too limited and that the occurrence of drug-resistant HIV-1 variants has been overlooked. In the present study, we examined HIV-1 variants isolated from 28 children with HIV-1 infection who were enrolled into the phase I/II study of tenofovir disoproxil fumarate (TDF) (Hazra et al., 2005) and a study of therapeutic drug monitoring and viral resistance testing. The present work represents the first extensive study to characterize the virological profiles of Q151M-carrying HIV-1 variants isolated from children receiving HAART.

2. Materials and methods

2.1. Patients

Twenty-eight children with AIDS, who had been on long term anti-HIV therapy and were not responding to existing regimens of combinations of anti-HIV-1 agents, were enrolled into one of the following two clinical trials to receive salvage therapy: a phase I/II study of TDF (Hazra et al., 2005) and a study of therapeutic drug monitoring and viral resistance testing (R.H., personal communication). Subjects on the former study must have failed at least two prior regimens to be eligible, and subjects on the latter study could be receiving their first HAART regimen on the study, but most had failed several previous regimens. On the phase I/II study of TDF, children received TDF-containing HAART, while on the therapeutic drug monitoring study, they could have received TDF but did not have to. Parents or guardians of the subjects agreed to and signed the informed consent form. The Institutional Review Board of the National Cancer Institute approved the study.

2.2. Cells

MT-2 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT), 50 U of penicillin/ml, and 50 μg of streptomycin/ml. Peripheral blood mononuclear cells (PBMC) obtained from HIV-1-negative healthy donors were stimulated with phytohemagglutinin (PHA) in RPMI 1640-based medium containing recombinant interleukin-2 (5 ng/ml; R&D Systems, Minneapolis, MN) for 2 days (PHA-PBMC) prior to HIV-1 exposure. HIV-1 strains were isolated as previously described (Shirasaka et al., 1993) by culturing PBMC obtained from patients, passaged once or twice in PHA-PBMC, and stored at −80 °C until used.

2.3. Determination of nucleotide sequence of HIV-1

HIV-1 RNA was isolated from plasma samples of the patients as previously described (Tamiya et al., 2004). In brief, viral RNA was extracted from plasma samples by using QIAamp Viral RNA minikit (Qiagen, Valencia, CA) and the RT-encoding region of HIV-1 was amplified using RT-PCR with QIAGEN OneStep RT-PCR kit (Qiagen). Thus obtained PCR products were directly sequenced using Applied Biosystems model 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA).

2.4. Generation of molecular infectious HIV-1 clones containing RT of clinical HIV-1 isolates

Molecular infectious HIV-1 clones carrying RT of HIV-1 were generated as previously described (Gatanaga et al., 2002; Tamiya et al., 2004). In brief, viral RNA extracted from plasma samples was subjected to RT-PCR using OneStep RT-PCR kit (Qiagen) to amplify the RT-encoding region of HIV-1. Primers used were EagI-1 (5′-CAACTCCCTCTCAGAAGCA-GCGGCCGATAGACAAGGAACTG-3′) and EcoRI-R (5′-CA-GCAGTTGTTGCAGAATTCT TATTATGGCTTCCAC-3′). RT-PCR products (1 μl) were used directly in the nested-PCR with XmaI-F (5′-CCAGTAAAATTAAAGCCCG-GGATGGATGGCCCAAAAG-3′) and ClaI-R (5′-GTTAAA-ATCACTAGCCATCGATCTCCAATTACTGTG-3′). XmaI-F primer had an *Xma*I site, whereas ClaI-R primer a *Cla*I site. The PCR products obtained were digested with *Xma*I and *Cla*I, and the largest fragments were introduced into pHIV-1_{NL4-3} (Tamiya et al., 2004). Each recombinant plasmid was transfected into COS-7 cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and the infectious virions obtained were harvested 48 h after transfection and stored at −80 °C until use. Thus obtained molecular infectious HIV-1 clones containing the RT region (aa 21–560) of HIV-1 from Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3} were designated as rHIV-1_{ERS1}, rHIV-1_{ERS2}, and rHIV-1_{ERS3}, respectively. Titration assays for these molecular infectious clones obtained were titrated as previously described (Shirasaka et al., 1993; Reed and Muench, 1938) in six replicates.

2.5. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to determine the cytopathic effect (CPE) in MT-2 cells exposed to each virus preparation as previously described (Gatanaga et al., 2002; Matsumi et al., 2003). All assays were performed in triplicate.

2.6. Drug sensitivity assays

The sensitivities of the rHIV-1_{NL4-3} and infectious molecular HIV-1 clones to various drugs were determined as previously described with minor modifications (Shirasaka et al., 1995).

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Briefly, MT-2 cells (2×10^3) were exposed to 100 $CCID_{50}$ 50% cell culture infectious dose of rHIV-1_{ERS1}, rHIV-1_{ERS2}, or rHIV-1_{ERS3} and were cultured in the presence of various concentrations of each reverse transcriptase inhibitor (RTI) in 96-well microculture plates and incubated at 37 °C for 7 days. The 50% inhibitory concentration (IC_{50}) values were determined using the MTT assay in triplicate. The value in parentheses represents a fold difference of the IC_{50} value of each RTI against each recombinant clone as compared to the IC_{50} value against rHIV-1_{NL4-3}.

The IC_{50} values were determined using the target PHA-PBMC exposed to HIV-1_{LAI} or HIV-1_{ERS2} (50 $CCID_{50}/10^5$ PBMC) in the presence of each RTI in 96-well microtiter culture plates and the inhibition of p24 Gag protein production by the drug as an endpoint, as previously described (Shirasaka et al., 1995). All values were determined in triplicate and those shown are representative of three separate experiments. The numbers in parentheses represent fold differences compared to the IC_{50} against HIV-1_{LAI}.

