Impact of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Inhibitor Drug Resistance Mutation Interactions on Phenotypic Susceptibility

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Abstract

The role specific reverse transcriptase (RT) drug resistance mutations play in influencing phenotypic susceptibility to RT inhibitors in virus strains with complex resistance interaction patterns was assessed using recombinant viruses that consisted of RT-PCR-amplified *pol* fragments derived from plasma HIV-1 RNA from two treatment-experienced patients. Specific modifications of key RT amino acids were performed by site-directed mutagenesis. A panel of viruses with defined genotypic resistance mutations was assessed for phenotypic drug resistance. Introduction of M184V into several different clones expressing various RT resistance mutations uniformly decreased susceptibility to abacavir, lamivudine, and didanosine, and increased susceptibility to zidovudine, stavudine, and tenofovir; replication capacity was decreased. The L74V mutation had similar but slightly different effects, contributing to decreased susceptibility to abacavir, lamivudine, and didanosine and increased susceptibility to zidovudine and tenofovir, but in contrast to M184V, L74V contributed to decreased susceptibility to stavudine. In virus strains with the nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations K101E and G190S, the L74V mutation increased replication capacity, consistent with published observations, but replication capacity was decreased in strains without NNRTI resistance mutations. K101E and G190S together tend to decrease susceptibility to all nucleoside RT inhibitors, but the K103N mutation had little effect on nucleoside RT inhibitor susceptibility. Mutational interactions can have a substantial impact on drug resistance phenotype and replication capacity, and this has been exploited in clinical practice with the development of fixed-dose combination pills. However, we are the first to report these mutational interactions using molecularly cloned recombinant strains derived from viruses that occur naturally in HIV-infected individuals.

Introduction

HIV DRUG RESISTANCE has become more prevalent over the past decade, both in patients with primary infection^{1,2} and as a consequence of long-term treatment in many patients with chronic HIV infection.^{3–8} Accumulation of the reverse transcriptase (RT) mutations M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E, termed thymidine analog-associated mutations (TAMs), can markedly limit the number of drugs available that are likely to have significant activity.^{9–11} Although initially associated with resistance to ZDV, TAMs have been shown to affect phenotypic susceptibility of all nucleoside RT inhibitors (NRTI).¹¹ The accu-

mulation of TAMs has substantial effects on virologic response to zidovudine (ZDV), stavudine (d4T), and abacavir (ABC), but both didanosine (ddI) and tenofovir disoproxil fumarate (TDF) retain at least partial antiviral activity against viruses with limited numbers of TAMs.^{12–17} Overall, the T215Y mutation appears to have the greatest effect on diminished susceptibility to NRTI.

Some *pol* mutations selected by reverse transcriptase inhibitors (RTI), such as the lamivudine (3TC)/emtricitabine (FTC)/ABC resistance mutation M184V, are well characterized, and have been shown to correlate with decreased virologic response.^{18–20} In addition, they can modulate phenotypic effects of other mutations. For example, M184V can

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improve apparent phenotypic susceptibility to ZDV, d4T, or TDF in HIV strains with one or more TAMs,^{11,21} but other potential interactions between clinically relevant RTI mutations, as investigated in the current study, have not been as rigorously explored.

While there is evidence that TAMs can result in increased susceptibility or hypersusceptibility to nonnucleoside RTI (NNRTI),^{22–25} the effects of NNRTI mutations on NRTI susceptibility are less well characterized. This is the first report presenting phenotypic drug resistance in viruses derived following the reconstruction of full-length proviral clones containing patient virus-derived *pol* sequences, with alteration of the nucleotide sequence to introduce type-specific drug resistance mutations or to restore mutated codons to wild type. This approach allows assessment of the effect of specific mutations on phenotypic susceptibility to RTI in a background of complex resistance mutations. The clinical relevance of our studies is exploration of various mutational interactions using virus strains that occur naturally in HIV-infected patients, which may represent a more appropriate strategy to study resistance associated with complex drug resistance patterns.

Materials and Methods

Patient samples

Frozen plasma stored from two patients was accessed for the study; patient 8 (Pt 8) was receiving d4T + ddI combination therapy and patient 19 (Pt 19) was receiving d4T + ddI + nevirapine (NVP); viral loads on these samples were 26,420 and 45,102 copies/ml, respectively. These plasma samples were previously collected during clinical monitoring for plasma HIV RNA testing and were assessed because they contained key RTI resistance mutations selected during antiretroviral therapy.

