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Genotypic Correlates of Phenotypic Resistance to Efavirenz in Virus Isolates from Patients Failing Nonnucleoside Reverse Transcriptase Inhibitor Therapy

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Efavirenz (also known as DMP 266 or SUSTIVA) is a potent nonnucleoside inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) activity and of HIV-1 replication in vitro and in vivo. Most patients on efavirenz-containing regimens have sustained antiviral responses; however, rebounds in plasma viral load have been observed in some patients in association with the emergence of mutant strains of HIV-1. Virus isolates from the peripheral blood mononuclear cells (PBMCs) of patients with such treatment failures, as well as recombinant viruses incorporating viral sequences derived from patient plasma, show reduced in vitro susceptibility to efavirenz in association with mutations in the RT gene encoding K103N, Y188L, or G190S/E substitutions. Patterns of RT gene mutations and in vitro susceptibility were similar in plasma virus and in viruses isolated from PBMCs. Variant strains of HIV-1 constructed by site-directed mutagenesis confirmed the role of K103N, G190S, and Y188L substitutions in reduced susceptibility to efavirenz. Further, certain secondary mutations (V106I, V108I, Y181C, Y188H, P225H, and F227L) conferred little resistance to efavirenz as single mutations but enhanced the level of resistance of viruses carrying these mutations in combination with K103N or Y188L. Viruses with K103N or Y188L mutations, regardless of the initial selecting nonnucleoside RT inhibitor (NNRTI), exhibited cross-resistance to all of the presently available NNRTIs (efavirenz, nevirapine, and delavirdine). Some virus isolates from nevirapine or delavirdine treatment failures that lacked K103N or Y188L mutations remained susceptible to efavirenz in vitro, although the clinical significance of this finding is presently unclear.

The reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) is both critical to the life cycle of HIV and is without a homologue in eukaryotic organisms. As such, it is an attractive target for selective antiviral therapy. Among inhibitors of RT, a large class of chemically diverse, generally HIV-1-specific nonnucleoside RT inhibitors (NNRTIs) has been identified. These inhibitors act by binding to a site on the RT that is distinct from the polymerase catalytic site. While NNRTIs can be potent inhibitors of HIV-1 replication, with favorable safety and pharmacokinetic parameters, rapid emergence of resistant viruses both in vitro (17, 22) and in vivo (4, 21, 27), often as the result of single-nucleotide changes, has limited the utility of these compounds as monotherapy. However, recent clinical trials of the use of NNRTIs in combination with other antiretroviral agents have demonstrated significant added benefit from inclusion of an NNRTI in such combination regimens (3, 19, 26; S. Green, M. F. Para, P. W. Daly, et al., XII World AIDS Conf., abstr. 12219, p. 55, 1998). A variety of mutations in the HIV-1 RT gene associated with resistance to NNRTIs have been identified (20, 23). In models of the three-dimensional structure of HIV-1 RT (25), these mutations cluster around the NNRTI binding site in the p66 subunit of HIV-1 RT.

Efavirenz (also known as SUSTIVA, formerly as DMP 266) is a potent nonnucleoside inhibitor of HIV-1 RT and of HIV-1 replication in vitro and in vivo. It has shown potent antiviral activity and significant clinical efficacy (26). In cell culture, efavirenz is a potent inhibitor of HIV-1 replication and retains significant activity against a variety of mutant strains of HIV-1 with single-amino-acid substitutions in the RT gene which have been associated with resistance to other NNRTIs (32). Cell culture selection experiments (31) demonstrated that passage of the RF strain of HIV-1 in MT-2 or peripheral blood mononuclear cell (PBMC) culture in the presence of efavirenz led to the selection of mutations encoding substitutions at amino acid positions 100, 108, 179, and 181 of the HIV-1 RT gene. Young et al. (32) reported the selection in cell culture of an L100I/K103N double mutant that was highly resistant to efavirenz. In both cases, high-level resistance to efavirenz (a ≥100-fold increase in 90% inhibitory concentration [IC_{90}]) appeared to be associated with multiple mutations in the RT gene of HIV-1.

In order to assess the role of viruses with reduced in vitro susceptibility to efavirenz in virologic treatment failure and the genotypic basis of such phenotypic resistance, we derived replicating virus isolates from the PBMCs of patients virologically failing efavirenz combination therapy in two phase II clinical studies. In addition, recombinant viruses incorporating HIV-1

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protease and RT genes from these PBMC isolates or directly from patient plasma were constructed and tested. The in vitro phenotypic susceptibility of these isolates and recombinant viruses was determined, either in PBMC culture or during replication in established T-cell lines. Site-directed mutants were constructed and tested to assess the contribution of specific RT gene mutations and combinations of mutations to phenotypic resistance.

MATERIALS AND METHODS

Compounds. Efavirenz, delavirdine, and nevirapine were dissolved in dimethyl sulfoxide at a concentration of 5 mg/ml, stored at −20°C, and diluted on the day of use in RPMI 1640 containing 10% fetal calf serum.

Cell culture. Human T-cell lines MT-2 and MT-4 were maintained in Dulbecco's modified Eagle's medium (MT-2) or RPMI medium (MT-4) supplemented with 10% fetal calf serum and 50 μg of gentamicin (Gibco-BRL)/ml at 37°C and 5% CO_2.

Clinical studies. Virus isolates and/or recombinant virus constructs were derived from patients participating in two phase II clinical studies of efavirenz combination therapy. Study DMP 266-003 enrolled NNRTI- and protease inhibitor (PI)-naïve patients with plasma HIV RNA levels of ≥20,000 copies/ml (Amplicor HIV-1 Monitor assay, v 1.0; Roche) and between 100 and 500 CD4 cells/mm^3. Patients received 800 mg of indinavir every 8 h (later raised to 1,000 mg every 8 h) and 200 mg of efavirenz once a day (q.d.) (later raised to 600 mg q.d.) or placebo. In this study, two cohorts began with a 2-week monotherapy lead-in period prior to the initiation of combination therapy. Study DMP 266-004 enrolled NNRTI- and PI-naïve patients with ≥8 weeks of prior zidovudine (ZDV)/lamivudine (3TC) combination therapy who had ≥2,500 copies of HIV RNA/ml of plasma. Patients received efavirenz (400 or 600 mg q.d.) or placebo and continued on ZDV/3TC. While representing the standard of care at the time that this study was initiated, this trial design is presently recognized as suboptimal therapy since a single agent was added to a failing drug regimen.