3. Results

3.1. Amino acid substitutions in the RT-encoding region of the genome of HIV-1 isolated from pediatric patients

Viral RNA samples isolated from patients' plasma samples were subjected to RT-PCR for identifying amino acid substitutions in the RT-encoding region of HIV-1 genome. Approximately 700 bp of the *pol* gene, spanning codons 30–260 of RT, were determined in all HIV-1 samples. Table 1 shows all amino acid substitutions identified, which have been reported to associate with drug resistance as we examined the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu) (Shafer et al., 1999; Rhee et al., 2003). Among them were the M184V substitution in 13 of the 28 HIV-1 isolates (46%), K70R in 8 of 28 (29%), T69D in 7 of 28 (25%), and V118I in 6 of 28 (21%). The 69 insertion complex conferring multidideoxynucleoside resistance was also identified in 2 of 28 (7%). It was noted that of the 28 patients, three patients (Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3}: 10.7%) were found to harbor HIV-1 that contained one or more of the Q151M complex substitutions (Shirasaka et al., 1993, 1995; Shafer et al., 1994) (Table 1). In the viruses isolated from 3 of 28 patients (10.7%), only amino acid substitutions that are not known to confer detectable resistance on HIV-1 were identified.

3.2. Amino acid substitutions identified in the protease-encoding region of HIV-1 genome

All of the 28 HIV-1 isolates were found to contain amino acid substitutions in the protease-encoding region, which have been shown to be associated with viral resistance against protease inhibitors (PIs) (Shafer et al., 1999; Rhee et al., 2003), as illustrated in Table 1. Among them were L90M (Eberle et al., 1995) in 15 of 28 (54%), V82A (Molla et al., 1996) in 14 of 28 (50%), and M46I (Kaplan et al., 1994) in 9 of 28 (32%). Isolates from 3 of 28 patients (10.7%) had no significant amino acid substitutions in the protease-encoding region, although there were substitutions that are not known to confer detectable resistance on HIV-1.

3.3. Clinical profiles of three children harboring HIV-1 that contained one or more of the Q151M complex

Clinical profiles of the three patients who harbored HIV-1 variants containing one or more of the Q151M-associated sub-

Table 1
Amino acid substitutions identified in HIV-1 isolates from 28 pediatric patients

Amino acid substitutions in RT	No. of patients	Amino acid substitutions in protease	No. of patients
Q151M complex	3	D30N	2
E44D, M184V	1	D30N, M46I	1
E44D, T69D, 184V	1	D30N, L90M	1
E44D, T69D, V118I, M184V	1	M46I	1
E44D, T69D, V118I, Y181C	1	M46I, V82A	1
E44D, K70R, M184V, T215Y	1	M46I, V82A, I84V, L90M	1
T69D, K70R	2	M46I, V82A, L90M	2
T69D, K70R, M184V	1	M46I, V82S, L90M	1
T69D, M184V, G190A	1	M46I, V82T, L90M	1
K70R, K103N, V118I, Y181C	1	M46I, I84V, L90M	1
K70R, M184V	1	V82A	3
K70R, M184V, T215Y	1	V82A, I84V, L90M	1
K70R, T215Y	1	V82A, L90M	6
L74V, K103N, V118I, M184V, T215Y	1	I84V	1
L74V, V118I, M184V, G190S	1	L90M	2
L74V, Y181C, Y188L	1	No primary mutations identified	3
K103N	1		
K103N, V108I, M184V, T215Y	1		
V108I, V118I, M184V	1		
M184V	1		
T69S + SG insertion	2		
No primary mutations identified	3		

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Table 2
Clinical profiles of Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3}

Patient	Sex	Age	$CD4^+$ (cells/mm^3)	HIV-1 RNA (copies/ml)	Year on antiviral therapy
Pt_{ERS1}	Female	11.3	42	150,000	8.6
Pt_{ERS2}	Male	10.3	2	544,000	7.6
Pt_{ERS3}	Male	10.7	0	364,000	4.3

stitutions, are shown in Table 2. The three subjects (two males and one female) were 10 or 11 years old, were severely immunocompromised, and had very low $CD4^+$ T cell counts. Pt_{ERS2} was first treated with ZDV monotherapy, followed by administration of ZDV + lamivudine (3TC), and later with various PIs and non-nucleoside RT inhibitors (NNRTIs), as shown in Fig. 1. Pt_{ERS1} initially received didanosine (ddI) monotherapy, followed by ddI + stavudine (d4T) combination, and later received various drugs, while Pt_{ERS3} initially received ZDV + 3TC combination and subsequently ddI, d4T, nevirapine (NVP), and ritonavir (RTV). It is of note that all these three patients were initially treated with NRTI monotherapy or NRTI combination therapy and received one or more NRTIs throughout the treatment periods of 4.3–8.6 years together with other classes of antiviral agents.

The earliest available $CD4^+$ T cell counts of Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3} were 473, 288, and 62 mm^{-3}, respectively. Although various antiviral regimens had been administered, CD4 counts had continued to decline until their enrolment into the present salvage therapy trial (Hazra et al., 2005). In the present salvage therapy trial, Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3} received ZDV/3TC/saquinavir (SQV)/ritonavir-boosted lopinavir (LPVr)/enfuvirtide (ENF)/TDF, ZDV/3TC/d4T/LPVr/TDF, and ZDV/emtricitabine (FTC)/EFV/RTV/SQV/atazanavir (ATV)/TDF, respectively. With the implementation of TDF-containing therapy, in Pt_{ERS1} and Pt_{ERS2}, CD4 cell counts increased up to 576 and 1018 mm^{-3} and stayed at these levels, and HIV-1 RNA copies decreased by 5.7 and 5.5 logs, respectively. In Pt_{ERS3}, there were no changes in CD4 counts or HIV-1 RNA copies, but with ENF administration, a significant decrease in HIV-1 RNA copy number by 3 logs was achieved, although no increase in CD4 counts occurred (Fig. 1). It is of note that there was a rebound seen later in HIV-1 RNA copy numbers in Pt_{ERS1} and Pt_{ERS3}.

