The Human Immunodeficiency Virus Type 1 Nonnucleoside Reverse Transcriptase Inhibitor Resistance Mutation I132M Confers Hypersensitivity to Nucleoside Analogs

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We previously identified a rare mutation in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), I132M, which confers high-level resistance to the nonnucleoside RT inhibitors (NNRTIs) nevirapine and delavirdine. In this study, we have further characterized the role of this mutation in viral replication capacity and in resistance to other RT inhibitors. Surprisingly, our data show that I132M confers marked hypersusceptibility to the nucleoside analogs lamivudine (3TC) and tenofovir at both the virus and enzyme levels. Subunit-selective mutagenesis studies revealed that the mutation in the p51 subunit of RT was responsible for the increased sensitivity to the drugs, and transient kinetic analyses showed that this hypersusceptibility was due to I132M decreasing the enzyme’s affinity for the natural dCTP substrate but increasing its affinity for 3TC-triphosphate. Furthermore, the replication capacity of HIV-1 containing I132M is severely impaired. This decrease in viral replication capacity could be partially or completely compensated for by the A62V or L214I mutation, respectively. Taken together, these results help to explain the infrequent selection of I132M in patients for whom NNRTI regimens are failing and furthermore demonstrate that a single mutation outside of the polymerase active site and inside of the p51 subunit of RT can significantly influence nucleotide selectivity.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is a key target for antiretroviral drug development. To date, 12 RT inhibitors have been approved for the treatment of HIV-1 infection and can be classified into two distinct therapeutic groups. These include the nucleoside/nucleotide RT inhibitors (NRTIs) that block HIV-1 replication by acting as chain terminators in DNA synthesis and the nonnucleoside RT inhibitors (NNRTIs) that are allosteric inhibitors of HIV-1 RT DNA polymerization reactions. Although combination therapies that contain two or more RT inhibitors have profoundly reduced morbidity and mortality from HIV-1 infection, their long-term efficacy is limited by the selection of drug-resistant variants of HIV-1.

Antiviral drug resistance is defined by the presence of viral mutations that reduce drug susceptibility compared with the drug susceptibilities of wild-type (WT) viruses. Whether or not a particular drug-resistant mutant develops depends on the extent to which virus replication continues during therapy, the ease of acquisition of the particular mutation, and the effect that the mutation has on drug susceptibility and viral fitness. In this regard, we recently detected a novel but rare NNRTI resistance mutation at codon 132 (I132M) in RTs of clinical isolates from patients for whom NNRTI therapy was failing (6, 16). In vitro analyses showed that the I132M mutation in HIV-1 RT conferred high-level resistance to nevirapine and delavirdine (>10-fold that of the WT) and low-level resistance (~2- to 3-fold that of the WT) to efavirenz (18). In fact, the levels of resistance conferred by I132M in RT were essentially similar to those conferred by the Y181C mutation (J. Radzio, C. W. Sheen, and N. Sluis-Cremer, unpublished results). According to the Stanford University HIV Drug Resistance Database, combination therapies that contain nevirapine select for the Y181C mutation in approximately 35% of patients for whom the therapies are failing. However, these therapies select for the I132M mutation in less than 0.5% of these patients. Accordingly, the primary objective of the present study was to determine why the I132M mutation in HIV-1 RT is infrequently selected in patients for whom NNRTI-containing therapies are failing.

MATERIALS AND METHODS

Enzymes and viruses. The I132M, A62V, and L214I mutations were introduced into the WT HIV-1_aoo molecular clone or the p6HRT-Prok proviral expression vector (21) by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Full-length sequencing of mutant RTs was performed to confirm the presence of the desired mutations and to exclude adventitious mutations introduced during mutagenesis. WT and mutant recombinant HIV-1 RTs were expressed and purified to homogeneity as described previously (12, 13). For subunit-selective mutagenesis, the p66 and p51 RT genes were cloned into the pET-DUET vector (Novagen-EMD Biosciences Inc., San Diego, CA) and enzymes were purified using a double-tag strategy as described...
previously (14, 15). The RT concentration was determined spectrophotometrically at 280 nm by using an extinction coefficient (ε280) of 260,450 M⁻¹ cm⁻¹. Virus stocks were made by the transfection of 293T cells with proviral plasmids by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The titers of the viruses were determined using GHOST cells expressing the human CD4 and CXC4 receptors (27) under single-cycle conditions, and the cells were analyzed for infection by flow cytometry with a FACS-Calibur instrument (BD Biosciences, San Jose, CA).