Virus isolation from patient PBMCs. PBMCs from patients in studies DMP 266-003 and -004 were separated from whole blood collected before the start of study medications and at various intervals during the study by using Ficoll-Hypaque density centrifugation. PBMCs were cryopreserved for subsequent virus isolation. Virus isolations were performed using the PBMCs of selected patients who experienced rebounds in viral load on efavirenz combination therapy (i.e., patients who became virologic treatment failures) by cocultivation with uninfected phytohemagglutin/interleukin-2-stimulated donor PBMCs (10).

Recombinant virus construction. Recombinant viruses incorporating viral protease and RT gene sequences derived from patient plasma and PBMC isolates were constructed by Virco NV (Mechelem, Belgium) as previously described (8).

Genotyping. Genotyping of PBMC isolates was performed on DNA isolated from infected PBMCs. Cell pellets from virus cocultivation in PBMCs were lysed, and DNA was precipitated (Puregene DNA Isolation; Gentra Systems). Viral DNA amplification was performed using a nested PCR procedure (GeneAmp XL PCR Kit) as previously described (16). For three samples, no product was obtained with this PCR procedure. A nested amplification to yield a smaller product of 0.80 kbp including only the RT region was used for these samples. Two methods of DNA sequencing were used on bulk PCR products. The first used cycle sequencing with dye-labeled primers (Thermo Sequenase; Amersham) followed by gel electrophoresis on an automated sequencer (A.L.F.; Pharmacia). The second used cycle sequencing with unlabeled primers and dye-labeled terminators (A.B.I. Prism BigDye; PE Biosystems) followed by gel electrophoresis on an automated sequencer (A.B.I. Prism 377). Files of sequences were exported to Sequencher (GeneCodes) for alignment and contiguity building with HIV-1 reference sequences (NL4-3 and HXB2 consensus sequences).

Genotyping of recombinant virus constructs derived from patient plasma or PBMC isolates was accomplished by direct sequencing of the pool of PCR products used for construction of each recombinant virus. A single consensus sequence was derived for each recombinant construct. Differences from the consensus sequence of HXB2 were reported when detected, even when present as part of a mixed sequence. Some PBMC isolates were resequenced by this method after an additional expansion in uninfected donor PBMCs (8).

Genotyping of plasma virus was accomplished by ABI-based dideoxy sequencing of multiple (typically eight) independently amplified and cloned viral genomes as previously described (2).

Construction of HIV-1 RT mutants. Variant viruses created by site-directed mutagenesis with single- or multiple-amino-acid substitutions in the NL4-3 wild-type background (see Tables 2 and 4) were obtained from E. Emini and William Schleif, Merck Research Laboratories. Additional site-directed mutants were constructed in the HXB2 background using the Altered Sites In Vitro Mutagenesis System (Promega, Madison, Wis.) based on modifications of a method previously described (30). In brief, mutagenesis was performed in a shuttle vector containing the *Apa*I-*Sal* fragment of HXB2. The mutated fragment was then subcloned into a plasmid containing the 5′ half of HXB2 (p5′R). p5′R was linearized with *Nco*I and ligated to a corresponding *Nco*I-linearized plasmid (p3′R) containing the 3′ half of HXB2. Ligations were transfected into MT-4 cells via Lipofectin (Gibco-BRL, Grand Island, N.Y.). Viral cultures were grown to complete lysis (4 to 6 days), stored as supernatants at −80°C, and sequenced to confirm the presence of the desired mutation.

In vitro drug susceptibility assays. The in vitro drug susceptibility of PBMC-derived virus isolates was tested during cultivation in donor PBMCs according to a modified AIDS Clinical Trials Group/Department of Defense consensus protocol (10) as noted. Virus stock was generated using the standard ACTG quantitative microculture method (Division of AIDS and National Institutes of Health, virology manual for HIV laboratory [http://www.niaid.nih.gov/daids/vir_manual/]). Determination of viral stock titers and drug susceptibility testing were performed as described by Johnson and Byington (11, 12). Recombinant viruses derived from PBMC isolates or patient plasma were tested in a recombinant virus assay (RVA; AntiVirogram) using an MT-4-derived indicator cell line (8). The IC_{50} was defined as the concentration of compound that reduced the level of accumulated p24 antigen (PBMC assay) or indicator gene expression (RVA) by 50%. Site-directed mutants were tested following acute infection of MT-4 cells as previously described (9). The IC_{90} was defined as the concentration of compound that reduced the level of accumulated viral p24 antigen by 90%.

Nucleotide sequence accession numbers. Nucleic acid sequences of virus isolates and recombinant virus constructs from the DMP-266-003 study described in this report have been deposited in GenBank with accession numbers AF349317 to AF349374. Sequences from the DMP 266-004 study are accession numbers AF349375 to AF349403. Sequences of plasma viruses described in this report have been previously deposited (1).

RESULTS

In vitro drug susceptibility of virus isolates from patients failing efavirenz combination therapy. Genotypic and phenotypic resistance was characterized for virus isolates from 14 patients entering the DMP 266-003 study and for isolates from 17 patients failing efavirenz combination therapy in this study. From the DMP 266-004 study, 7 baseline isolates and 12 efavirenz treatment failure isolates were characterized both genotypically and phenotypically. Genotypic characterization of 13 additional virologic treatment failure isolates from patient PBMCs is presented and compared to viral sequences detected in plasma collected at the same time point. Two types of in vitro drug susceptibility tests were performed. PBMC isolates were tested in a PBMC-based phenotypic assay. In addition, a series of recombinant viruses were constructed from patient plasma virus or from viruses initially isolated from patient PBMCs. The susceptibility of these recombinant viruses to efavirenz was determined in a high-throughput assay format utilizing an MT-4-based indicator cell line. Results of in vitro susceptibility tests and corresponding viral genotypes are summarized in Tables 1 (study DMP 266-003, efavirenz plus indinavir) and 2 (study DMP 266-004, efavirenz added to ZDV/3TC). Virus isolates from NNRTI-naïve subjects at study entry showed high susceptibility to efavirenz; the median IC_{50} for eight PBMC isolates tested in PBMC culture (including three baseline isolates from patients later exposed to nevirapine) (Table 4) was 1 nM, with a range from 0.2 to 3.0 nM. Similarly, the median IC_{50} for 22 recombinant viruses incorporating baseline protease and RT gene sequences from patient plasma

virus or from PBMC isolates was 1.8 nM, with a range from 0.4 to 3.5 nM.

