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A Mutation in the 3′ Region of the Human Immunodeficiency Virus Type 1 Reverse Transcriptase (Y318F) Associated with Nonnucleoside Reverse Transcriptase Inhibitor Resistance

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The Y318F substitution in the 3′ region of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) has been linked to nonnucleoside RT inhibitor (NNRTI) resistance in vitro. A systematic search of a large phenotypic-genotypic database (Virco) linked the Y318F substitution with a >10-fold decrease in NNRTI susceptibility in >85% of clinically derived isolates. There was a significant association between Y318F and use of delavirdine ($P = 10^{-11}$) and nevirapine ($P = 10^{-6}$) but not efavirenz ($P = 0.3$). Site-directed HIV-1 Y318F mutants in an HXB2 background displayed 42-fold-decreased susceptibility to delavirdine but <3-fold-decreased susceptibility to nevirapine or efavirenz. Combinations of Y318F with K103N, Y181C, or both resulted in decreased efavirenz susceptibility of 43-, 3.3-, and 84-fold, respectively, as well as >100- and >60-fold decreases in delavirdine and nevirapine susceptibility, respectively. These results indicate the importance of the Y318F substitution in HIV-1 drug resistance.

The nonnucleoside reverse transcriptase inhibitors (NNRTIs) are potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) replication. These are used in combination with other antiretroviral agents in the treatment of HIV infection, partly due to the ease of selection of NNRTI-resistant viruses (6). NNRTIs currently in clinical use (nevirapine [NVP], delavirdine [DLV], and efavirenz [EFV]) exhibit at least partially overlapping genotypic resistance patterns, often resulting from selection of the K103N substitution in the HIV-1 reverse transcriptase (RT) (1). Additional mutations are also commonly selected. Phenotypic and/or genotypic testing for HIV-1 drug resistance is now recommended in a variety of circumstances (8).

The RT mutation P236L has been linked to high-level in vitro resistance to DLV but not other NNRTIs (4). In response to DLV monotherapy, however, the K103N and Y181C mutations developed rapidly while the P236L mutation was only rarely observed (3). Genotypic analyses of HIV-1 RT from patients failing EFV combination therapy revealed the accumulation of viruses with multiple linked NNRTI mutations following the emergence of K103N mutant viruses (1), suggesting the presence of drug selection pressure even after the emergence of K103N. Despite the high degree of cross-resistance among NNRTIs, a separate study reported that nearly one-third of resistant isolates carrying the single NNRTI-resistance-associated Y181C mutation remained susceptible to EFV (2), suggesting that additional uncharacterized mutations may play a role in NNRTI resistance.

An RT structure-function study by Pelemans et al. (13), which used site-directed mutagenesis to investigate HIV-1 RT codon Y318 (which forms a part of the lipophilic NNRTI-binding pocket), found that Y318W and Y318F mutants maintained substantial RT activity and exhibited some degree of resistance to NNRTIs in vitro. This region is not included in many genotypic or phenotypic resistance assays and it remains to be seen whether mutations at RT codon 318 could be selected in vivo (13).

In our study, the prevalence of mutations at codon 318 was examined in two large, clinically derived databases: Virco (Mechelen, Belgium) and the British Columbia Centre for Excellence in HIV/AIDS (Vancouver, Canada). The Virco database contains >20,000 matched drug resistance phenotypes and genotypes obtained during routine drug susceptibility testing, with the samples in this study originating mainly from the United States and Europe. Although treatment history is generally not available for these samples (due to the nature of the services), it may be assumed that most samples are derived from treatment-experienced individuals.

Patient samples with known treatment histories were derived from the B.C. Centre for Excellence in HIV/AIDS (B.C. Centre) Drug Treatment Program, which distributes antiretroviral drugs free of charge to all eligible HIV-infected individuals in British Columbia. In addition, the B.C. Centre provides genotypic resistance testing for HIV-infected individuals in British Columbia as well as the rest of Canada. The B.C. Centre database contains genotypes from >7,000 patient isolates, as well as therapy history. There is a small overlap (<500 samples) between the Virco and B.C. Centre databases.