**Antiviral assays.** Antiviral assays were performed with TZM cells by using different concentrations of zidovudine (AZT; Sigma, St. Louis, MO), lamivudine (3TC; Moravek, Brea, CA), and tenofovir (NIH AIDS Research and Reference Reagent Program, Rockville, MD) as described previously (1). Briefly, TZM-bl cells (4) seeded into 24-well plates were infected in duplicate in the presence or absence of the drug. After 48 h, cells were lysed and analyzed for luciferase expression. Results were expressed as luciferase counts per second and are shown as the percentage of cells infected with each virus at each dilution of the drug relative to the proportion of cells infected without the drug.

**Inhibition of WT and mutant HIV-1 RTs by nucleoside analogs.** Fixed-time-point assays were used to evaluate HIV-1 RT-associated RNA-dependent DNA polymerase activity, as reported previously (18). Assays were carried out using both heteropolymeric and homopolymeric template/primer (TP) substrates. For the heteropolymeric TP, the sequences of the DNA primer and template were 5'-TGGGCGGCGACTGCTAGAA-3' and 5'-TCTAGACCCTTTATGCTACG AATGTTGAAAAGTCTTCAAGTGCGCGCCAGAAGGCA-3', respectively. Poly(A)-oligo(dT)₁₈ was used as the homopolymeric TP substrate. Both the heteropolymeric and oligo(dT)₁₈ primers were synthesized with a biotin label on their 5' terminus. DNA polymerase reactions using heteropolymeric TP were carried out in a mixture of 50 mM Tris-HCl, pH 7.5 (37°C), 50 mM KCl, and 10 mM MgCl₂ containing 600 nM T/P, 10 mM MgSO₄, and 0.5 M EDTA. Streptavidin scintillation proximity assay beads (GE Healthcare) were added to each reaction mixture. The extent of radionuclide incorporation was determined by scintillation spectrometry using a 1450 Microbeta liquid scintillation counter (PerkinElmer, Waltham, MA). DNA polymerase assays using homopolymeric TP were carried out using identical experimental conditions except that 600 nM poly(A)-oligo(dT)₁₈ and 10 μM [³H]dUTP were used as substrates.

**Pre-steady-state kinetic experiments.** A rapid-quench instrument (model RQF-3, KinTek Corporation, Claremont, PA) was used for pre-steady-state experiments with reaction times ranging from 5 ms to 30 min. The typical experiment was performed at 37°C with solutions of 50 mM Tris-HCl (pH 8.0) containing 50 mM KCl, 10 mM MgCl₂, and various concentrations of nucleotides. All concentrations reported refer to the final concentrations after mixing. HIV-1 RT at 300 nM was preincubated with 50 nM DNA T/P substrate, prior to rapid mixing of nucleotides and divalent metal ions to initiate the reactions which was quenched with 0.5 M EDTA. The sequences of the primer and template were 5'-TGGGCGGCGACTGCTAGAA-3' and 5'-TCTAGACCCTTTATGCTACG AATGTTGAAAAGTCTTCAAGTGCGCGCCAGAAGGCA-3', respectively. The quenched samples were then mixed with an equal volume of gel loading buffer (98% deionized formamide, 10 mM EDTA, and 1 mg/ml of each of bromophenol blue and xylene cyanol) and denatured at 85°C for 5 min, and the products were separated from the substrates on a 7 M urea-16% polyacrylamide gel. The disappearance of the substrate (20-mer) and the formation of the product (21-mer) were quantified using a GS525 molecular imager polyacrylamide gel. The turnover rate of the product (21-mer) were quantified using a GS525 molecular imager polyacrylamide gel. The apparent burst rate constant (kₐ) for each particular concentration of dNTP was determined by fitting the time courses for the formation of the product (21-mer) with the following equation: [21-mer] = A[1 - exp(-kₐt)] where A represents the burst amplitude and t represents time. The turnover rate (kₐ) and apparent dissociation constant (K₀) for dNTP were then obtained by plotting the apparent catalytic rates (kₐobs) values against dNTP concentrations and fitting the data with the following hyperbolic equation: kₐobs = (kₐ[ddNTP])/(K₀ + [ddNTP]) + kₐ₀.