Cloning and genotypic sequence analysis

The amplification and cloning strategy is shown in the Fig. 1. Pt 8 and Pt 19 *pol* mutations were selected naturally by RTI therapy, as mentioned above. The region of the RT gene encoding amino acids 13–491 was amplified from RNA purified from plasma (Pt 8 and 19) by nested reverse transcriptase polymerase chain reaction (RT-PCR). The primers used for PCR amplification contained restriction enzyme sites not found in field isolates of HIV, *Xma*I in the sense primer and *Xba*I in the antisense primer.²⁶ These restriction sites generated a 1.4-kb fragment as shown in Fig. 1. Amplicons were cloned into a TA-cloning vector (Invitrogen, Carlsbad, CA) and were sequenced with ABI 377XL Automated DNA Sequencer (Applied Biosystems, Foster City, CA). The plasma-derived *Xma*I–*Xba*I HIV-1 fragment was isolated, purified (Gel Purification Kit, Qiagen, Valencia, CA), and subcloned into a modified pALTER vector, provided by Dr. John Mellors, University of Pittsburgh.²⁶ A chimeric proviral construct was generated using a larger fragment of HIV sequence (*Sph*I to *Sal*I, 4.3 kb) encoding a patient RT sequence bounded by HIV-III_B sequences by substitution into a full-length proviral clone, pNL4-3.²⁷

Site-directed mutagenesis and virus stock generation

Specific predicted NRTI or NNRTI amino acid resistance mutations were introduced or were restored to wild type by

modification of *pol* sequences encoding RT in pALTER clones with mutagenic oligonucleotides by site-directed mutagenesis kit (Stratagene, La Jolla, CA). Clones obtained at each step of the construction scheme (Fig. 1), TA vector, pALTER, and full-length proviral clone, were sequenced in the region encoding RT to confirm the modifications. To make a full-length HIV clone, pNL4-3 was modified with deletion of a 2-kb *Bal*I–*Bal*I fragment in *pol* to give rise to pNL–*Bal*IΔ. An *Sph*I–*Sal*I fragment (4.3 kb) from pALTER HIV RT that included HIV *pol* with a defined combination of RT resistance mutations was isolated and substituted into pNL4-3 *Bal*IΔ to give rise to a chimeric clone encoding patient-derived and modified *pol* sequences. Then 293 T cells (1×10^6) were transiently transfected with the full-length recombinant proviral clone by calcium phosphate transfection (Profection Mammalian Transfection System Kit, Promega, Madison, WI) and were cocultured with phytohemagglutinin (PHA)-stimulated, HIV-seronegative blood lymphocytes after 48 h. Culture supernatants were collected 2–3 days after lymphocyte addition and stored at -80°C . The p24 antigen content of virus stocks was determined by enzyme-linked immunosorbent assay (ELISA) (Immunodiagnostics, Woburn, MA).

Phenotypic drug susceptibility analysis of molecularly cloned HIV-1 strains

Drug susceptibility of recombinant virus (encoding patient-derived or modified *pol* sequences encoding RT, amino acid positions 13–491) to NRTI (ABC, ddI, 3TC, d4T, TDF, and ZDV) and NNRTI [delaviridine (DLV), efavirenz (EFV), and NVP] was assessed using the PhenoSense HIV assay (Monogram Sciences, Inc.).^{28,29} In this assay, recombinant virus is obtained from polymerase chain reaction (PCR)-generated, RT-protease encoding fragments through ligation to a luciferase-expressing proviral vector with deletions in *pol* and *env*. Virus is pseudotyped with an amphotropic MLV envelope and used to infect target cells in the presence or absence of various concentrations of each of the HIV RT inhibitor drugs. The 50% inhibitory concentration (IC_{50}) is calculated for test virus and for a reference wild-type strain, HIV-NL4-3. Results are expressed as fold-change (FC) difference in IC_{50} for test virus, as compared with wild-type virus. Clinically relevant cut-offs for determining resistance were established by correlating virologic response to baseline FC values in controlled clinical trials; in the absence of suitable data sets (e.g., for ZDV or the NNRTIs) the cut-off is based on variability of FC in the population (biological cut-off) and/or assay reproducibility (Table 1). In comparison to NL4-3, the ability of the virus strains to replicate in culture in the absence of drug was determined by the replication capacity (RC) assay, which measures single cycle virus infection in the same assay system.³⁰

Results

PhenoSense assay reproducibility

The cloned RT gene from Pt 19 encoded the TAMs M41L and T215Y, the ddI resistance mutation L74V, and NNRTI resistance mutations K101E and G190S. The cloned RT gene from Pt 8 encoded 3 TAMs (M41L, L210W, and T215Y), but no NNRTI or other NRTI resistance mutations.