3.4. Genotypic analysis of HIV-1 isolates containing the Q151M complex

HIV-1 isolated from Pt_{ERS1} had all five Q151M complex substitutions (A62V, V75I, F77L, F116Y, and Q151M), while Pt_{ERS2} had four amino acid substitutions (A62V, V75I, F116Y, and Q151M), and Pt_{ERS3} had two substitutions (F116Y and Q151M). These HIV-1 isolates also had substitutions associated with resistance to NRTIs and non-nucleoside

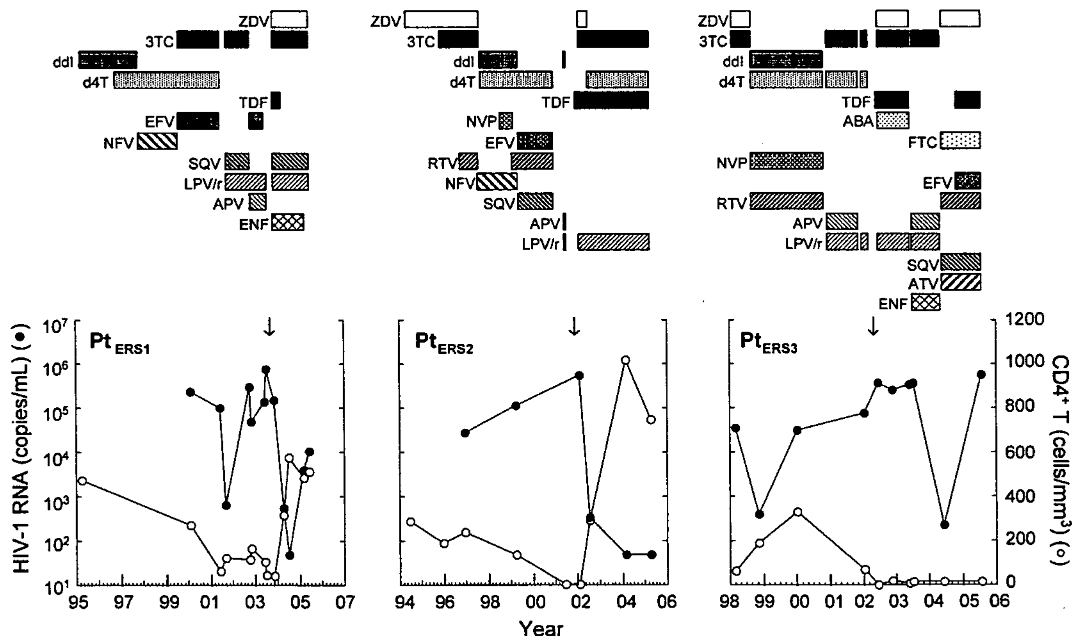

Fig. 1. $CD4^+$ cell counts and HIV-1 RNA copy numbers and the antiviral regimens administered. $CD4^+$ cell counts, HIV-1 RNA copy numbers in plasma, and antiviral regimens the three patients received are illustrated. Each arrow indicates the date of study entry when blood samples were drawn from patients that were subjected to virological analysis reported in this work. It is of note that there is uncertainty in the treatment regimens Pt_{ERS1} received from 1998 to 1999 due to conflicting reports from her clinic record. ZDV, zidovudine; ddI, didanosine; d4T, stavudine; 3TC, lamivudine; RTV, ritonavir; SQV, saquinavir; APV, amprenavir; NFV, nelfinavir; LPV/r, ritonavir-boosted lopinavir; NVP, nevirapine; EFV, efavirenz; TDF, tenofovir disoproxil fumarate; ABA, abacavir; FTC, emtricitabine; ATV, atazanavir; and ENF, enfuvirtide.

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Table 3
Amino acid substitutions identified in HIV-1 isolated from the 3 patients and the mother of one of them

Patient	MDR substitutions in RT[a]					Other substitutions in RT[a]											
	A62	V75	F77	F116	Q151	D67	T69	K70	K103	Y181	M184	G190	H208	L210	F214	T215	K219
Pt_{ERS1}	V	I	L	Y	M	–	–	N	–	–	–	A	H/Y[b]	V/F[b]	–	–	–
Pt_{ERS2}	V	I	–	Y	M	N/S[b]	–	–	–	I	–	E	–	–	L	V	Q
Mother of Pt_{ERS2}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pt_{ERS3}	–	–	–	Y	M	E/del[b]	G	K/R[b]	N	I	–	–	–	–	L/M[b]	V	E

[a] Amino acids and their positions in HIV-1_{LAI} are shown as a reference at the top; "–" indicates that the amino acid is the same as in the reference sequence.
[b] Amino acids seen mixed. del: deletion.

reverse transcriptase inhibitors: Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3} had K70N/G190A/H208Y/L210V(F), D67N(S)/Y181I/G190E/F214L/T215V/K219Q, and D67E(del)/T69G/K70R/K103N/Y181I/F214L(M)/T215V/K219E, respectively. Despite the history of long-term or intermittent 3TC administration in these three patients, no amino acid substitutions were identified at position 184. In this respect, at the time of enrollment and sample collection from Pt_{ERS1}, Pt_{ERS2}, and Pt_{ERS3}, 3TC administration had been discontinued for 12, 52, and 4 months, respectively (Fig. 1). Thus it is likely that 3TC resistance-associated amino acid substitutions, if they existed, had disappeared from peripheral blood in these patients (Kavlick and Mitsuya, 2001). It is worth noting that two patients had T215V together with the Q151M complex. Although we attempted to determine whether the Q151M complex-containing HIV-1 was transmitted to these children from their mothers, only one plasma sample from mother of Pt_{ERS2} was available. The HIV-1 of the mother of Pt_{ERS2} was isolated from her blood taken in July 2004, when the child was enrolled into the clinical trial. She was found to harbor HIV-1 lacking significant resistance-associated substitutions and the Q151M complex (Table 3).

All three HIV-1 isolates were found to contain primary substitutions associated with PI resistance. These substitutions were thought to be highly related to PIs each patient received during antiviral therapy. Among them were M46I (Kaplan et al., 1994) in HIV-1_{ERS3}, V82A (Molla et al., 1996) in HIV-1_{ERS2} and HIV-1_{ERS3}, and L90M (Eberle et al., 1995) in all three isolates (Table 4).

The Gag-encoding gene contained genetic alterations including a 453-PEPTAP-459 insertion in HIV-1_{ERS2} and HIV-1_{ERS3}, 127-SS-128 insertion plus 472-deletion in HIV-1_{ERS3}.