**Yeast two-hybrid assays.** Protein-protein interactions were quantified using the β-galactosidase (β-Gal) liquid assay performed with permeabilized Saccharomyces cerevisiae cells grown from at least three independent transformants, as described previously (25). Briefly, individual transformants were grown in 1 ml of SC-His-Leu (synthetic complete medium lacking histidine and leucine and containing 2% [wt/vol] glucose) at 30°C with aeration for 16 h before being diluted to an absorbance (at 600 nm) of 0.2 in 2.5 ml of SC-His-Leu. Cells were then allowed to grow with aeration at 30°C until the absorbance (at 600 nm) reached 0.5 to 0.8 before they were pelleted and stored at ~20°C. Thereafter, they were permeabilized in 50 μl of Y-PER yeast protein extraction reagent (Pierce) and assayed for β-Gal activity by adding a 1 ml mixture of 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, and 1 mM MgSO₄ containing 40 mM 2-mercaptoethanol and then adding 200 μl of a 4-μg/ml stock of ortho-nitrophenyl-β-D-galactopyranoside. Reaction mixtures were incubated at 30°C, reactions were quenched by the addition of 0.5 ml of 1 M Na₂CO₃, and the absorbance at 420 nm was read. β-Gal activity (in Miller units) was calculated by using the following equation: Miller units = (A₂₄₅ × 1,000) incubation time (in minutes).

**Protein expression in yeast.** To prepare yeast protein extracts for Western blot analysis, yeast transformants were grown and pelleted as described above. To extract protein, the cells were lysed at room temperature in 160 μl of Yeast-Buster protein extraction reagent (Novagen) containing 1-μg/ml concentrations of pepstatin, leupeptin, and aprotinin. The cells were subjected to a vortex for 10 s and allowed to mix on a rotating wheel for 20 min at room temperature. The supernatant was clarified after centrifugation at 13,000 × g for 15 min. The total protein concentration for each protein extract was determined using Bradford reagent (Bio-Rad), and to 15 μg of total protein per well was loaded for Western blot analysis. Fusion protein expression in yeast was evaluated by Western blot analyses of lysates with Gal4 activation domain (Gal4AD) polyclonal antibodies (Upstate Biotechnology) and anti-LexA polyclonal antibodies (Invitrogen). Immunodetection was accomplished using an ECL-Plus kit (Amersham).
antiviral data presented in Fig. 1. In comparison with the WT enzyme, I132M RT showed 0.3-, 0.08-, and 0.2-fold increases in susceptibilities to inhibition by AZT-TP, 3TC-TP, and tenofovir-TP, respectively.

Residue 132 forms part of the β7-β8 loop (residues 132 to 140) in HIV-1 RT. In the 66-kDa (p66) subunit of RT, this loop is situated in the fingers domain and resides a substantial distance from both the DNA polymerase active site and the NNRTI-binding pocket. In the 51-kDa (p51) subunit of RT, the β7-β8 loop is located at the dimer interface and contributes to the formation of the base of the NNRTI-binding pocket. We previously demonstrated that I132M in the p51 subunit of HIV-1 RT was responsible for the observed NNRTI resistance (18). To delineate the effects of I132M in each of the RT subunits on 3TC-TP susceptibility, we performed a subunit-selective analysis using purified enzyme that harbored I132M in either the p66 or p51 subunit. The data (Table 1) clearly demonstrate that 3TC-TP resistance is conferred by the mutation present in the p51, and not the p66, subunit.