HIV-1 RNA extraction, RT-PCR, and genotyping were performed as previously described (7, 11, 12). Phenotypic drug susceptibility analysis was performed using a recombinant virus assay (10) (Virco, Mechelen, Belgium) with modifications as described previously (R. Pauwels, K. Hertogs, B. A. Larder, et

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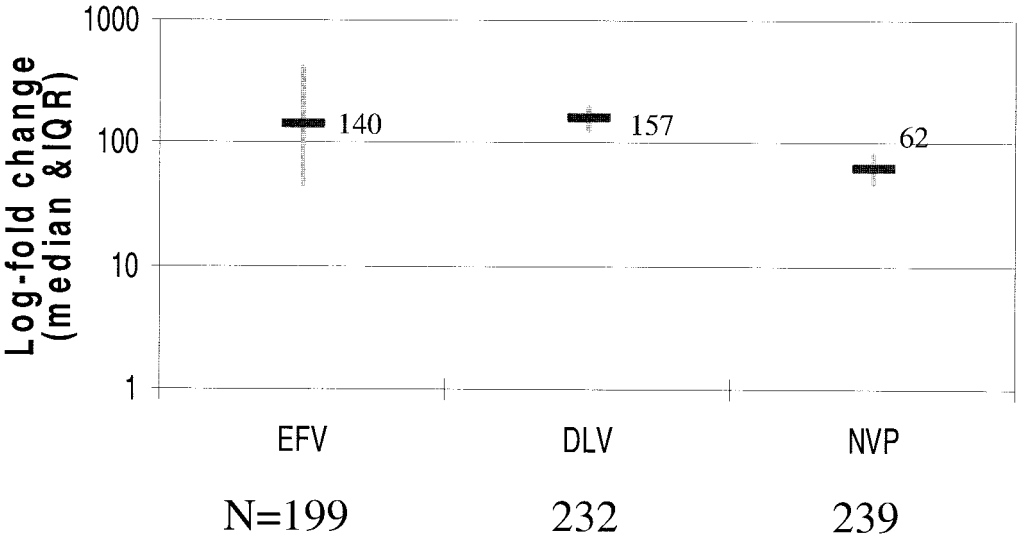

FIG. 1. Log-fold change in NNRTI phenotypic resistance to EFV, DLV, and NVP in clinical samples harboring the Y318F mutation. The median log-fold change is indicated by the horizontal bar, and the interquartile range (IQR) is indicated by the vertical shaded line. Slight differences in sample size for each drug are due to the fact that not every isolate was successfully phenotyped for each of the drugs.

al., Abstr. 2nd Int. Workshop HIV Drug Resist. Treatment Strategies, abstr. 51, 1998). Results are expressed as the geometric mean fold increase in the 50% inhibitory concentration IC_{50} of the sample (or site-directed mutant) compared to results with a wild-type control (HIV IIIB) run at the same time. For information purposes, the mean IC_{50}s for the wild-type IIIB control for samples with 318F were 0.02 ± 0.008 μM for NVP, 0.007 ± 0.003 μM for DLV, and 0.0007 ± 0.0002 μM for EFV.

A systematic search of the Virco database (containing >20,000 genotypes and phenotypes) revealed that 479 isolates (1.3%) harbored the Y318F mutation. The Y318F mutation was 7.5 times more common than P236L. In contrast, no examples of Y318W were observed. The presence of Y318F, irrespective of other mutations, was associated with a >10-fold decrease in susceptibility to EFV, DLV, or NVP in 87, 98, and 95% of cases, respectively. In addition, the geometric mean fold changes in phenotypic susceptibilities to EFV, DLV, and NVP for recombinant viruses harboring Y318F were significant and dramatic compared to results with wild-type controls (Fig. 1).

The Y318F mutation was usually observed in association with other recognized NNRTI resistance mutations. For example, 79% of isolates with the Y318F also had K103N, while 21% had either Y181C, Y181I, or Y181V and 2% had P236L. (Many recombinant viruses had multiple mutations, so this total is greater than 100%). Conversely, 14% of all the samples with the P236L mutation ($n = 64$) also had Y318F, compared to 4% of samples with K103N ($n = 10{,}113$) and 2% of samples with either a Y181C, Y181I, or Y181V mutation ($n = 5885$).