Most virus isolates from patients for whom efavirenz combination therapy failed showed reduced in vitro susceptibility to efavirenz. This phenotypic resistance to efavirenz was associated with the presence of mutations in the RT gene encoding one or more substitutions of amino acids forming the NNRTI binding pocket of HIV-1 RT, notably K103N, Y188L, or G190S or -E. In a previous study of plasma virus genotypes in patients failing efavirenz-containing regimens, K103N was the most frequently observed mutation, occurring in more than 90% of patients with virologic treatment failure (2). In the present study, post-efavirenz treatment failure isolates with K103N as the only NNRTI resistance mutation had median IC_{50}s for efavirenz of 42 nM (seven recombinant viruses) or 48 nM (two PBMC isolates). One isolate from patient 100, with K103N as the only detectable NNRTI resistance mutation, had unusually high resistance to efavirenz ($IC_{50} > 310$ nM) in both assay systems; the genetic basis of this high resistance is unknown. Among K103N recombinant viruses, median fold resistance relative to the resistance of the HXB2 reference wild type was 63-fold (range, 2- to 100-fold; seven recombinant viruses). Among K103N recombinant virus isolates for which a matching baseline isolate was available (six pairs), the median fold resistance conferred by the K103N mutation relative to each individual patient's baseline virus was 26-fold (range from 2.3- to 40-fold).

Post-efavirenz treatment failure isolates that carried the K103N mutation in combination with other NNRTI resistance mutations demonstrated higher levels of resistance to efavirenz, with a median IC_{50} of >190 nM (16 recombinant viruses). Five of the 16 recombinant viruses had IC_{50}s of >310 nM, the highest concentration of efavirenz tested. The additional mutations observed in virus isolates along with K103N included L100I, K101E/Q, V108I, G190A or -S, P225H, and K238T. These combinations of mutations were similar to those observed in the virus in the plasma of patients failing efavirenz combination therapy (2).

Among post-efavirenz treatment failure isolates without a K103N substitution, three recombinant virus isolates from two subjects showed high-level resistance to efavirenz. In one case, the isolate carried a G190E mutation, while in two isolates from a second patient, a Y188L mutation, with or without V106I, was found. The full extent of resistance of these isolates could not be determined based on the range of efavirenz concentrations tested. One isolate, from patient 69, showed only modest reductions in susceptibility to efavirenz: it was threefold less susceptible than the HXB2 wild-type reference strain in the RVA system and twofold less susceptible as a PBMC isolate than the baseline isolate from the same patient. The only NNRTI resistance mutation detected in either the PBMC isolate or the recombinant virus was a V108I mutation. Sequencing of plasma virus from this patient collected at the same interval revealed the presence of additional NNRTI resistance mutations, notably, the K103N/V108I and K103N/P225H double NNRTI mutations (Table 1).

In a number of cases, in vitro susceptibility testing of virus isolates was performed in PBMCs and also in recombinant viruses derived from such isolates. Figure 1 illustrates the excellent correlation of IC_{50}s ($r^2 = 0.89$ for 65 pairs of IC_{50}s) determined by testing multiple inhibitors (NNRTIs and in some cases NRTIs and PIs as well) against 14 pairs of PBMC isolate recombinant virus constructs.

Among efavirenz/ZDV/3TC treatment failures in study DMP 266-004, high-level resistance to 3TC, in association with the M184V mutation, was observed in nine of nine baseline virus isolates (data not shown). This was not unexpected, since patients in study DMP 266-004 were required, as a study entry criterion, to have had at least 8 weeks of prior ZDV/3TC therapy and to have a viral load of >10,000 copies of HIV RNA/ml of plasma. Resistance to ZDV was variable, both at baseline and following treatment failure, ranging from no reduction of in vitro susceptibility (relative to HXB2) to >49-fold reduction with a median fold resistance of 24-fold at baseline (34-fold after treatment failure) and a median IC_{50} of 0.64 μM at baseline (0.99 μM after treatment failure).

Correlation of plasma virus and virus isolate genotypes. The genotype of virus RNA amplified from plasma collected from patients at the same time as the PBMCs was determined (Tables 1 and 2). In general, the genotypes of virus isolates derived from patient PBMCs were similar to the predominant viral genotypes detected in plasma. The plasma virus genotyping, which was performed by sequencing of multiple, independently amplified and cloned viral genomes, often detected greater heterogeneity than did the virus isolate genotyping, which was performed by direct sequencing of pools of PCR products. This could be a consequence of differential sensitivities of the two genotyping methods to the presence of minor species.

In vitro drug susceptibility of variant viruses with site-directed mutations in HIV-1 RT. In order to confirm that the RT gene mutations observed in clinical isolates were responsible for the observed phenotypic resistance, a panel of site-directed mutants of HIV-1 was constructed in the genetic backgrounds of two common laboratory-adapted strains, NL4-3 and HXB2. Antiviral potency was assessed in an assay measuring accumulation of viral p24 antigen 4 days after acute infection of MT-4 cells (9). Efavirenz was a potent inhibitor of HXB2 and NL4-3 with IC_{90}s of 3.0 to 3.2 nM. There was a less-than-twofold difference in the efavirenz IC_{90} for mutant viruses with the substitution K103R, V106I, V108I, E138K, Y181C, P225H, F227L, or P236L (Table 4). Viruses with the substitution A98G, K101Q, K101E, V106A, Y188C, Y188H, or G190A showed less than 10-fold reduction in susceptibility to efavirenz (average $IC_{90} \leq 14$ nM). Moderate losses in efavirenz susceptibility were noted for viruses with L100I (24-fold less susceptible than the wild type; average $IC_{90} = 73$ nM), or K103N (19- to 36-fold less susceptible; average IC_{90} of 57 to 110 nM depending on genetic background). The greatest reductions in efavirenz susceptibility for single-amino-acid variants of HIV-1 were observed for Y188L (140-fold) or G190S (97-fold) mutant viruses (Table 3). These amino acid substitutions each require two nucleotide changes from the viral sequences present in most patients before NNRTI treatment.