Recombinant Pt 19 virus (M41L, L74V, T215Y, and NNRTI mutations) showed moderately reduced sensitivity to ABC,

FIG. 1. Molecular cloning of plasma HIV-1 reverse transcriptase sequences and construction of full-length molecular clones. HIV RNA was purified from frozen plasma samples containing specific RTI resistance mutations. The region of RT encoding amino acids 13–491 was amplified by nested RT-PCR. The PCR amplification primers contained unique restriction enzymes, *Xma*I (sense) and *Xba*I (antisense), and generated a 1.4-kb fragment. Amplicons were cloned into TA vector (Invitrogen, Carlsbad, CA) and were sequenced (Department of Biochemistry and Cell Biology, DNA core facility, University of Texas Medical Branch). The RT gene was subcloned into the vector pALTER HXB2 10 provided by Dr. John Mellors, University of Pittsburgh, which contains HIV-III sequences between *Sph*I (nt 1490) and *Sal*I (nt 5757) that allowed subsequent construction of full-length proviral clones encoding patient RT sequences. NRTI or NNRTI amino acid resistance mutations were then introduced or restored to wild type by site-directed mutagenesis of *pol* sequences encoding RT in pALTER clones with mutagenic oligonucleotides (Stratagene, La Jolla, CA). pNL4-3 was modified with deletion of a 2-kb *Bal*Δ–*Bal*I fragment in *pol* to give rise to pNL *Bal*Δ. A *Sph*I to *Sal*I fragment (1.4 kb) containing patient sequences *Xma*I to *Xba*I was substituted into pNL *Bal*Δ to generate a full-length clone. Infectious virus generated following transfection of the full-length clones was subjected to phenotypic HIV drug resistance testing (PhenoSense HIV assay, Monogram Sciences, South San Francisco, CA).

ddI, 3TC, d4T, and ZDV (IC₅₀ FC 2.6, 1.6, 2.4, 1.9, and 3.7, respectively; Table 1). The chimeric, full-length clone for Pt 19 was generated twice, over 1 year apart, and each clone was tested independently at the time of isolation. The reproducibility of the PhenoSense assay was demonstrated by the nearly identical FC results for NRTI and NNRTI from the independent clones of the same virus isolate generated and tested separately (Table 1). The FC phenotypic cut points needed for resistance to NRTI and NNRTI for this assay are also shown.

Effect of M184V, together with various TAM patterns, on NRTI drug resistance

The M184V mutation is selected during treatment failure of 3TC, FTC, and ABC and has been reported to have predictable effects on sensitivity to NRTI.^{11,21,31,32} This mutation has little effect on NNRTI susceptibility, and a tendency to diminish kinetics of replication of the recombinant virus, formally defined by this assay as replication capacity (RC). Introduction of mutation M184V into the Pt 19 clone resulted

TABLE 1. PT 19 PHENOTYPE ON TWO INDEPENDENT CLONES AND VIROLOGIC PHENOTYPIC CUT POINTS [FOLD-CHANGE (FC) IC₅₀] FOR REDUCED SUSCEPTIBILITY TO NRTI AND NNRTI

Pt 19 genotype 98S, 101E, 190S 41L, 74V, 215Y (date tested)	Phenotype (FC IC ₅₀)									
	NRTI						NNRTI			
	ABC	ddl	3TC	d4T	TDF	ZDV	DLV	EFV	NVP	RC%
November 2002	2.6	1.6	2.4	1.9	NT ^a	3.7	0.2	200	<< ^b	100
January 2004	2.6	1.7	3.0	2.1	1.0	3.7	0.3	240	<<	100
Monogram Sciences phenotypic resistance cut points	4.5 ^c	1.3 ^c	3.5 ^c	1.7 ^c	1.4 ^c	1.9 ^d	2.5 ^d	2.5 ^d	2.5 ^d	

^aNT, not tested.

^bIndicates FC over the maximal limit detected by the PhenoSense assay. In this assay, recombinant virus is obtained from PCR-generated, RT-protease encoding fragments through ligation to a luciferase-expressing proviral vector with deletions in *pol* and *env*. Virus is pseudotyped with amphotropic MLV envelope and used to infect target cells in the presence or absence of various concentrations of each of the HIV RT inhibitor drugs. The 50% inhibitory concentration (IC₅₀) is calculated for test virus and for a reference wild-type strain, HIV-NL4-3. Results are expressed as fold-change (FC) difference in IC₅₀ for test virus, as compared with wild-type virus.

^cClinical cut-off. Clinically relevant cut-offs for determining resistance were established by correlating virological response to baseline FC values in controlled clinical trials.