3.5. Susceptibility of the Q151M-containing HIV-1 variants to RTIs

We propagated a clinical HIV-1 isolate from Pt_{ERS2} by culturing his PBMC with PHA-PBMC from an HIV-1-negative, healthy donor. However, we failed to propagate HIV-1 using samples from Pt_{ERS1} and Pt_{ERS3}. Thus, to study the replication characteristics of these patients' viruses in the presence and absence of antiviral agents, we generated recombinant infectious clones by incorporating the RT-encoding region and a part of integrase-encoding region (aa 21–560 plus aa 1–110) into pHIV-1_{NL4-3} and examined each infectious clone for its susceptibility against various NRTIs and NVP (Table 5). The recombinant infectious clones were confirmed to have the RT-region containing the Q151 complex mutations as well as other mutations (D67N, K70R, and K219Q/E) shown in Table 3. As expected, all three recombinant infectious clones were substantially resistant to ZDV, ddI, and d4T as previously described for other Q151M-containing infectious clones (Shirasaka et al., 1993, 1995). Each clone was only moderately resistant to 3TC with 5–6 fold increase in IC_{50} values in agreement with previous reports (Shirasaka et al., 1993, 1995; Shafer et al., 1994). All three clones (rHIV-1_{ERS1}, rHIV-1_{ERS2}, and rHIV-1_{ERS3}) were sensitive or only slightly resistant to TDF with 2–4.2-fold increase in IC_{50} values, which probably explain why the regimens containing TDF effectively reduced peripheral HIV RNA levels in Pt_{ERS1} and Pt_{ERS2}. The reason no reduction in HIV-1 RNA copies was seen in Pt_{ERS3} is not clear (Fig. 1). Two clones (rHIV-1_{ERS2} and rHIV-1_{ERS3}) and HIV-1_{ERS2}, all of which contained NVP resistance-associated amino acid substitutions K103N and/or Y181I (Table 3), proved to be highly resistant to NVP, in agreement with previous reports (Richman et al., 1991, 1994).

Table 4
Genetic alterations in protease and Gag of HIV-1 isolated from the 3 patients and the mother of one of them

Patient	Substitutions in protease[a]													Alterations in Gag[a]		
	L10	K20	V32	L33	M36	M46	I54	K55	L63	A71	V77	V82	L90	Q127	P453	P472
Pt_{ERS1}	–	R	I	–	I	–	–	–	P	V	–	L	M	–	–	–
Pt_{ERS2}	–	–	–	–	–	–	–	–	P	V	I	A	M	–	*ins*PEPTAP[b]	–
Mother of Pt_{ERS2}	I	–	–	–	–	I	V	–	P	–	I	–	–	–	–	–
Pt_{ERS3}	F	–	–	F	–	I	V	R	P	V	–	A	M	*ins*SS[b]	*ins*PEPTAP[b]	del

[a] Amino acids and their positions in HIV-1_{LAI} are shown as a reference at the top; "–" indicates that the amino acid is the same as in the reference sequence.
[b] An insertion identified. del: deletion.

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Table 5
Susceptibility against RTIs of various infectious clones and HIV-1_{ERS2}

HIV-1	IC_{50} (μM)					
	ZDV	ddI	d4T	3TC	TDF	NVP
rHIV-1_{NL4-3}[a]	0.011 (1)	1.6 (1)	0.36 (1)	1.0 (1)	0.88 (1)	0.091 (1)
rHIV-1_{ERS1}[a]	0.44 (40)	52 (33)	7.2 (20)	6.1 (6)	1.8(2)	0.2 (2)
rHIV-1_{ERS2}[a]	0.15 (14)	62 (39)	5.6 (15)	5.5 (5.5)	3 (3.4)	>10 (>110)
rHIV-1_{ERS3}[a]	0.14 (13)	64 (40)	4.3 (12)	5.2 (5.2)	3.7 (4.2)	>10 (>110)
HIV-1_{LAI}[b]	0.004 (1)	0.69 (1)	0.09 (1)	0.3 (1)	0.06 (1)	0.017 (1)
HIV-1_{ERS2}[b]	0.046 (12)	14 (20)	1.1 (12)	0.9 (3)	0.2 (3.3)	>10 (>588)

[a] MT-2 cells were exposed to rHIV-1_{ERS1}, rHIV-1_{ERS2}, or rHIV-1_{ERS3} and IC_{50} values were determined using the MTT assay. The value in parentheses represents a fold difference of the IC_{50} value of each RTI as compared to that against rHIV-1_{NL4-3}.

[b] The IC_{50} values were determined using PHA-PBMC exposed to HIV-1_{LAI} or HIV-1_{ERS2} in the presence of each RTI and the inhibition of $p24^{Gag}$ protein production by the drug as an endpoint. The numbers in parentheses represent fold differences compared to the IC_{50} against HIV-1_{LAI}.

4. Discussion

In the present study, we identified a set or subset of the Q151 complex (A62V, V75I, F77L, F116Y, and Q151M), which confers MDR on HIV-1 in 3 of 28 (10.7%) children enrolled into a phase I/II study of TDF (Hazra et al., 2005) and a study of therapeutic drug monitoring and viral resistance testing. This rate of the emergence of the Q151M complex is greater than the rates reported by others. For example, in a study conducted between 1991 and 1995 by Englund et al., of 52 children with AIDS who were drug-naïve or had received antiviral therapy for less than 6 weeks and subsequently received ZDV, ddI or both drugs combined for 8.6 months (range, 2.6–28.9 months), only one child developed Q151M (1.9%) (Englund et al., 2004). Larder et al. reported that of 180 patients who had <300 $CD4^+$ cells per mm^3 and had received <4 weeks (range, 0–0.93 months) of ZDV therapy and subsequently ZDV monotherapy or combination of ZDV plus ddI or ZDV plus zalcitabine for 48 weeks, none developed the Q151 complex (Larder et al., 1996). The duration of treatment with NRTI regimens varied from 2.6 to 28.9 months in these studies, but was shorter than the duration of NRTI-containing therapy administered to the 28 pediatric patients of the present study. The duration of therapy for the pediatric patients ranged from ~4.8 to ~13.5 years (mean, 9.7 years), so that these patients had received antiviral therapy with multiple NRTIs for significantly longer periods of time, which may relate to the high rate of the emergence of the Q151M complex seen in the present study. It is of note that the duration of treatment with NRTI-monotherapy or dual combination regimens in patients who developed the Q151M complex in our previous study which enrolled those who had received >36 months of ZDV plus ddI (Kavlick et al., 1998) was >52 to >93 months (mean: 68.3 months or longer), and of 36 such patients, 6 patients (17%) were found to harbor HIV-1 variants containing the Q151M complex. Zaccarelli et al., between June 1999 and December 2002, examined as many as 470 patients who had treatment failure and found the Q151 complex in 17 patients, a relatively low ratio of 3.6%. However, these authors found a high association of the emergence of the Q151M complex with younger age, lower CD4 counts, greater HIV-1 RNA copy numbers, and treatment with >2 years prior antiviral therapy (Zaccarelli et al., 2004). Considering that all three patients in the present study are at younger ages with lower CD4 counts and substantially high HIV-1 RNA copy numbers, and >4.3 years of therapy, these factors may possibly explain why the high ratio of the Q151M emergence was seen in our cohort. It is apparent that long-term NRTI monotherapy and/or dual NRTI combination therapy, without other classes of antiviral agents, might be the reason for the high incidence of the Q151M emergence. Another possible reason is that HIV-1-infected children, especially young children, have higher viral loads than adults do. Hence, it is harder to drive the viral loads to undetectable levels with HAART, so that some residual viral replication will continue, even during HAART, rendering it more likely that resistance will develop (Shearer et al., 1997), although further studies are needed to definitively determine the factor(s) responsible for the emergence of the Q151M complex.