I132M impairs HIV-1 heterodimer formation, but this phenotype does not account for the observed NNRTI hypersensitivity. Mutations of residues I135, N136, N137, and E138 in the β7-β8 loop of the p51 subunit of RT significantly decrease the dimeric stability of the enzyme (18). To determine whether the I132M mutation also had an impact on RT dimerization, we analyzed the ability of the I132M RT to form functional heterodimers by using the yeast two-hybrid assay (25). In this assay system, p66 is fused to the LexA DNA-binding domain (the bait) and p51 is fused to Gal4AD (the prey). Specific interaction between the two subunits results in the transactivation of the lacZ reporter gene in the yeast strain CTY10-5d, which permits the interaction of the RT subunits to be quantified by assaying for β-Gal activity. Initially, β-Gal activity in yeast coexpressing mutant p66 bait and p51 prey fusions engineered with the same mutations in each subunit was measured. This analysis revealed that I132M significantly decreased β-Gal activity compared with that in yeast expressing the WT p66 bait and p51 fusion proteins (Fig. 2A). The observed defect in I132M HIV-1 RT dimerization was similar to that in I135A HIV-1 RT dimerization. Consistent with previously published data, the E138K mutation did not have an impact on RT dimerization (18). To delineate the effects of I132M in each of the subunits on RT dimerization, we performed a subunit-selective analysis. To analyze the effects of mutations in the p66 subunit, we cotransformed yeast cells with mutant p66 bait and WT p51 prey, and to analyze the effects of mutations in the p51 subunit, we cotransformed yeast cells with WT p66 bait and mutant p51 prey. Our data show that I132M in both subunits contributes to the observed decrease in β-Gal activity (Fig. 2A).

Decreases in β-Gal activity, as measured in a yeast two-hybrid assay, may represent a true diminution in RT subunit interaction or may be due to decreased expression levels of either the bait or prey fusions compared with that of the WT protein. To distinguish between these two possibilities, we compared the steady-state protein levels in yeast expressing I132M p66 bait and p51 prey fusion proteins with those in yeast expressing the WT protein. Yeast protein extracts from exponentially growing cells were prepared and subjected to Western blot analysis. The p66 bait was detected using LexA antibodies, and p51 prey was detected using Gal4AD antibodies. No differences in the steady-state levels of the p66 bait and p51

| RT composition | IC50a (μM) of: | | |
|----------------|----------------|----------------|
|                | AZT-TP         | 3TC-TP         | Tenofovir-TP |
| WT p66/WT p51  | 0.06 ± 0.01 (1.0) | 7.3 ± 0.7 (1.0) | 1.3 ± 0.2 (1.0) |
| I132M p66/I132M p51 | 0.02 ± 0.01 (0.3) | 0.6 ± 0.1 (0.88) | 0.2 ± 0.1 (0.2) |
| I132M p51/WT p66 | 10.8 ± 2.8 (1.5) | 1.4 ± 0.3 (0.2) |
| WT p66/I132M p51 | 1.4 ± 0.3 (0.2) | 1.4 ± 0.3 (0.2) |

a The IC50 values are reported as the means ± standard deviations of results from three independent experiments. Values in parentheses indicate the change in resistance (n-fold) compared to that of the WT.

FIG. 1. Inhibition of WT and I132M HIV-1 by AZT, 3TC, and tenofovir. Assays were carried out as described in Materials and Methods, and each curve represents the average of results from three to four independent experiments. Error bars correspond to standard deviations. Virus infectivity is expressed as the percentage of cells infected with each virus at each concentration of the drug relative to the proportion of cells infected without the drug. The EC50 of AZT calculated for the WT and I132M viruses were 0.04 and 0.02 μM, respectively, those of 3TC were 0.1 and 0.2 μM, respectively, and those of tenofovir were 2 and 0.3 μM, respectively.
prey mutants and the WT subunits were observed (data not shown). This result indicates that the observed decreases in β-Gal activity were entirely due to a diminution in RT subunit interactions.