A search of the B.C. Centre database, containing a substantially independent panel of HIV-1 isolates from antiretroviral drug-treated individuals in British Columbia, Canada (Fig. 2), revealed that 138 of 7,611 samples (1.8%) harbored the Y318F mutation. Y318F appeared at ~2-fold-lower prevalence than the NNRTI-associated mutations L100I, V106A, and V108I, a 5- to 10-fold-lower prevalence than the K103N and Y181C/I mutations, and a 6-times-higher prevalence than the P236L mutation previously associated with DLV resistance (Fig. 2).

Current therapy information was available for 5,680 of 7,611 Canadian samples (74.6%). Of these, 110 samples harbored the Y318F mutation (1.9%). Of the samples with Y318F, 36, 25, and 22% originated from patients who were receiving DLV, EFV, and NVP, respectively, at the time of the genotyping request (rarely, patients may have been concurrently prescribed more than one NNRTI). Although 34% of patients were not prescribed any NNRTIs at time of the genotype request, complete therapy history data were not available in all cases, and at least some of these patients may have been previously prescribed NNRTIs. Of the 15 specimens originating from patients whose complete therapy histories were available, 3 had been previously exposed to all three NNRTIs, 3 had previously been prescribed EFV and NVP, 1 had been previously prescribed DLV and NVP, and 8 had never been prescribed any NNRTI before the genotype request date. Chi-square analysis of these 5,680 isolates revealed a highly statistically significant association between the Y318F mutation and DLV use ($P = 10^{-11}$), a slightly smaller association between Y318F and NVP use ($P = 10^{-6}$), and no correlation with EFV use ($P = 0.3$).

To confirm the effect of the Y318F mutation on decreased drug susceptibility, mutations in the RT-coding region were generated by site-directed mutagenesis of a wild-type HXB2-D *Eco*RI-*Pst*I restriction enzyme fragment encompassing the HIV-1 *pol* gene and cloned into pGEM3 (Promega). Single and multiple nucleotide changes were introduced into RT with the QuikChange site-directed mutagenesis kit (Stratagene). All mutant clones were verified by DNA sequence analysis of the RT to codon 400. PCR fragments were prepared from the mutated clones, the altered RT-coding regions were transferred into the HIV-1 HXB2-D genetic background by homologous recombination, and the susceptibility to nonnucleoside