Additional recombinant viruses were constructed to characterize the effects of combinations of amino acid substitutions in HIV-1 RT observed in specimens from patients failing NNRTI combination therapy. A number of substitutions which conferred little or no resistance to efavirenz as a single-amino-acid substitution caused substantial resistance when present in combination with a K103N substitution, particularly the K101Q/

TABLE 1. Genotypic and phenotypic characterization of virus isolates from patients failing an efavirenz/indinavir combination regimen in study DMP 266-003[d]

Patient no.	Study day	Source	RT genotype[b]	In vitro IC_{50} in nM (fold resistance)		
				Elavirenz	Nevirapine	Delavirdine
Reference strain						
Wild-type HXB2				0.9	22	8
Patient isolate						
3	0	PBMCs	M41L T215Y	1.8 (2.4)	59 (2.7)	17 (2.1)
		Plasma	M41L T215Y			
	134	PBMCs	M41L K103N			
		Plasma	M41L **K103N** T215Y			
	273	PBMCs	None			
		Plasma	M41M/L **A98A/G** **K103N/K** M184M/V T215T/Y			
7	0	Plasma	None	0.6 (0.7)	21 (1.0)	13 (2.1)
	502	Plasma	**L74L/V** **G190E**	>310 (>437)	>1,250 (>57)	310 (39)
21	0	Plasma	None	1.8 (2.0)	48 (2.3)	20 (2.5)
	111[c]	Plasma	**Y188L**	280 (432)	>1,250 (>50)	730 (111)
	503	Plasma	V106I M184V **Y188L**	>310 (>475)	>1,250 (>50)	670 (102)
24[a]	328[a]	PBMCs	**K103N**	31	1,100	1,200
		Plasma	**K103N**			
26	0	Plasma	M41L/M,D 67D/N K70R/K T215Y	0.9 (1.0)	37 (1.8)	19 (2.4)
		PBMCs	D67N K70R T215Y	1.2	45	14
	14[c]	Plasma	D67D/N K70R **K103N/K** **G190S/G** T215Y	7.9 (9)	>1,250 (>61)	66 (8)
				29 (31)	>1,250 (>61)	110 (15)
	125[c]	PBMCs	K70R **K103N** **Y188L**	5,400	41,000	220,000
		Plasma	D67N K70R **L100I/L** **K103N** **Y188L/Y** T215T/Y			
28	0	Plasma	M184V	1.8 (2.0)	41 (2.0)	34 (4.4)
				0.8 (0.9)	94 (4.6)	59 (7.5)
	321[c]	PBMCs	**K103N** **P225H**			
		Plasma	**K103N** **P225H**	240 (180)	>1,250 (>57)	320 (97)
	419[c]	PBMCs	None			
		Plasma	**K103N/K** **V108/V** **P225H/P**			
44	0	PBMCs	**A98S**	2 (2.2)	150 (7.3)	50 (6.4)
		Plasma	**A98S**			
	259[c]	PBMCs	**A98S** **K101Q** **K103N**	160 (178)	>1,250 (>61)	>1,250 (>160)
		Plasma	**A98S** **K101Q** **K103N**			
47[a]	0	Plasma	K67D/N K70R T215X	1 (1.1)	42 (2.0)	28 (3.6)
	226	Plasma	**K70R** **K103N**	42 (63)	>1,250 (>50)	>1,250 (>190)
	497	Plasma	K70R **K103N** P225H	180 (247)	>1,250 (>57)	1,000 (140)
50	0	Plasma	y215y	0.6 (0.7)	76 (3.7)	10 (1.3)
	14[c]	Plasma	**K103K/N** G190S T215Y KW19K/E	97 (150)	>1,250 (>50)	12 (2)
	182[c]	PBMCs	**K103N** T215S/T	2.3 (2)	1,240 (60)	130 (7)
		Plasma	K103N P225P/H			
55	126[c]	PBMCs	None			
		Plasma	K70K/R **L100L/I** **K103N/K** T215T/Y			
63	0	PBMCs	None			
		Plasma	V106V/I			
	126	PBMCs	**K103N**			
		Plasma	K103N			
69	0	PBMCs	D57D/N K70R K219Q	2.1 (2.3)	89 (4.3)	150 (19)
		PBMCs	D67N K70R K219Q	0.74	36	24
		Plasma	D67D/N K70K/R			
	183[c]	PBMCs	K70R V108I	2.1 (3)	200 (9)	8 (1)
				1.7	25	19
		Plasma	D67D/N K70R **K103N** V108V/I P225H/P			
70[a]	0	PBMCs	None	2.3 (3.5)	48 (1.9)	78 (12)
		Plasma	None			

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TABLE 1—*Continued*

Patient no.	Study day	Source	RT genotype[b]	In vitro IC_{50} in nM (fold resistance) of:		
				Efavirenz	Nevirapine	Delavirdine
	290	PBMCs	K103N K219R/K-P225H	>310 (>475)	>1,250 (>50)	1.8 (1)
		Plasma	**K103N P225H**			
73	0	PBMCs	None	0.8 (1.2)	9 (0.4)	5.8 (0.9)
		Plasma	None			
	290[c]	PBMCs	**K103N V108I**			
89	0	Plasma	None	1.9 (2.1)	43 (2.1)	17 (2.1)
	56	Plasma	**K103N G190G/S P225P/H**	>310 (>339)	>1,250 (>61)	1,100 (140)
	503	Plasma	**K101E/Q K103N/K G190G/S P225P/H**	>310 (>230)	1,110 (51)	440 (140)
94	0	PBMCs	M41L	2.6 (2.8)	190 (9.3)	40 (5.1)
		Plasma	M41L/M	1.3 (1.4)	51 (2.5)	9.8 (1.2)
	242	Plasma	M41L **K103N V108V/I**	130 (148)	990 (46)	820 (100)
99	0	PBMCs	M184V	1.1 (2.1)	19 (0.8)	16 (2.4)
				1	28	11
		Plasma	M184V			
	114	PBMCs	**K103N** M184V	5.1 (77)	>1,250 (>50)	>1,250 (>190)
				65	2,600	1,900
		Plasma	**K103N/K** M184V/M			

[a] Indinavir monotherapy followed by addition of EFZ/d4T; study day is after addition of EFZ/d4T.
[b] Mutations associated with NNRTI resistance are in boldface.
[c] Patient had a 14-day efavirenz monotherapy lead-in before beginning combination therapy.
[d] Underlined genotypes were determined by sequencing of multiple independently amplified and cloned viral genomes (2). All other genotypes were determined by population-based sequencing of PCR products amplified from plasma or virus isolates as noted. Underlined phenotypes were determined by testing of PBMC-derived isolates in PBMCs. All other phenotypes were determined by AntiVirogram assay of recombinant viruses derived from patient plasma or PBMC virus isolate as noted. For viruses tested in the AntiVirogram assay, the fold resistance relative to the resistance of a reference HXB2 wild-type strain is shown in parentheses.