To determine whether this decrease in dimerization for I132M RT accounted for the observed NRTI hypersusceptibility, we next evaluated the susceptibilities of recombinant I132M, I135A, and E138K HIV-1 mutants to 3TC-TP. As described above, I135A decreases RT dimer stability whereas E138K in RT has no effect. Interestingly, all three mutations (i.e., I132M, I135A, and E138K) confer NNRTI resistance (17). The data from this study demonstrated that neither the I135A nor the E138K mutation in RT had an impact on 3TC-TP susceptibility (Fig. 2B). Taken together, these results suggest that decreases in RT dimerization do not cause NRTI hypersusceptibility. Therefore, the hypersusceptibility of I132M HIV-1 RT to NRTIs must be due to another mechanism.

Pre-steady-state kinetic analyses of dCTP and 3TC-TP incorporation by WT, I132M, and M184V HIV-1 RTs. To determine the mechanisms responsible for I132M HIV-1 RT hypersusceptibility to 3TC-TP, pre-steady-state analyses were carried out to elucidate the interactions of dCTP and 3TC-TP with the polymerase active sites of WT, I132M, and M184V HIV-1 RTs (Fig. 3; Table 2). M184V RT was included as a control in this study because the M184V mutation confers significant resistance to 3TC (3, 26). The pre-steady-state kinetic experiments, the results of which are presented in Table 2, defined the maximum rates of nucleotide incorporation (k_{pol} values), the nucleotide K_d values, and the catalytic efficiencies of incorporation (k_{pol}/K_d values). The k_{pol}/K_d value for the incorporation of dCTP by I132M RT was ~5-fold less than the k_{pol}/K_d value calculated for the WT enzyme. This change was driven primarily by a decrease in affinity (K_d) for dCTP at the polymerase active site of I132M HIV-1 RT. In contrast, the k_{pol}/K_d value for the incorporation of 3TC-TP by I132M RT was ~3-fold higher than the k_{pol}/K_d value calculated for the WT enzyme, and this effect was driven by an increase in K_d for 3TC-TP at the polymerase active site of I132M HIV-1 RT. As reported previously, 3TC-TP was not efficiently incorporated by M184V HIV-1 RT due to weak binding of the nucleoside analog at the mutant enzyme’s active site (5).
RT, which is defined by the ratio of the $k_{pol}$/$K_d$ value for the incorporation of dCTP to the $k_{pol}$/$K_d$ value for the incorporation of 3TC-TP, is an indication of the ability of the WT or mutant RT to discriminate between dCTP and 3TC-TP. The WT enzyme favors the incorporation of the natural dNTP over the nucleoside analog (selectivity = 11.9). Whereas the M184V mutation increased this selectivity ~170-fold, the I132M mutation decreased selectivity 0.06-fold. This value is consistent with the changes in EC$_{50}$s and IC$_{50}$s for the WT and I132M viruses or RTs reported in Fig. 1 and Table 1.

TABLE 2. Pre-steady-state kinetic constants for binding and incorporation of dCTP and 3TC-TP by WT, I132M, and M184V HIV-1 RTs

<table>
<thead>
<tr>
<th>RT</th>
<th>dCTP</th>
<th>3TC-TP</th>
<th>Selectivity $^b$</th>
<th>Change in resistance ($n$-fold)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{pol}$ (s$^{-1}$)</td>
<td>$K_d$ (µM)</td>
<td>$k_{pol}/K_d$ value (µM$^{-1}$ s$^{-1}$)</td>
<td>$k_{pol}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>WT</td>
<td>1.2 ± 0.7</td>
<td>1.1 ± 0.4</td>
<td>1.09</td>
<td>0.012 ± 0.001</td>
</tr>
<tr>
<td>I132M mutant</td>
<td>1.1 ± 0.2</td>
<td>5.0 ± 0.8</td>
<td>0.22</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td>M184V mutant</td>
<td>1.3 ± 0.4</td>
<td>3.0 ± 1.5</td>
<td>0.43</td>
<td>0.005 ± 0.001</td>
</tr>
</tbody>
</table>

$^a$ Data are the means ± standard deviations of results from at least three independent experiments.