^dBiological or assay/technical cut-off. In the absence of suitable data sets (e.g., for ZDV or the NNRTIs) the cut-off is based on variability of FC in the population.

in decreased susceptibility to ABC, ddI, and especially 3TC, improved susceptibility to d4T, TDF, and ZDV, and diminished RC (Table 2). The same trends were seen when M184V was introduced into several different resistance mutation backgrounds. Introduction of L210W into Pt 19 virus (with

M41L, L74V, and T215Y) diminished susceptibility to all NRTI except for 3TC (Table 2); introduction of M184V in this background showed the characteristic changes for phenotype FC across NRTI as well as for a decrease in RC. Introduction of M184V into various modified Pt 19-derived

TABLE 2. NRTI MUTATION 184V INCREASES PHENOTYPIC SUSCEPTIBILITY TO d4T, ZDV, AND TDF AND DECREASES PHENOTYPIC SUSCEPTIBILITY TO ABC, 3TC, AND DDI

Genotype	Phenotype (FC IC ₅₀)									
	NRTI						NNRTI			
	ABC	ddl	3TC	d4T	TDF	ZDV	DLV	EFV	NVP	RC%
Pt 19										
101E, 190S	2.6	1.7	3.0	2.1	1.0	3.7	0.3	240	>> ^a	100
41L, 74V, 215Y										
101E, 190S	5.8	2.6	>>	1.4	0.4	1.0	0.2	95	220	41
41L, 74V, 184V, 215Y										
101E, 190S	3.3	2.0	2.5	2.9	1.2	12	0.3	282	>>	117
41L, 74V, 210W, 215Y										
101E, 190S	7.8	3.1	>>	1.6	0.5	1.1	0.1	92	161	29
41L, 74V, 184V, 210W, 215Y										
NNRTI reverted ^b	2.0	1.5	2.7	1.9	0.6	1.8	0.1	0.2	0.2	39
41L, 74V, 210W, 215Y										
NNRTI reverted	6.3	2.5	>>	1.7	0.3	0.7	0.1	0.1	0.1	18
41L, 74V, 184V, 210W, 215Y										
NNRTI reverted	1.7	1.1	2.2	1.8	1.1	6.8	0.1	0.3	0.2	62
41L, 210W, 215Y										
NNRTI reverted	3.8	1.2	>>	1.0	0.5	0.9	0.01	0.2	0.2	30
41L, 184V, 210W, 215Y										
Pt 8										
41L, 210W, 215Y	2.7	1.3	2.1	2.3	3.3	197	0.5	0.4	0.7	51
41L, 184V, 210W, 215Y	5.4	1.5	>>	1.6	1.2	13	0.3	0.3	0.5	48

^aIndicates maximal limit of FC detected by phenotype assay, PhenoSense.^bIndicates NNRTI resistance mutations K101E and G190S have been modified to confer wild-type amino acids at these positions.

clones, including restoration of the three NNRTI resistance mutations to wild type, alone or together with restoration of L74V to wild type (74L), showed a remarkably consistent pattern of altered phenotypic susceptibility and RC despite differing background resistance mutations. Specific effects of restoration of L74V to wild type or to reversion of the three NNRTI resistance mutations to wild type are discussed below.

A recombinant clone derived from Pt 19 modified to express the resistance mutation pattern identical to that in the Pt 8 clone (M41L, L210W, and T215Y) was shown to differ substantially in the magnitude of FC resistance for TDF and ZDV, suggesting that other mutations not known to be important for RTI drug resistance can affect measured phenotypic susceptibility. A trend in IC_{50} FC was observed for introduction of M184V in Pt 8-derived virus similar to that seen for introduction of M184V in various Pt 19-derived viruses, with diminished sensitivity to ABC, ddI, and especially 3TC,

and improved susceptibility to d4T, TDF, and ZDV; however, in this case, there was essentially no change in RC (Table 2).

Effect of L74V on RTI susceptibility and RC

The L74V mutation was selected by d4T and ddI therapy in Pt 19, and the effect of restoration of this mutation to wild-type 74L was examined (Table 3). The effects of L74V appear to be similar to those seen with M184V, with some important differences. In this case, the presence of L74V appeared to modestly decrease susceptibility to ABC, ddI, and 3TC and to increase susceptibility to TDF and ZDV, but in contrast to M184V, susceptibility to d4T appeared to diminish (Table 3). L74V did not have substantial effects on NNRTI susceptibility. In a clone with M184V introduced, ABC, ddI, and 3TC FC increased further, and d4T, TDF, and ZDV FC decreased further (Table 3), suggesting that phenotypic con-

TABLE 3. NRTI MUTATION 74V DECREASES PHENOTYPIC SUSCEPTIBILITY TO ABC, ddI, 3TC, AND d4T AND INCREASES PHENOTYPIC SUSCEPTIBILITY TO ZDV AND TDF