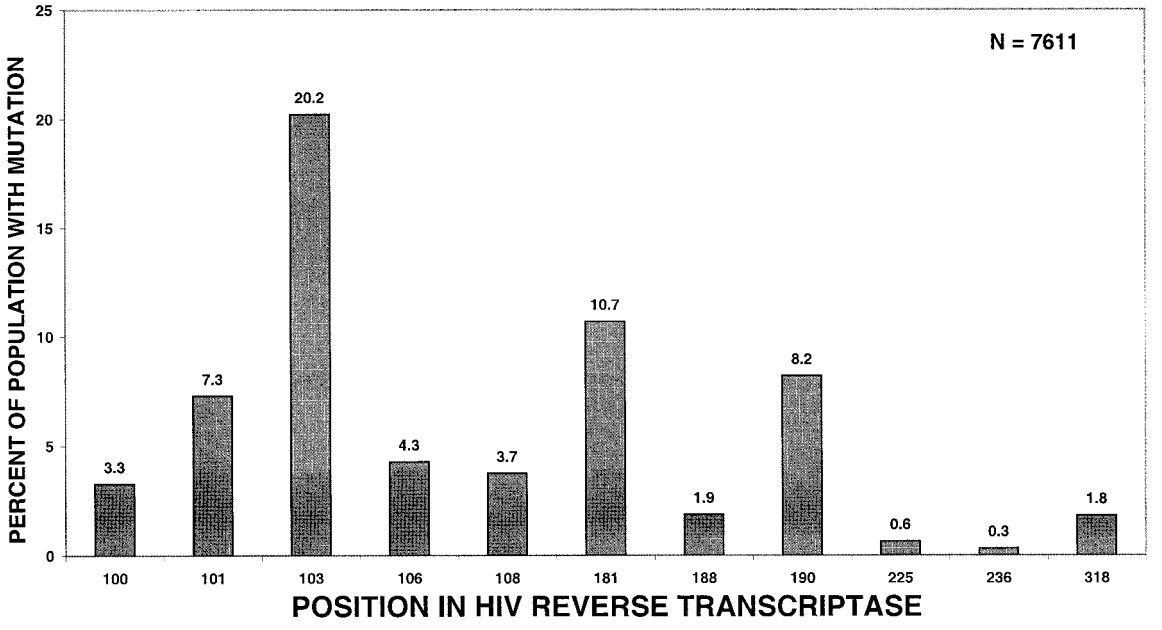

FIG. 2. The frequency of resistance mutations commonly associated with NNRTI resistance in a large panel of HIV-infected, antiretroviral drug-treated Canadian patients (n = 7611). Mutations presented include the following: L100I, K101E/Q, K103N, V106A, V108I, Y181C/I, Y188C, G190S/A/E, P225H, P236L, and Y318F in the HIV-1 RT.

RT inhibitors was determined as described above. Reported values represent the geometric mean of two independent assays.

The alteration of Y318F alone in HIV-1 HXB2 by site-directed mutagenesis was sufficient to confer a 42-fold change in susceptibility to DLV, a level similar to that conferred by either the Y181C or the K103N mutation (Table 1). Any of these mutations present as double or triple combinations were sufficient to confer greater than 100-fold decreases in DLV susceptibility, the highest value assessed in the assay. Neither Y318F nor Y181C conferred a discernible impact on EFV susceptibility alone. However, the presence of the Y318F mutation resulted in a modest decrease in EFV susceptibility (3.3-fold) when present in combination with Y181C. The addition of Y318F to a viral background with the K103N mutation resulted in a dramatic decrease in EFV susceptibility, from 15-fold for the K103N to 43-fold for the combination. This decrease was greater than that conferred by the K103N/Y181C combination. The triple combination K103N/Y181C/Y318F decreased EFV susceptibility 84-fold (Table 1). The Y318F mutation alone conferred a threefold decrease in susceptibility to NVP when present alone, but further effects on NVP resistance could not be assessed because all combinations were greater than the 60-fold upper limit which could be measured in the assay (Table 1). DLV susceptibility of the Y318F site-directed mutant was broadly similar to that reported by Pelemans et al. (13), although the level of NVP resistance observed here was slightly lower (they did not examine EFV or the effects of multiple NNRTI mutations).

The highly conserved wild-type residue Y318 forms part of the NNRTI-specific binding pocket of the HIV-1 RT (Fig. 3), and a structural rationalization of the effect of the Y318F mutation on DLV binding may be proposed. The X-ray crystal structure of HIV-1 RT in complex with DLV has been determined to a 2.65-Å resolution (5), revealing detailed information about the nature of its interactions with HIV-1 RT. DLV protrudes from the NNRTI pocket and makes a number of protein contacts that are distinct from those of other NNRTIs, including interactions with P236. The indole ring of DLV makes a number of van der Waals interactions with the side chain of P236, consistent with inhibition data that show that the RT mutation P236L results in a 70-fold reduction in DLV susceptibility (4). An adjacent residue, H235, forms an interaction via its main chain carbonyl with the side chain hydroxyl of Y318 (Fig. 3). The Y318F substitution would result in the loss of this hydrogen bond, which in turn could introduce more flexibility into the loop and reduce the interaction of the DLV indole ring with P236. It is known that the region containing

TABLE 1. NNRTI susceptibilities of HIV-1 variants constructed by site-directed mutagenesis[a]

Mutation(s)	Fold increase in IC_{50} of:		
	DLV	EFV	NVP
K103N	56	15	>60
Y181C	36	1.1	>60
Y318F	42	1.1	3.0
K103N/Y181C	>100	23	>60
K103N/Y318F	>100	43	>60
Y181C/Y318F	>100	3.3	>60
K103N/Y181C/Y318F	>100	84	>60

[a] Mutations were introduced in HIV-1 RT by site-directed mutagenesis, and the mutant RT was transferred into the HXB2-D wild-type background. The susceptibilities to the three NNRTIs were assessed as described in the text. The data represent the geometric mean for two independent assays. In some cases (indicated by ">") observed fold changes exceeded the maximum that could be assessed in the assay.