In the RT sequence from Pt_{ERS1}, all five substitutions (A62V, V75I, F77L, F116Y, and Q151M) were identified, while Pt_{ERS2} had A62V, V75I, F116Y, and Q151M, and Pt_{ERS3} had F116Y and Q151M (Table 3). However, these two latter HIV-1 populations (Pt_{ERS2} and Pt_{ERS3}) had ZDV resistance-associated substitutions (T215V plus K219Q or K219E) (Table 3). While several previously reported cases of HIV-1 having the Q151M substitution carried both the Q151M complex and a substitution at position 215, such dual mutations are relatively rarely found (Shirasaka et al., 1993, 1995; Kavlick et al., 1998). Indeed, in the Stanford HIV Drug Resistance Database (Shafer et al., 1999; Rhee et al., 2003) compiled to date, of 2642 cases in which a substitution(s) at position 215 was identified, only 33 cases (1.25%) have been found to carry both 215 substitution(s) and the Q151M complex mutation(s). The T215V substitution has been reported in an HIV-1-infected child who developed ZDV resistance with D67N/K70R/T215V/K219Q, although its virologic significance is not completely understood (Orlandi et al., 1998). The examination of the Stanford University HIV Drug Resistance Database revealed that of 2642 persons having substitutions at position 215, only 57 persons had T215V (2.2%), suggesting that although T215V substitution could be related to ZDV resistance, the level of its contribution to ZDV resistance and/or viral replication appears to be low in comparison with that of T215Y (~73.8% seen in the Database) and T215F (~20% in the Database). It is not clear at this time as to why Pt_{ERS2} and Pt_{ERS3} had these dual amino acid substitutions.

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We generated recombinant infectious HIV-1 clones from all three patients by incorporating into HIV-1_{NL4-3} the RT-encoding region of HIV-1 using viral RNA from their plasma, and examined each clone for its susceptibility to various NRTIs and NVP. In this study, however, we chose not to incorporate amino acid substitutions in protease, since the introduction of amino acid substitutions in protease without amino acid substitutions seen in Gag proteins often lead to greatly compromised replicative ability of the resultant recombinant infectious clones (Gatanaga et al., 2002). We found that all three infectious clones (rHIV-1_{ERS1}, rHIV-1_{ERS2}, and rHIV-1_{ERS3}) were substantially resistant to ZDV, ddI, and d4T, and only moderately resistant to 3TC in agreement with previous reports (Shirasaka et al., 1993, 1995; Shafer et al., 1994). These three clones were sensitive or only slightly resistant to TDF, which probably explains why the TDF-containing regimens reduced the numbers of HIV RNA copy numbers in Pt_{ERS1} and Pt_{ERS2}. However, no reduction occurred in Pt_{ERS3} with the implementation of TDF-containing regimen (Fig. 1). In this regard, Pt_{ERS3} had previously been reported to have the K65R substitution that has been shown to be associated with viral resistance to TDF (Wainberg and Turner, 2004), although K65R was not detected at baseline in the present study. Thus, re-emergence of K65R-containing variants may explain his lack of response. It is also of note that Pt_{ERS3} was intermittently non-adherent to the salvage regimen. Such non-adherence might have been the major reason for his treatment failure.

HIV-1 with mutations conferring drug resistance are likely less fit than the wild-type virus and are transmitted relatively inefficiently (Leigh Brown et al., 2003), although there is a growing body of literature indicating increasing cases of transmission of drug-resistant HIV-1 variants (Salomon et al., 2000; Masquelier et al., 2005), posing a formidable threat to those at risk. Nevertheless, there has been no report to date that HIV-1 carrying the Q151M complex was transmitted in the form of either sexual or vertical transmission. Thus, we attempted to ask whether the Q151M complex was transmitted from their mothers to the children. However, only a blood sample from Pt_{ERS2}'s mother was available and this mother's HIV-1 had no significant amino acid substitutions in RT or protease (Tables 3 and 4).

In conclusion, of 28 pediatric patients examined in the present study, the Q151M complex was identified in three patients (10.7%), all of who had received ZDV monotherapy and/or combination regimens with multiple NRTI over 4.3–8.6 years. This work represents the first extensive study to characterize the virological profiles of Q151M-carrying HIV-1 variants isolated from children receiving HAART and suggests that the Q151M emergence rate could be high in pediatric patients who are treated with long-term NRTI monotherapy and/or dual NRTI regimens. The present data also suggest that TDF- or ENF-containing regimens may be meritorious in such patients with the Q151M complex.

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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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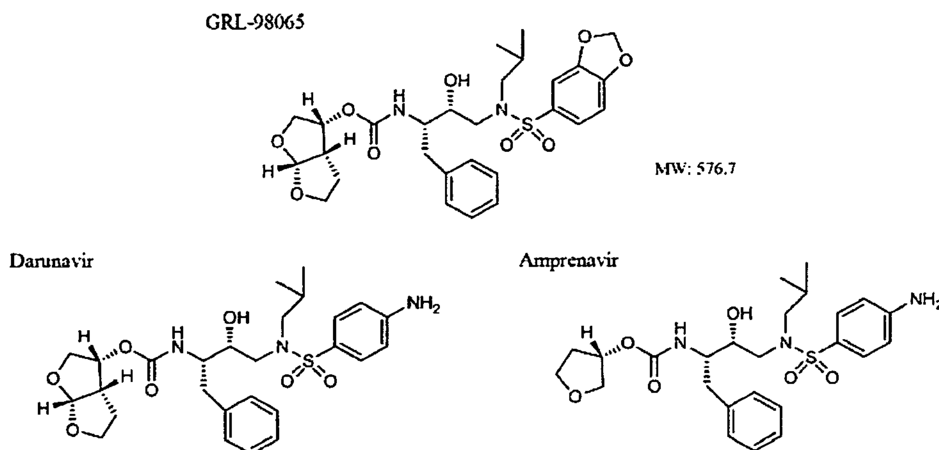

FIG. 1. Structures of GRL-98065, darunavir, and amprenavir.