Genotype	Phenotype (FC IC_{50})									
	NRTI						NNRTI			
	ABC	ddI	3TC	d4T	TDF	ZDV	DLV	EFV	NVP	RC%
Pt 19										
101E, 190S	2.6	1.7	3	2.1	1.0	3.7	0.3	240	>>	100
41L, 74V, 215Y										
101E, 190S	1.6	1.0	1.7	1.6	1.3	7.9	0.2	174	>>	56
41L, 215Y										
101E, 190S	5.8	2.6	>>	1.4	0.4	1.0	0.1	95	220	41
41L, 74V, 184V, 215Y										
101E, 190S	3.0	1.0	>>	1.0	0.4	1.5	0.1	51	104	4.9
41L, 184V, 215Y										
101E, 190S	3.3	2.0	2.5	2.9	1.2	12	0.3	282	>>	117
41L, 74V, 210W, 215Y										
101E, 190S	2.0	1.0	1.7	1.6	1.5	36	0.2	192	>>	49
41L, 210W, 215Y										
101E, 190S	7.8	3.1	>>	1.6	0.5	1.0	0.1	92	161	29
41L, 74V, 184V, 210W, 215Y										
101E, 190S	3.6	1.0	>>	1.0	0.6	1.9	0.1	49	93	5.4
41L, 184V, 210W, 215Y										
NNRTI reverted ^a	6.3	2.5	>>	1.7	0.3	0.7	0.1	0.1	0.1	18
41L, 74V, 184V, 210W, 215Y										
NNRTI reverted	3.8	1.2	>>	1.0	0.5	1.0	0.1	0.1	0.1	30
41L, 18V, 210W, 215Y										
NNRTI reverted	2.0	1.5	2.7	2.0	0.6	1.8	0.1	0.2	0.2	39
41L, 74V, 210W, 215Y										
Pt 8										
41L, 210W, 215Y	2.7	1.3	2.1	2.3	3.3	197	0.5	0.4	0.7	51
41L, 74V, 210W, 215Y	3.0	1.7	2.8	2.4	1.6	24	0.2	0.3	0.3	78
41L, 67N, 210W, 215Y	2.6	1.6	4.6	2.8	2.7	>> ^b	0.2	0.3	0.4	93
41L, 67N, 74V, 210W, 215Y	2.9	1.8	4.1	2.1	1.2	11	0.2	0.2	0.2	— ^c
41L, 65R, 184V, 210W, 215Y	7.9	2.4	>>	1.2	1.0	0.8	0.1	0.2	0.2	13
41L, 74V, 184V, 210W, 215Y ^a	6.6	2.8	>>	1.6	0.6	3.8	0.2	0.3	0.3	91
41L, 65R	2.2	1.8	12	1.5	2.6	0.8	0.9	0.6	0.6	69
41L, 74V	1.5	1.6	1.2	1.1	0.8	0.7	0.9	0.6	0.6	97

^aIndicates NNRTI resistance mutations K101E and G190S have been modified to confer wild-type amino acids at these positions.

^bIndicates maximal limit of FC detected by phenotype assay, PhenoSense.

^cReplication capacity is low and cannot be reliably measured.

sequences of L74V and M184V are additive. As shown in Table 3, the same general trend with and without L74V was seen in other patient 19 clones, including clones modified to encode the TAM L210W and in clones with L210W and M184V.

In all clones expressing the NNRTI resistance mutations K101E and G190S (as well as the polymorphism A98S), the L74V mutation was associated with improved RC (Table 3), which is consistent with earlier findings that demonstrated improvement of RC in virus strains encoding the NNRTI resistance mutation G190S.³¹ To test the importance of the interaction of L74V and G190S on RC, we examined Pt 19-derived clones having the two NNRTI resistance mutations (K101E and G190S) restored to wild type by site-directed mutagenesis. We also restored the polymorphism A98S to wild type. In every clone without the G190S mutation, L74V was associated with diminished RC, as compared with viruses having 74L (Table 3).

The effects of L74V were also examined in Pt 8, where this mutation was not naturally selected. Similar to findings with Pt 19, introduction of L74V caused a decrease in susceptibility to ABC, ddI, and 3TC, improved susceptibility to TDF and ZDV, and a modest decrease or no change in d4T susceptibility (Table 3). We examined the effect of L74V together with three TAMs with or without M184V, and compared the effects of L74V and K65R. When compared with Pt 8 virus encoding mutations M41L, M184V, L210W, and T215Y, introduction of L74V caused a modest decrease in ABC and ddI susceptibility, improvement in TDF and ZDV susceptibility, and essentially no change in susceptibility to d4T or NNRTIs (Table 3). Interestingly, the introduction of K65R instead of L74V in the same context caused similar changes in NRTI susceptibility, including a striking improvement in ZDV susceptibility to a level greater than wild-type virus (FC 0.8), in spite of the presence of three TAMs. In combination with only M41L, the effects of K65R or L74V were again similar, except for diminished susceptibility to TDF with K65R (Table 3). In both comparisons, the K65R mutation appeared to have effects greater than L74V on decreasing viral RC.