K103N, K103N/V108I, and K103N/P225H double mutations (Table 3). Substitutions K101E or -Q, V106I, V108I, P225H, and F227L have been observed in the virus in the plasma of patients failing efavirenz combination therapy largely or exclusively in combination with other NNRTI resistance mutations, usually K103N or Y188L (2). The in vitro susceptibility of site-directed mutants carrying these amino acid substitutions alone or in combination with K103N or Y188L suggests an explanation for this observation. It appears that these substitutions are unlikely to be selected by efavirenz in vivo as single-amino-acid variants, since the level of drug resistance that they confer is minimal. Rather, selection of these substitutions as secondary mutations is probably due to the enhanced phenotypic resistance to efavirenz that they confer to viruses carrying a K103N or Y188L substitution.

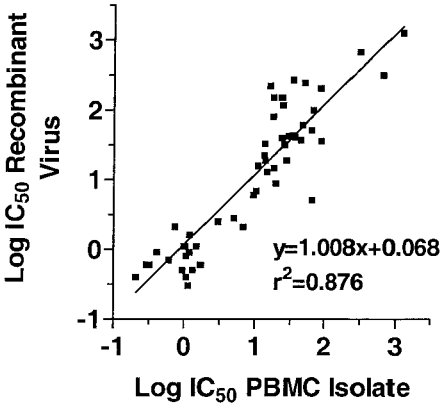

FIG. 1. Correlation of phenotypic resistance to antiretroviral drugs of clinical isolates tested in PBMCs and in RVAs (AntiVirogram). The IC_{50}s of clinical isolates derived from patient PBMCs were determined in vitro in PBMC culture and in MT-4 cells following construction of a recombinant virus incorporating the protease and RT genes of the PBMC isolate into an HXB2 background. Sixty-five phenotypic susceptibility values for 14 pairs of virus isolates are included.

A L100I site-directed mutant both conferred resistance to efavirenz as a single substitution and enhanced resistance to efavirenz when combined with a K103N substitution (2,400-fold resistance for the double mutant). An L100I-plus-K103N combination of mutations was present in a recombinant virus isolate from patient 132 (Table 2) and conferred high-level resistance to efavirenz (>480-fold resistance), consistent with the resistance of the site-directed double mutant. While the L100I substitution has frequently been selected in efavirenz-resistant virus in vitro (31) (M. M. Rayner, S. Garber, K. Logue, J. Corbett, D. Baker, S. Lukac, D. Powell, L. Bacheler, and S. Erickson-Viitanen, Abstr. XIII Int. AIDS Conf., abstr. A347, 2000), it has never been detected as a single NNRTI resistance mutation in patients failing efavirenz combination therapy (2). Rather, L100I has been observed in vivo exclusively in combination with the K103N mutation. As demonstrated by the site-directed mutants, the phenotypic resistance conferred by the L100I mutation is similar to that of the frequently observed K103N mutation; reasons for the failure to select L100I as an initial NNRTI resistance mutation in NNRTI-treated patients are not apparent.

Cross-resistance to NNRTIs selected in vivo during failure of NNRTI combination therapy. In order to assess the degree of cross-resistance to multiple NNRTIs of mutant strains of HIV-1 selected either by efavirenz, nevirapine, or delavirdine,

TABLE 2. Genotypic and phenotypic characterization of virus isolates from patients failing an efavirenz/ZDV/3TC combination regimen in study DMP 266-004[a]

Patient no.	Study day	Source	RT genotype	In vitro IC_{50} in nM (fold resistance) of:		
				Efavirenz	Nevirapine	Delavirdine
Reference strain						
Wild-type HXB2				0.9	22	8
Patient isolate						
100	0	PBMCs	M184V	3	51	68
		Plasma	D67N/D K70K/R M184V	2.5 (4)	243 (10)	99 (15)
	139	PBMCs	D67N K70R **K103N** M184V K219E	>310 (>437)	>1,250 (>57)	>1,250 (>160)
		Plasma	D67N K70R **K103N** M184V K219K/E	670	≥38,000	≥18,000
101	0	Plasma	M41L M184V L210W T215Y	0.4 (0.4)	10 (0.5)	1.7 (0.2)
107	0	PBMCs	M41L M184V T215Y			
		Plasma	M41L M184V T215Y/F K219K/E			
	149	PBMCs	M41L M184V **Y188L** T215S/F			
		Plasma	M41L M184V **Y188L** T215F/Y			
	230	PBMCs	M41L M184V **Y188L** T215F			
		Plasma	M41L M184V **Y188L** T215F			
121	0	PBMCs	M41L/M M184V T215Y	0.6 (0.9)	32 (1.3)	5.1 (0.8)
		Plasma	M41L/M M184V T215Y/T			
	167	PBMCs	M41I **K103N** M184V T215Y			
		Plasma	M41L **K101K/E** K103N V108V/I M184V T215Y			
	251	PBMCs	M41I **K103N V108I** M184V T215Y			
		Plasma	M41I A98A/G **K103N** **V108I/V** M184V T215Y			
126	112	PBMCs	D67N K70R **K103N** M184V/M **G190A** K219Q	180 (250)	>1,250 (>57)	1,200 (150)
		Plasma	D67N K70R **K101K/E** K103N/R V108V/I M184V/M **G190A** K219Q **P225P/H**			
132	0	Plasma	M41L D67N T69D K70R M184V T215F K219Q	1.9 (2.3)	460 (2.1)	64 (8.2)
	41	Plasma	D67N T69D K70R **K101E/K K103N V108V/I** M184V **G190G/A** T215F K219Q	41 (58)	>1,250 (>57)	>1,250 (>160)
	162	Plasma	M41L/M D67N T69D K70R **L100I K103N** M184V T215F K219Q	>310 (>440)	>1,250 (>57)	>1,250 (>160)
135	0	Plasma	K70R M184V	3.3 (2.4)	68 (3.1)	25 (7.4)
	55	Plasma	K70R **K103N** M184V	92 (100)	>1,250 (>61)	>1,250 (>160)
	115	Plasma	K70R K103N M184V **P225P/H**	140 (210)	1,200 (47)	1,100 (175)
141	0	PBMCs	M41L M184V L210W T215Y	3.5 (3.8)	89 (4.3)	22 (2.7)
		Plasma	M41L M184V L210W T215Y	2 (2.2)	100 (5.0)	19 (2.4)
	41	Plasma	M41L **K103N** M184V L210W T215Y	82 (89)	>1,250 (>61)	1,200 (154)
	116	PBMCs	M41L **K103N** M184V L210W T215Y **K238T**	220 (160)	>1,250 (>57)	>1,250 (>380)
		Plasma	M41L **A98A/G** **K103N** V108V/I M184V L210W/L T215Y			
	171	Plasma	M41L **A98A/G** **K103N** **V108I** M184V L210W T215Y			
143	0	PBMCs	M184V	3 (4.6)	190 (7.4)	170 (26)
		Plasma	M184V			
	113	PBMCs	**K103N** M184V			
		Plasma	**K103N** M184V			
146	0	Plasma	M41L V75X M184V L210L/W T215Y	0.6 (0.7)	520 (2.6)	25 (3.2)
	43	Plasma	M41L T69D V75M **K103N** M184V L210L/W T215Y	24 (25)	>1,250 (>61)	>1,250 (>160)
	113	Plasma	M41L **A98A/G** **K103N** **V108V/I** M184V L210W T215Y	>310 (>480)	>1,250 (>50)	>250 (>190)
147	295	Plasma	T69D/N K70R **K103N**	8.4 (9.1)	210 (10)	410 (52)