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_{Bu-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. ^1H 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

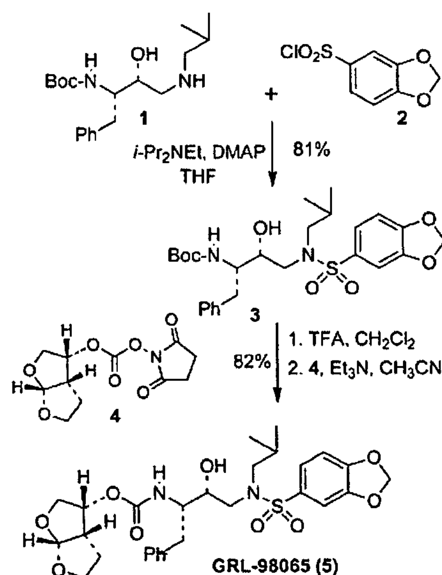

FIG. 2. Synthesis of GRL-98065.

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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-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_{NL4-3}), 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_{NL4-3} and PI-resistant HIV-1_{NL4-3} 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_{NL4-3} (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_{NL4-3} variant and wild-type HIV-1_{NL4-3}. 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_{NL4-3} 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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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, F53I, K55R, I62V, L63P, A71V, G73S, V82A, L90M, and I93L in HIV-$1_{MDR/B}$; L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, and L89M in HIV-$1_{MDR/C}$; 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.

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

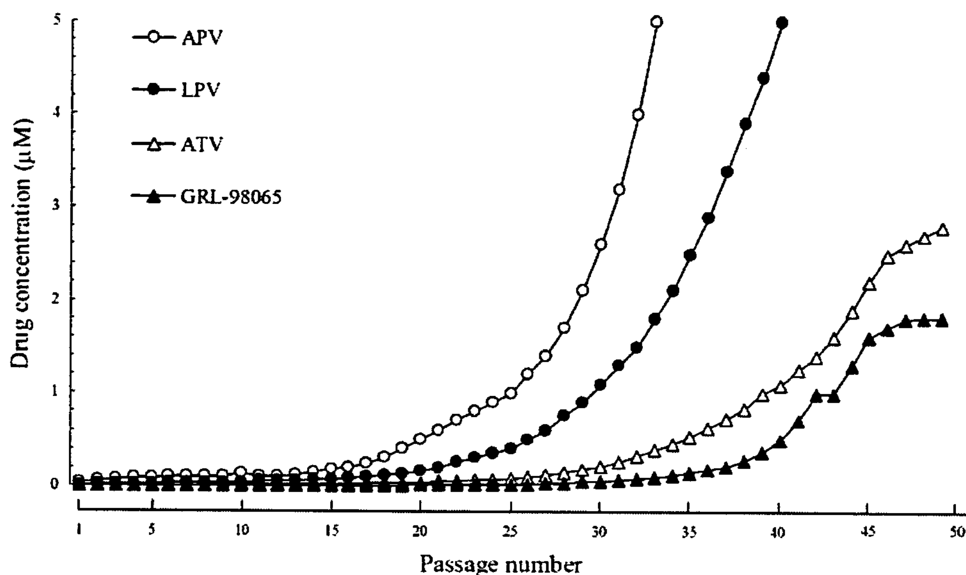

FIG. 3. In vitro selection of PI-resistant HIV-1 variants. HIV-1_{NL4-3} was propagated in MT-4 cells in the presence of increasing concentrations of amprenavir (○), lopinavir (●), atazanavir (△), or GRL-98065 (▲). Each passage of virus was done in a cell-free fashion.

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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                  10         20         30         40         50         60         70         80         90        99
pNL4-3 PR  PQITLWQRPL VTIKIGGQLK EALLDTGADD TVLEEMNLPG RWKPKMIGGI GGFIKVRQYD QILIEICGHK AIGTVLVGPT PVNIIGRNLL TQIGCILNF
5P-1       .......... .......... .......... .......... .......... .......... .......... .......... .......... ......... 9/13
5P-2       .......... .......... .......... .......... .......... ....E..... .......... .......... .......... ......... 1/13
5P-3       .......... .......... .....A.... .......... .......... .......... .......... .......... .......... ......... 1/13
5P-4       .......... .......... .......... .......... ......V... .......... .......... .......... .......... ......... 1/13
5P-5       .......... .......... .......... .........E .......... .......... .......... .......... .......... ......... 1/13

10P-1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ......... 8/13
10P-2      .......... .......... .......... .......... .......... .......... .......... .......... .......... .H....... 1/13
10P-3      .....C.... .......... .......... .......... .......... .......... .......... .......... .......... ......... 1/13
10P-4      ....P..... .......... .......... .......... .......... .......... .......... .......... .......... ......... 1/13
10P-5      .......... .......... .......... .........E .......... .......... .......... .......... .......... ......... 1/13
10P-6      .......... .......... .......... .......... .L........ .......... .......... .......... .I........ ......... 1/13

15P-1      .......... .......... .......... .......... ..I....... .......... .......... .......... .......... ......... 5/11
15P-2      .......... .......... .......... .......S.. ..I....... .......... .......... .......... .......... ......... 1/11
15P-3      .......... .......... .......... .......... ..I....... .......... .......... .......... ....V..... ......... 1/11
15P-4      .......... .......... .......... .......... ..I....... .D........ .......... .......... .......... ......... 1/11
15P-5      ..V....... .......... .......... .......... ..I....... .......... .......... .......... .......... ......... 1/11
15P-6      .......... .......... .......... .......... ..I....... .......... .......... .......... .......... ....Y.... 1/11
15P-7      .......... .......... .......... .......... ..I....... .......... .......... .L........ .......... ......... 1/11