Effect of NNRTI resistance mutations on RTI sensitivity

Virus derived from Pt 19, which contain the NNRTI mutations K101E and G190S, showed high level resistance to EFV and NVP (FC >240) but susceptibility to DLV (Table 4). This pattern has been seen previously in NNRTI-resistant viruses harboring G190S.^{33–35} The appearance of NRTI mutations (TAMs) can result in markedly improved susceptibility to NNRTI, with at times hypersusceptibility, which is defined as IC_{50} FC <0.4.^{11,22–24} To determine if there might be a reciprocal effect, whereby restoration of NNRTI resistance mutations to wild type influences NRTI susceptibility, we restored K101E and G190S (as well as A98S) to wild type using site-directed mutagenesis. Although the FC differences were not dramatic, there was a fairly consistent increase in susceptibility to all NRTI (Table 4). This suggests that NNRTI mutations A98S, K101E, and G190S enhanced resistance to the NRTI ZDV, d4T, ABC, 3TC, and TDF. Virus derived from these clones exhibited NNRTI hypersusceptibility (FC 0.1–0.3), consistent with the effects of TAMs on NNRTI susceptibility.^{11,23} Although NNRTI mutations are typically thought to have little consequence on viral fitness, there was an effect seen on RC related to the presence of A98S, K101E,

and G190S, shown in multiple paired Pt 19 clones. The RC of clones with or without NNRTI mutations (NNRTI/NNRTI reverted) showed M41L + T215Y (100% / 28%), with M41L + T215Y + K219Q/E (104% / 69%), with M41L + D67N + T215Y + K219Q (77%/50%), and M41L + M184V + L210W + T215Y (29%/18%), respectively. In these cases, the presence of the NNRTI resistance mutations appeared to improve RC, independent of the NRTI mutation patterns. It is possible that these mutations were compensating for TAMs in order to improve viral fitness in the patient-derived virus strain. In every case in which the A98S, K101E, and G190S mutations were reverted to wild type, there was a decrease in RC.

To determine if susceptibility to NRTI was affected by another NNRTI mutation, we tested the effect of the introduction of the K103N mutation in the Pt 8 clones. There appeared to be a modest 2-fold decrease in ZDV susceptibility in each pair of matched viruses with introduction of K103N. However, there was no consistent effect on the susceptibility of other NRTIs or on viral RC. As expected, there was markedly diminished NNRTI susceptibility associated with K103N (Table 4), as shown previously.^{31,36}

Discussion

We have developed a model system to evaluate the effect of mutation interactions in viruses generated from recombinant molecular clones containing *pol* gene sequences derived from patient plasma HIV RNA. This approach is novel in several ways: (1) use of patient-derived virus sequences, with examination of interactions of mutations that actually occur in patients, rather than introducing them into molecular clones of laboratory-derived strains, (2) assessment of specific modifications in molecularly cloned strains in the same viral genetic background, (3) a high level of phenotypic test reproducibility, (4) the ability to assess RC together with resistance for each cloned virus, and (5) assessment of the phenotypic effect of interactions between NRTI and NNRTI mutations that have not been well explored previously.

We demonstrated the effects of interactions of specific RTI drug resistance mutations, which can modulate susceptibility to RT inhibitors, particularly for drugs generally not thought to be affected by the primary mutation. Mutations associated with resistance to single drugs are fairly well characterized, but monotherapy is no longer used; the treatment standard is combination therapy with at least three or four antiretroviral drugs. Combination therapy has been shown to select for more complex mutation patterns, which are commonly seen in treatment-experienced patients. Thus, it is important to understand the consequences of these patterns of mutations.

Our study has confirmed several previous observations and presented some new ones. We have confirmed effects of multiple TAMs on decreasing susceptibility to all NRTI, specifically d4T, ZDV, ABC, and TDF.^{25,37,38} We also showed that the M184V mutation reduces phenotypic susceptibility to ABC, ddI, and 3TC, but can enhance susceptibility to d4T, ZDV, and TDF, and is associated with decreased RC capacity.^{11,21,32} Even in the presence of M184V and a marked decrease in phenotypic susceptibility to 3TC and FTC, 3TC appears to contribute to suppression of HIV replication in patients.³⁹ An accumulation of TAMs is associated with broad class resistance by accelerating the rate at which ter-

TABLE 4. NNRTI MUTATIONS 101E AND 190S TEND TO DECREASE SUSCEPTIBILITY TO NRTI, AS WELL AS NNRTI, BUT NNRTI MUTATION 103N HAS MODEST EFFECT ON NRTI SUSCEPTIBILITY WHILE MARKEDLY DECREASING NNRTI SUSCEPTIBILITY