[a] Underlined genotypes were determined by sequencing of multiple independently amplified and cloned viral genomes (2). All other genotypes were determined by population-based sequencing of PCR products amplified from plasma or virus isolates. Underlined phenotypes were determined by testing of PBMC-derived isolates in PBMCs. All other phenotypes were determined by AntiVirogram assay of recombinant viruses derived from patient plasma or PBMC virus isolate as noted. For viruses tested in the AntiVirogram assay, the fold resistance relative to the resistance of a reference HXB2 wild-type strain is shown in parentheses.

the in vitro susceptibility of clinical isolates from patients failing one of these drugs was determined to all three NNRTIs. Cross-resistance of site-directed mutants was also assessed. Isolates from efavirenz failures carrying K103N, either alone or in combination with other mutations, were 93- to >740-fold resistant to nevirapine and 170- to >15,000-fold resistant to delavirdine (Tables 1 and 2). These results are consistent with resistance data for site-directed mutant viruses (Table 3) and

TABLE 3. Resistance to NNRTIs of HIV-1 variants created by site-directed mutagenesis[a,b]

Substitution	Fold resistance to:		
	Efavirenz	Nevirapine	Delavirdine
Single-amino-acid substitutions			
A98G[d]	5.2	6.2	ND[c]
L100I[a]	24	5.3	30
K101E[a]	6.9	14	3.5
K101Q	5.6	3.2	6.0
K103N/NL4-3[a]	19	44	24
K103N/HXB2	36	>50	68
K103R	0.6	0.9	1.0
V106A[a]	3.8	120	13
V106I	1.1	1.9	1.3
V108I	1.6	2.8	0.9
E138K	1.1	ND	ND
Y181C[a]	1.1	100	33
Y188H	3.8	1.9	5.0
Y188L[d]	140	>1,500	17
Y188C[a]	4.0	44	2.3
G190A	4.6	41	0.2
G190S[d]	97	290	0.7
P225H	1.2	1.7	0.4
F227L	0.9	3.1	ND
P236L	0.60	1.7	66
Multiple-amino-acid substitutions			
S48T/G190S	110	590	0.80
L100I/K103N[a]	2,400	110	870
K101E/K103N[a]	210	310	130
K101Q/K103N	250	136	36
K103N/V108I	84	270	39
K103N/Y181C[a]	32	>1,600	270
K103N/Y188H	404	ND	ND
V106I/Y188L	>1,400	2,600	170
S48T/K103N/G190S	3,800	1,100	97
K103N/P225H	100	140	11
K103N/F227L	57	260	2.0
K103N/V108I/P225H	625	268	12

[a] Designated mutants were constructed in a wild-type NL4-3 background. All other viruses were constructed in a wild-type HXB2 background.

[b] The average IC_{90} of each mutant strain was divided by the corresponding wild-type average to obtain the average fold resistance. The IC_{90}s (in nanomolar units) ± standard deviations of efavirenz, nevirapine, and delavirdine for wild-type HXB2 were as follows: 3.2 ± 0.32; 150 ± 59; and 36 ± 15, respectively. For wild-type NL4-3, the IC_{90}s (in nanomolars) ± standard deviations of efavirenz, nevirapine, and delavirdine were 3.0 ± 1.3, 120 ± 52, and 44 ± 15, respectively.

[c] ND, not done.

[d] Single-amino-acid substitution which required 2-nucleotide substitution.

support the conclusion that many viruses selected by efavirenz in vivo will demonstrate in vitro cross-resistance to nevirapine and delavirdine.

The in vitro susceptibility to NNRTIs of PBMC isolates from patients failing nevirapine or delavirdine combination therapy was determined during coculture in PBMCs from healthy donors (Table 4). Five post-nevirapine treatment isolates from patients who failed ZDV, dideoxyinosine, and nevirapine treatment were tested. Resistance to nevirapine was high in post-treatment failure isolates (over 1,000-fold greater for three post-treatment failure isolates than for baseline isolates from the same patient). Cross-resistance to efavirenz and delavirdine varied. Viruses with a single-amino-acid substitution (V106A or Y188L) or the mutation combination of K101E plus Y181C plus G190A were highly resistant to efavirenz and delavirdine, while a virus with A98G plus Y181C mutations was susceptible to efavirenz but highly resistant to delavirdine. An isolate with A98S and G190A mutations was susceptible to both efavirenz and delavirdine despite substantial nevirapine resistance. Among patient isolates from delavirdine treatment failures, an isolate with the P236L mutation was susceptible to efavirenz and only moderately resistant to nevirapine. Two virus isolates from delavirdine treatment failures, each of which carried K103N in combination with various mutations associated with resistance to ZDV, were cross-resistant to both efavirenz and nevirapine.