20P-1      .......... .......... .......... .......... ..I....... .......... .......... .......... .I..V..... ......... 8/19
20P-2      .......... .......... .......S.. .......... ..I....... .......... ......Y... .......... .I......M. ......... 2/19
20P-3      .......... .......... .......S.. .......... ..I....... .......... R.....Y... .......... .I......M. ......... 1/19
20P-4      .......... .......... .......S.. .......... ..I....... .......... ......Y... .......... .I..V...M. ......... 1/19
20P-5      .......... ...R...... ........E. .......... S.I....... .......... .......... .......... .I..V..... ......... 1/19
20P-6      .......... .......... .......... .......... ..I....... .......... ......Y... .......... .I......M. ......... 1/19
20P-7      .......... .......... .......... .......... ..I....... ........C. .......... .......... .I..V..... ......... 1/19
20P-8      .......... .......... .......... .......... ..I....... .......... .......... .......... .I......M. ......... 1/19
20P-9      .......... .......... .......... .......... ..I....... ......G... .......... .......... .I..V..... ......... 1/19
20P-10     .......... .......... .......... I......... ..I....... .......... .......... .......... .I..V..... ......... 1/19
20P-11     .......... .......... .......... .......... ..I...L... .......... .......... .......... .I..V..... ......... 1/19

25P-1      .......... .......... .......S.. .......... ..I..I.... .......... .......... .......... .I..V..... ......... 4/20
25P-2      .......... .......... .......S.. .......... ..I....... .......... .......... .......... .I......M. ......... 3/20
25P-3      .......... .......... .......... .......... ..I....... .......... .......... V......... .I........ ......... 2/20
25P-4      .......... .......... .......... .......... ..I..I.... .......... .......... .......... .I..V..... ......... 2/20
25P-5      .......... .......... .......S.. .......... ..I....... .......... .......... T......... .I......M. ......... 1/20
25P-6      .......... .A........ .......S.. .......... ..I..I.... .......... .......... .......... .I..V..... ......... 1/20
25P-7      .......... .......... .......... .......... ..I..I.... .......... .......... .......... .I......M. ......... 1/20
25P-8      .......... .......... .......... .......... ..I....... ...T...... .......... V......... .I........ ......... 1/20
25P-9      .......... .......... .......... .......... ..I....... .......... .......... .......... .I......M. ......... 1/20
25P-10     .......... .......... .......... .......... ..I..I.... .......... .......... .......... .T..V..... ......... 1/20
25P-11     .......... .......... .....A.... .......... ..I..I.... .......... .......... .......... .I......M. ......... 1/20
25P-12     .......... .......... .......... .......... ..I..I.... .......... ...M...... .......... .I..V..... ......... 1/20
25P-13     .......... .......... .......... .......... ..T..I.... .......... .......... .......... .I..V..... ......... 1/20

30P-1      .......... .I........ .......... ...K...... ..I....... .......... .......... .......... .I..V...M. ......... 2/10
30P-2      .......... .I........ K......S.. .......... ..I..I.... .......... .......... .......... .I..V..... ......... 2/10
30P-3      .....R.... .......... .......S.. .......... ..I....... ...V...... .......... V......... .I........ ......... 1/10
30P-4      .......... .......... K......S.. ...K...... ..I..I.... .......... .......... .......... .I..V..... ......... 1/10
30P-5      .......... .......... K......S.. .......... ..I....... .......... .......... .......... .I..V..... ......... 1/10
30P-6      .......... .......... .......S.. .......... ..I..I.... .......... .......... .......... .I......M. ......... 1/10
30P-7      .......... .I........ .......... A..K...... ..I....... .......... .......... .......... .I..V...M. ......... 1/10
30P-8      .......... .......... .......... .......... ..I....... .......... .......... .......... .I..V...M. ......... 1/10

33P-1      .......... .......... .......... .......... ..I..I.... .......... .......... .......... .I......M. ......... 2/11
33P-2      .......... .......... K......S.. .......... ..I..I.... .......... .......... .......... .I..V...M. ......... 2/11
33P-3      .......... .......... K......S.. .......... ..I..I.... ...T...... .......... .......... .I..V...M. ......... 1/11
33P-4      .......... I......... K......S.. A......... ..I..I.... .........N .......... .......... .I......M. ......... 1/11
33P-5      .......... .......... .......... .......... ..I..I.... .........N .......... .......... .I..V...M. ......... 1/11
33P-6      .......... .......... .......... .......... ..I..I.... ....N....G .......... .......... .I......M. ......... 1/11
33P-7      .......... .......... .......... .......... ..I..I.... .......... .......... .......... .I..V...M. ......... 1/11
33P-8      .......... .......... .......... .......... ..I..I.... .......... .......... .......... .I..V..... ......... 1/11
33P-9      .......... .......... .......... .......... ..I....... .......... .M........ .......... .I..V...M. ......... 1/11