Genotype	Phenotype (FC IC ₅₀)									
	NRTI						NNRTI			
	ABC	ddl	3TC	d4T	TDF	ZDV	DLV	EFV	NVP	RC%
Pt 19										
101E, 190S	2.6	1.7	3.0	2.1	1.0	3.7	0.3	240	>> ^a	100
41L, 74V, 215Y										
NNRTI reverted ^b	1.9	2.0	2.1	1.4	0.6	1.1	0.1	0.1	0.1	28
41L, 74V, 215Y, 215Y										
101E, 190S	2.6	1.6	2.6	2.1	0.9	3.1	0.6	248	>>	104
41L, 74V, 215Y, 219Q										
NNRTI reverted	1.5	1.3	1.4	1.0	0.4	0.3	0.3	0.3	0.2	69
41L, 74V, 219Q										
101E, 190S	1.5	1.7	2.2	1.0	0.7	0.6	0.8	>>	>>	77
41L, 67N, 74V, 215Y, 219Q										
NNRTI reverted	1.1	1.4	1.2	0.9	0.4	0.4	0.2	0.2	0.2	50
41L, 67N, 74V, 215Y, 219Q										
101E, 190S	7.8	3.1	>>	1.6	0.5	1.1	0.1	92	161	29
41L, 74V, 184V, 210W, 215Y										
NNRTI reverted	6.3	2.5	>>	1.7	0.3	0.7	0.1	0.1	0.1	18
41L, 74V, 184V, 210W, 215Y										
Pt 8										
41L, 210W, 215Y	2.7	1.3	2.1	2.3	3.3	197	0.5	0.4	0.7	51
103N										
41L, 210W, 215Y	2.2	1.6	2.4	2.4	2.8	546	17	16	36	80
41L, 184V, 210W, 215Y	5.4	1.5	>> ^a	1.6	1.2	13	0.3	0.3	0.5	48
103N										
41L, 184V, 210W, 215Y	4.2	1.8	>>	2.0	1.0	43	12	13	27	60
41L, 67N, 210W, 215Y	2.6	1.6	4.6	2.8	2.7	>>	21	0.3	0.4	93
103N										
41L, 67N, 210W, 215Y	3.5	1.4	4.4	2.6	4.0	>>	16	16	39	46
41L, 67D, 210W, 215Y	3.4	1.7	4.3	2.5	2.6	75	0.4	0.4	0.6	92
103N										
41L, 67D, 210W, 215Y	3.3	1.6	4.8	2.4	2.8	167	21	18	44	92
41L, 210W, 215Y, 219Q	1.4	1.4	2.2	1.9	1.7	58	1.2	0.8	0.8	61
103N										
41L, 210W, 215Y, 219Q	2.2	1.1	1.7	1.8	2.6	101	58	14	28	46

^aIndicates over the maximal limit of FC detected by phenotype assay, PhenoSense.

^bIndicates NNRTI resistance mutations K101E and G190S have been modified to confer wild-type amino acids at these positions.

minimal monophosphates (MP) are removed through proteolytic cleavage, particularly for nucleoside analogues, allowing DNA polymerization to proceed.^{40,41} The appearance of the M184V mutation diminishes the ability of reverse transcriptase harboring TAMs to carry out the phosphorolytic excision of incorporated nucleoside analogs, mediated by ATP or PPi.^{42–44}

We have also extended our understanding of effects of L74V, which are similar to but distinct from those of M184V. The mutation was introduced into molecular clones of laboratory strains by site-directed mutagenesis, and the virus with L74V was shown to have a replicative disadvantage. L74V is a resistance mutation for ddI and ABC. We have shown that L74V reduces susceptibility to ddI and ABC, improves susceptibility to TDF and ZDV, but in contrast to M184V has only very modest effects on 3TC susceptibility; in addition, there is usually decreased susceptibility to d4T. In fact, L74V has been selected in patients failing d4T without coadministration of ddI or ABC.⁴⁵ The L74V resistance

mutation was described for ddI prior to its approval and widespread use.⁴⁶

In a later study, replication kinetics and reverse transcriptase processivity were examined, and reductions in both measures were found comparable to that seen with the introduction of M184V in the same backbone (HIV-HXB2).^{47,48} The L74V mutation was shown to counteract the enhanced excision of ZDV MP with reverse transcriptase harboring TAMs.^{43,49} When M184V and L74V were introduced together, there was more impairment in ZDV-MP excision than seen with either mutation alone.^{7,43} In addition to affecting excision, these mutations have also been shown to affect RT processivity, with decreases in initiation of primer-driven viral DNA synthesis. These studies were limited by the introduction of only a single predicted amino acid resistance mutation, generated in cell line-adapted virus strains, rather than primary virus isolates. In addition, L74V was found to be a contributing factor to an observed discordance between phenotypic and genotypic NRTI resistance assessments for ZDV.²⁹