The impact of single and multiple NNRTI resistance mutations on cross-resistance to each of the NNRTIs in vitro was examined by testing mutant strains of HIV-1 constructed by site-directed mutagenesis. Mutations reported to be associated with resistance to each of the NNRTIs, as well as novel mutations detected during genotypic characterization of plasma virus from efavirenz treatment failures (2), were tested. Some substitutions conferred significant resistance to a single NNRTI. A V106A mutant conferred high-level resistance to nevirapine but not to efavirenz or delavirdine. Similarly, a P236L mutant conferred resistance only to delavirdine. Some mutations conferred resistance to some, but not all, of the NNRTIs tested. L100I conferred significant resistance to efavirenz and delavirdine but less resistance to nevirapine. The Y181C mutation conferred significant resistance to nevirapine and delavirdine but not to efavirenz. In two cases, different amino acid substitutions at the same position in RT had different effects on resistance and cross-resistance. Y188L conferred more resistance to each of the NNRTIs than did Y188C or -H. A G190A substitution conferred significant resistance to nevirapine but not to efavirenz or delavirdine, while a G190S substitution conferred high-level resistance to both efavirenz and nevirapine while retaining susceptibility to delavirdine. Finally, some mutations conferred resistance to each of the NNRTIs tested. K101E conferred a low level of resistance (3.5- to 14-fold) to all NNRTIs tested. Importantly, the K103N mutation, which has been reported for virus in plasma and clinical isolates following treatment failure with efavirenz, nevirapine, delavirdine, HBY097 (14), and loviride (18), is a cross-class resistance mutation, conferring resistance (7.2- to >50-fold) to each of these NNRTIs.

Cross-resistance of the double mutants varied depending on the specific substitutions combined in a single virus. K101E, which conferred enhanced resistance to efavirenz in combination with K103N, also significantly enhanced resistance to each of the other NNRTIs tested. Similarly, L100I, when combined with K103N, enhanced resistance to efavirenz and delavirdine. V108I and P225H mutations, which conferred little if any resistance to NNRTIs as single mutations, enhanced the level of resistance to efavirenz and nevirapine when either was present together in the same viral genome with a K103N mutation. A K103N/Y181C double mutant, reflecting the most prevalent double mutant reported among samples with NNRTI resistance mutations submitted to Virco for resistance testing (13), was highly resistant to nevirapine and delavirdine and showed a twofold increase in resistance to efavirenz from that conferred by K103N alone. The K103N/Y181C double mutant is frequently seen in virus in plasma from patients failing nevirapine or delavirdine therapy but was extremely rare in that from patients failing efavirenz treatment (2). L100I and

TABLE 4. Viral genotype, resistance, and cross-resistance to NNRTIs of PBMC isolates from nevirapine or delavirdine treatment failures

Isolate used	Study week	IC_{50} (nM)[a]			RT gene mutations[b]
		Efavirenz	Nevirapine	Delavirdine	
Isolates from nevirapine-exposed patients					
070272f	0	1	36	10	M41L T69D L210W T215Y
	8	4	>50,000	>10,000	M41L T69D **A98G Y181C** L210W 2T15Y
071035g	0	0.25	21	1.2	M41L T69D L210W T215Y
	48	36	23,000	14	M41L T69D **V106A** L210W T215Y
071597j	16	2	21,000	<1	M41L L741 **A98S G190A** L210W T215Y
071611d	48	110	>50,000	120	D67N K70R **Y188L** T215Y
090146j	0	0.87	39	6.5	M41L T69D 2L10W Y215Y
	48	110	>50,000	120	M41L T69D **K101E Y181C G190A** L210W T215Y
Isolates resistant to delavirdine					
P&U 174		2.4	190	5,000	**P236L**
P&U 412		64	7,600	850	M41l, **K103N**, T215Y
P&U 463		35	6,300	4,100	D67N, K70R, **K103N**, K219Q
Laboratory strain					
HIV-1RF		1	12	9	Wild-type lab strain

[a] In vitro drug susceptibility of PBMC isolates to NNRTIs was determined during culture in donor PBMCs.
[b] Mutations indicated in boldface have been previously associated with resistance to one or more NNRTIs.

G190S, which conferred moderate to high levels of resistance to efavirenz as single-amino-acid substitutions in mutated virus, showed substantially increased resistance (>2,000-fold) when expressed in combination with K103N.

DISCUSSION

Among the set of NNRTIs assessed, it is apparent that certain cross-class mutations confer significant resistance to all three of the presently approved NNRTIs. A single-amino-acid substitution at position 103 in RT confers significant resistance to efavirenz, nevirapine, and delavirdine. The data suggest that there is also broad cross-resistance to combinations of NNRTI resistance mutations frequently observed in plasma or virus isolates from patients failing NNRTI combination therapy, such as the Y181C/K103N and V108I/K103N double mutants. These in vitro findings suggest that a number of resistance mutations that are commonly seen in vivo may confer clinically significant cross-resistance to all of the presently approved NNRTIs.

Baseline susceptibility to NNRTIs as measured in the RVA was somewhat variable compared to the susceptibility of the wild-type reference strain. Baseline isolates from 11 of 23 patients demonstrated modest (4- to 10-fold) reductions in susceptibility to one or more NNRTIs. Two baseline isolates showed 4- to 4.6-fold resistance to efavirenz. A number of recent studies have described similar variability in NNRTI susceptibility, including low-level resistance, among recently infected subjects (15, 29). Several recent reports (1, 6) have suggested that such low-level (4- to 10-fold) resistance does not negatively affect the virologic response of NNRTI-naïve patients when placed on NNRTI-containing regimens.