40P-1      .......... .......... K......S.. .......... ..I..I.... .........N .......... V......... .I..V...M. ......... 3/11
40P-2      .......... .......... K......S.. .......... ..I..I.... .......... .......... V.S....... .I..V...M. ......... 2/11
40P-3      .......... .......... K......S.. .......... ..I..I.... .........N .......... V...I..... .I..V...M. ......... 1/11
40P-4      .......... .......... K......S.A .......... ..I..I.... .......... .......... .......... .I..V...M. ......... 1/11
40P-5      .......... .......... .......... .......... ..I..I...V .........N .......... .......... .I......M. ......... 1/11
40P-6      .......... .......... .......... .......... ..I..I...V .......... .......... .......... .I..V...M. ......... 1/11
40P-7      .......... .......... .......V.. .......... ..I..I...V .......... .......... .......... .I..V...M. ......... 1/11
40P-8      .......... ...NT..... .......... .......... ..I..I...V .........N .......... .......... .I......M. ......... 1/11
```

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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                   10         20         30         40         50         60         70         80         90        100
pNL43 Gag  MGARASVLSG GELDKWEKIR LRPGGKKQYK LKHIVWASRE LERFAVNPGL LETSEGCRQI LGQLQPSLQT GSEELRSLYN TIAVLYCVHQ RIDVKDTKEA
 5P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
10P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
15P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
20P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
25P        .......... .......... .......... .......... .......... .......... ....P..... .......... .......... ..........
30P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
33P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
40P-1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  8/9
40P-2      .......... .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  1/9

                  110        120        130        140        150        160        170        180        190        200
pNL43 Gag  LDKIEEEQNK SKKKAQQAAA DTGNNSQVSQ NYPIVQNLQG QMVHQAISPR TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
 5P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
10P        .......... .......... ........X. .......... .......... .......... .......... .......... .......... ..........
                                         G/S
15P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
20P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
25P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
30P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
33P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
40P-1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  5/9
40P-2      .......... .......... .......... .......... .......... .......... .......A.. .......... .......... ..........  1/9
40P-3      .......... .......... .......... .......... .......... .......... .......... .......... .H........ ..........  1/9
40P-4      .......... .......... .......... .......... .......... .......... ....R..... ......G... .......... ..........  1/9
40P-5      ........Y. .......... .......... .......... .......... .......... .......... ......G... .......... ..........  1/9

                  210        220        230        240        250        260        270        280        290        300
pNL43 Gag  LKETINEEAA EWDRLHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM THNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
 5P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
10P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
15P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
20P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
25P        .......... .......... .......... .......... .......... .......... .......... ....X..... .......... ..........
                                                                                          K/R
30P        .......... .......... .......... .......... .......... .......... .......... ....K..... .......... ..........
33P        .......... ..X....... .......... .......... .......... .......... .......... ....K..... .......... ..........
                      N/D
40P-1      .......... .......... .......... .......... .......... .......... .......... ....K..... .......... ..........  5/9
40P-2      .......... .......... .......... .......... .........V .......... .......... ....K..... .......... ..........  1/9
40P-3      .......... ........Y. .......... .......... .......... .......... .......... ....K..... .......... ..........  1/9
40P-4      .........T .......... .......... .......... .......... .......... .......... ....K..... .......... ..........  1/9
40P-5      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  1/9

                  310        320        330        340        350        360        370        380        390        400
pNL43 Gag  YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPG ATLEEMMTAC QGVGGPGHKA RVLAEAMSQV TNPATIMIQK GNFRNQRKTV KCFNCGKEGH
 5P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
10P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
15P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
20P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
25P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
30P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
33P        .......... .......... .......... .......... .......... .......... .......... .......... .........X ..........
                                                                                                           D/V
40P-1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  3/9
40P-2      .......... .......... .......... .......... .......... .......... .......... .......V.. .......... ..........  1/9
40P-3      .......... .......... .......... .......... .......... R......... .......... .......... .......... ..........  1/9
40P-4      .......... .......... .......... .......... .......... .......... .......... .......... .......... ...D......  1/9
40P-5      .......... .......... .......... .......... T......... .......... .......... .......... .......... ..........  1/9
40P-6      .......... .......... .......... .......... .......... .......... ......K... .......... .......... ..........  1/9
40P-7      .......... .......... .......... .......... .S........ .......... .......... .......... .......... ..........  1/9

                  410        420        430        440        450        460        470        480        490        500
pNL43 Gag  IAKNCRAPRK KGCWKCGKEG HQMKDCTERQ ANFLGKIWPS HKGRPGNFLQ SRPEPTAPPE ESFRFGEETT TPSQKQEPID KELYPLASLR SLFGSDPSSQ
 5P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
10P        .......... .......... .......... ........X. .......... .......... .......... .......... .......... ..........
                                                    L/P
15P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
20P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
25P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
30P        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
33P        .......X.. .X........ .......... .X........ .......... .......... .......... .......... .......... ..........
                  H/P D/G                   I/N
40P-1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  2/9
40P-2      .......... .D........ .......... ......T... .......... .......... .......... .......... .......... ..........  2/9
40P-3      .......... .D........ .......... ......T... .......... .......... .......... .........E ...F...... ..........  1/9
40P-4      .......... .D........ .......... ......T... .......... .......... .......... .........E .......... ..........  1/9
40P-5      .......... .......... .......... .......... .......... .......... .......... ..P....... .......... ..........  1/9
40P-6      .......... .......... .......... .......... .......... .......... .......... ........M. .......... ..........  1/9
40P-7      .......... .......... .......... .....R.... .......... .......... .......... .......... R......... ..........  1/9
```

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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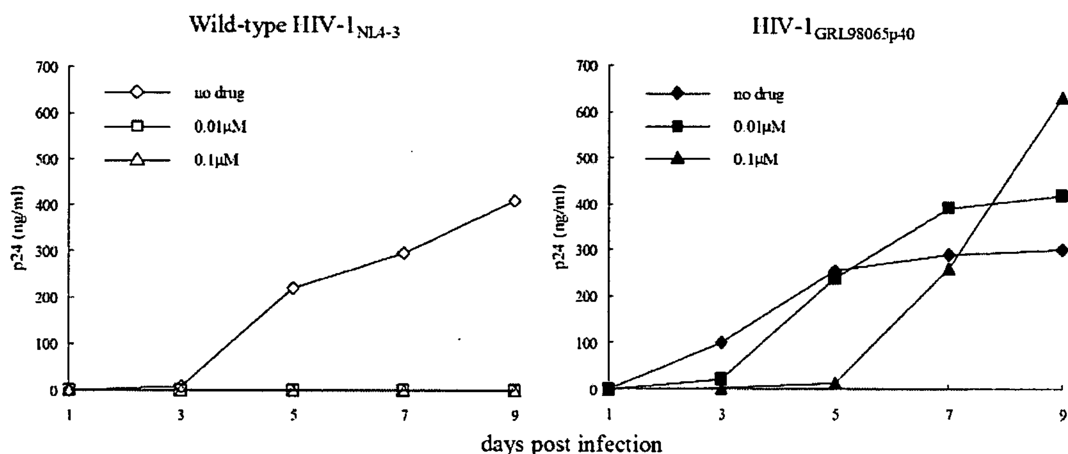

FIG. 6. Replication kinetics of GRL-98065-resistant HIV-1 variant and HIV-1_{NL4-3}. MT-4 cells (2.4 × 10^5) were exposed to an HIV-$1_{GRL98065p40}$ or wild-type HIV-1_{NL4-3} preparation containing 30 ng p24 in six-well culture plates for 3 h, and these MT-4 cells were divided into three fractions, 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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