We also confirmed that the K103N mutation, while conferring resistance to all NNRTIs, has little effect on NRTI susceptibility or RC.⁵⁰ Another NNRTI mutation pattern (A98S, K101E, and G190S) selected by NVP therapy and seen previously⁵⁰ can affect NRTI susceptibility. The L74V mutation can slightly diminish RC when seen in isolation or only with NRTI resistance mutations, but as shown previously, we demonstrated improved RC when L74V was introduced in the presence of G190S. G190S, as well as other mutations at codon 190, typically diminishes susceptibility to NVP and EFV, but generally has little effect on DLV susceptibility.^{33,34} RT codon 190 mutations are associated with a moderate to marked decrease in replication kinetics³³ and have also been found to have less polymerase activity with less processivity than RT enzymes that are wild type at that position.^{33,34} In these biochemical studies, L74V and V75I appeared to be compensatory in that they restored reverse transcriptase activity to levels similar to that seen with wild-type virus. In fact, L74V and V75L/I were selected *in vitro* by selective pressure of an NNRTI.⁵¹ Although initial studies identified G190E as a primary NVP resistance mutation that exhibited impairment of reverse transcriptase activity and virus replication,^{52,53} multiple substitutions at RT position 190 have been observed, including G190A, G190S, G190E, G190Q, and G190V.^{33,34} The G190A RT variant, while decreasing phenotypic susceptibility to NVP and EVF, has little effect on RT processivity or RC, and in some cases can increase polymerase activity. Viruses with 190S, 190T, 190Q, or 190V have markedly impaired RC when introduced into recombinant virus strains, as well as diminution in RT activity.³⁴ The G190S RT variant is intermediate between wild-type or G190A variants and the markedly impaired variants G190Q, G190V, and G190E. Introduction of L74V, which alone had no measurable effect on the replication efficiency of the reference virus or on the 190A virus, increased replication capacity viruses with 190S, or other variants.^{33,34,51,54} Review of the Monogram Sciences, Inc. database showed that the RC of viruses with 190E, 190Q, and 190V range from 20% to 60%, although viruses made by site-directed mutagenesis exhibited RC <1%. This strongly suggests that other mutations can compensate in order to improve RT enzyme activity.

Although not observed with the frequency of M184V, interaction of L74V with other RTI resistance mutations has implications in virus susceptibility as well as replication kinetics. This suggests a theoretical advantage in activity to several NRTIs for viruses that maintain this mutation. Just as ZDV was paired with 3TC because of theoretical advantages, ZDV may exhibit enhanced activity when used with drugs, such as ddI, that maintain L74V, while at the same time negatively impacting replication kinetics. Of note, viruses with K65R has been shown to have an even stronger effect on RC and on resensitization to ZDV, as previously described.⁵⁵ The K65R mutation is thought to affect susceptibility to all NRTI except ZDV through a mechanism of diminished efficiency of initiation of minus double-stranded HIV-DNA synthesis.⁴⁴ The decreased efficiency of initiation of HIV-DNA synthesis together with diminished RNA template usage when these mutations are seen together exceeds defects seen with either one alone, and may explain the diminished viral fitness observed in tissue culture when K65R/M184V-containing viruses are evaluated. It is noteworthy that clonal expression of K65R with either T215Y or

with L75V is rarely observed.^{56–58} TAMs appeared to antagonize the phenotypic effects of K65R, reducing resistance to tenofovir, whereas K65R results in resistance to all NRTIs except for ZDV. A further mechanistic explanation for the bidirectional antagonism of K65R and TAMs was shown to result from a decrease in NRTI MP excision activity of RT containing TAMs by K65R. Conversely, TAMs antagonized the ability of K65R RT to discriminate against the nucleotide analog.⁵⁷ This suggests the potential role for therapies that result in selection for both K65R and TAMs, since one or the other drug is likely to retain some activity, at least initially.

Importantly, we showed the effects of NNRTI resistance mutations on NRTI susceptibility. Although the presence of TAMs can markedly improve susceptibility to NNRTI,^{23–25} we now show that an NNRTI resistance mutation pattern (K101E and G190S) was shown to generally diminish NRTI susceptibility (Table 4). However, this observation did not exhibit the same effects as those seen for changes in NNRTI susceptibility in the presence of TAMs only, where hypersusceptibility is often observed.

Initial combination therapy is increasing based on an NNRTI and two NRTI drugs. Failure with this regimen results most commonly in the emergence of NNRTI resistance mutations, as well as NRTI resistance mutations,¹⁵ and is more prevalent than in patients failing ritonavir-boosted protease inhibitor-based regimens.⁵⁹ Use of genotypic HIV drug resistance testing at the time of treatment failure has been shown to result in improved short-term outcomes from subsequent therapy.^{60,61} Better delineation of the effect of complex mutation patterns will help to refine algorithms used to interpret genotypic resistance mutations that arise during antiretroviral therapy. Moreover, since combinations of NRTI and NNRTIs will be frequently used early in therapy, strategies of treatment predicted to result in mutation patterns and their interactions may improve resistance test algorithms and provide better guidance for selection of subsequent drug regimens.

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