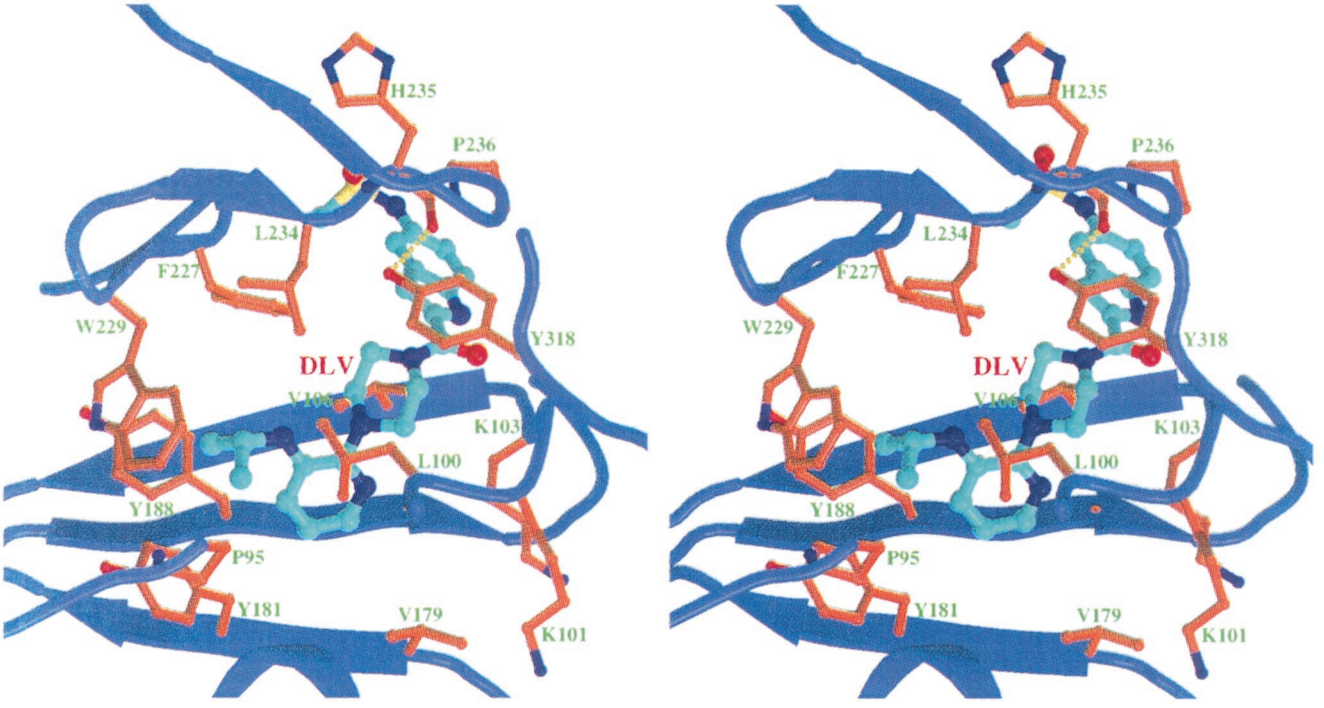

FIG. 3. Stereo image showing the major amino acids forming the NNRTI lipophilic pocket, in the crystal structure of the HIV-1 reverse transcriptase-delavirdine complex. Note that the functional groups of the wild-type Y318 and H235 residues form a hydrogen bond in close proximity to DLV. This hydrogen bond may play a part in DLV binding.

P236 is flexible and can adopt a number of different conformations (9). Unlike DLV, NVP does not interact with P236. Although NVP has a van der Waals contact with the CZ atom of the Y318 side chain (14, 16), this interaction would be unaffected by the Y318F mutation. EFV has no closer approach to the Y318 side chain than 4.2 Å and only a weak interaction with the side chain of P236 (15).

Regardless of the molecular mechanism, the data presented here indicate that a mutation in the 3′ region of HIV-1 RT (Y318F) is sufficient to confer high-level resistance to DLV and can increase EFV and NVP resistance in combination with other common NNRTI mutations. The Y318F mutation was observed in a significant proportion of clinically derived HIV-1 isolates and appears to be selected during NNRTI therapy. These results suggest that this region should be considered for incorporation in routine genotype and phenotype assays for NNRTI resistance.

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