Whitcomb et al. (J. Whitcomb, S. Deeks, W. Huang, T. Wrin, E. Paxinos, K. Limoli, R. Hoh, N. Hellmann, and C. Petropoulos, Abstr. 7th Conf. Retrovir. Opportunistic Infect., abstr. 234, 2000) have recently reported detecting hypersensitivity to NNRTIs among recombinant viruses from patients with extensive NRTI experience and resistance. Possible clinical relevance of this observation has been suggested by Haubrich et al. (7), who described a better short-term antiviral response to NNRTI-based therapy in the California Clinical Trials Group 575 study among subjects whose baseline virus demonstrated NNRTI hypersusceptibility. In our study, hypersusceptibility to NNRTIs was rare among the 25 baseline recombinant viruses derived from 23 patients, even though many of these virus isolates demonstrated both NRTI resistance mutations and phenotypic resistance to ZDV and/or 3TC. The recombinant virus from patient 101 demonstrated hypersusceptibility to delavirdine (0.2-fold resistance compared to that of the reference wild-type strain) and multiple NRTI resistance mutations. Borderline hypersusceptibility (0.37-fold resistance to nevirapine) in the baseline isolate from a second patient (patient 73) was not associated with NRTI resistance, either genotypic or phenotypic. Baseline samples in our study were derived from patients who subsequently failed efavirenz combination therapy and may not be representative of all patients in the study. In addition, the in vitro drug susceptibility assay utilized in our study (AntiVirogram) differs from the single-cycle assay utilized in the ViroLogic studies. It is unclear to what extent the observation of NNRTI hypersusceptibility may be dependent on assay methodology.

While broad cross-class resistance to the presently approved NNRTIs has been observed for some mutations or combinations of mutations, other mutations appear to confer reduced susceptibility in vitro more selectively. For example, the P236L mutation appears to confer reduced susceptibility in vitro only to delavirdine, remaining highly susceptible to nevirapine and delavirdine. The Y181C mutation, while conferring high-level resistance to nevirapine and delavirdine, is still susceptible in vitro to efavirenz. These in vitro results have led to the concept that sequential use of NNRTIs in combination therapy regimens might be efficacious for patients in whom only this second type of apparently non-cross-resistant NNRTI resistance mutation is detected. However, this treatment strategy is, as

yet, unsupported by clinical data. While there are presently relatively few data from controlled clinical trials on the sequential use of NNRTIs in patients selected by resistance testing, multiple reports of cohort studies have described poor virologic responses when efavirenz is used in NNRTI-experienced patients. ACTG 398 (S. Hammer, J. Mellors, F. Vaida, K. Bennett, V. Degruttola, L. Sheiner, and the ACTG 398 Study Team, 7th Conf. Retrovir. Opportunistic Infect., abstr. LB7, 2000) and CNAA 2007 (5) each demonstrated that prior NNRTI experience was significantly associated with failure of an efavirenz-containing regimen. Several reports have retrospectively examined the response of NNRTI-experienced patients to efavirenz-based salvage therapy as a function of baseline NNRTI resistance mutations. Bacheler et al. (L. T. Bacheler, D. Baker, M. Paul, S. Jeffrey, and K. Abremski, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2200, 1999) reported a poor short-term viral load response among nevirapine- and/or delavirdine-experienced patients who initiated efavirenz combination therapy as part of the SUSTIVA Expanded Access Program. Schulman et al. (24) also reported observing only a transient viral load reduction, which was lost at 12 and 24 weeks, among NNRTI-experienced patients treated with an efavirenz-plus-adefovir-based salvage therapy regimen. In both of these studies, even patients with viral genotypes associated with continued susceptibility to efavirenz in vitro, such as Y181C or G190A, did not achieve a sustained response to efavirenz-containing combination therapy. Similarly, MacArthur et al. (R. D. MacArthur, J. M. Kosmyna, L. R. Crane, L. Kovari, R. Podzorski, Abstr. 7th Int. Conf. Clin. Aspects Treat. HIV Infect., abstr. 208, 1999) and Keiser et al. (P. Keiser, W. Williams, L. Evans, W. O'Brien, and D. Skiest, Abstr. XIII Int. AIDS Conf., abstr. 4195, 2000) have described poor responses to efavirenz-containing salvage therapy in patients with any NNRTI resistance mutations, even those whose viral genotypes have been associated with phenotypic susceptibility to efavirenz in vitro. It is possible that detection of any NNRTI resistance mutations in patients failing an NNRTI-containing regimen is indicative of the selection of a variety of mutations, albeit at levels below the limits of detection of present technologies. Thus, patients failing a nevirapine- or delavirdine-containing regimen with mutations apparently susceptible to efavirenz (e.g., Y181C or G190A) may harbor minority variants, such as K103N mutants, that are likely to affect the response to a subsequent efavirenz-containing regimen (28). These studies were limited by the extensive prior nucleoside and PI experience of the patients studied. Highly experienced patients for whom an NNRTI regimen fails are also likely to harbor viral variants conferring resistance to NRTIs and PIs, making selection of a subsequent combination regimen containing multiple active drugs difficult. It is unknown whether NNRTI mutant viruses with retained efavirenz susceptibility might respond to a regimen including three or more active drugs, as defined by resistance testing. These results do not appear promising for the routine sequential use of the presently approved NNRTI drugs. There is a great need for new drug candidates with unique resistance characteristics for use in NNRTI-based combination therapy.

Comparison of the degree of phenotypic resistance measured by two in vitro assays of HIV drug susceptibility demonstrated that IC_{50}s determined during propagation of PBMC-derived virus isolates in stimulated PBMCs were highly correlated with IC_{50}s determined by testing recombinant viruses derived from such PBMC isolates in a commercial assay. While expected, this correlation is reassuring and emphasizes that the majority of genetic determinants of phenotypic drug susceptibility to inhibitors of HIV RT and protease are contained in the *gag*, protease, and RT segments of the HIV genome that are transferred from patient isolate to recombinant virus construct. A comparison of genotypic resistance detected in PBMC-derived virus isolates to resistance mutations detected in plasma virus collected at the same time also showed good correlation between these two compartments. Differences between the plasma virus and PBMC isolate genotypes in this study may be due in part to differing sensitiviies of the two genotyping methods to the presence of minority viral variants.

In summary, virus isolates from patients experiencing rebounds in plasma virus load in two phase II clinical studies of efavirenz combination therapy demonstrated reduced susceptibility to efavirenz and broad cross-resistance to nevirapine and delavirdine. Reduced phenotypic susceptibility was associated with specific NNRTI resistance mutations, particularly K103N or Y188L. Additional NNRTI resistance mutations observed in combination with K103N enhanced the degree of phenotypic resistance to NNRTIs.

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