$^b$ Selectivity is defined as follows: ($k_{pol}/K_d$ value for incorporation of dNTP)/($k_{pol}/K_d$ value for incorporation of NRTI-TP).

$^c$ The change in resistance was calculated as follows: selectivity of the mutant/selectivity of the WT.
RCs of WT and I132M HIV-1. Because viral replication fitness may substantially contribute to the pattern of resistance mutations selected during NNRTI therapy, we also measured the impact that the I132M mutation has on the HIV-1 RC. The RC was investigated by infecting the T-cell line HUT-CCR5 with WT HIV-1 or I132M HIV-1 at equal multiplicities of infection (Fig. 4A). Although approximately 100-fold more I132M HIV-1 particles than WT HIV-1 particles were used in the infection, the mutant virus exhibited a significantly delayed growth curve, with detectable replication occurring after 16 days postinfection (Fig. 4A). Therefore, these data suggest that the I132M mutation significantly diminishes viral RC.

Previously, a variant containing A62V and I132M was isolated from samples generated for the detection of minority drug-resistant variants (8, 17). Because studies have shown that A62V can partially eliminate the replication defect associated with the K65R or Q151M mutation (10, 24), we also introduced this mutation into our WT and I132M HIV-1 molecular clones. While A62V alone had no effect on RC, it significantly improved the replication of I132M HIV-1 (Fig. 4A). However, HIV-1 carrying mutations A62V and I132M (A62V/I132M HIV-1) still exhibited a replication lag of approximately 7 days compared to the WT virus.

To determine whether there was reversion at codon 132 or whether a compensatory mutation in I132M HIV-1 was selected, virus that eventually grew out from two independent replication assays was isolated and sequenced. In one experiment, M132 had reverted to the WT residue I132; in the second experiment, the L214I mutation was found to coexist with the I132M mutation. We introduced L214I into the WT and I132M molecular clones to further investigate the role of this mutation in viral RC. Both L214I HIV-1 and I132M/L214I HIV-1 replicated similarly to WT virus, suggesting that L214I is a compensatory mutation that improved the RC of virus containing the I132M mutation in RT (Fig. 4B).

DNA polymerase activities of recombinant purified WT and mutant HIV-1 RTs. To determine the mechanisms responsible for the decreased RCs of viruses containing I132M, we expressed and purified WT, I132M, A62V/I132M, and I132M/L214I HIV-1 RTs. The RNA-dependent DNA polymerase activities of these enzymes were evaluated. As reported previously, the I132M recombinant HIV-1 RT is 40 to 50% less active than the WT enzyme. However, both the A62V and L214I mutations restored the activities of the enzymes to near-WT levels (Fig. 5). We also introduced the A62V/I132M RT into yeast to perform the yeast two-hybrid assay for RT dimerization. However, the steady-state protein expression levels in yeast were significantly decreased compared to those of WT RT, thus preventing the quantification of RT dimerization.

DISCUSSION

The I132M mutation in HIV-1 RT was identified in a phenotypic screen of clinical HIV-1 isolates from patients for whom therapy containing an NNRTI was failing (6, 16), and we previously demonstrated that this mutation confers high-level resistance to nevirapine and delavirdine (18). Remarkably, in this study we have shown that I132M also leads to marked hypersusceptibility (0.06- to 0.1-fold-increased susceptibility compared to that of the WT) to the nucleoside analogs 3TC and tenofovir. While previous reports have demonstrated that NRTI resistance mutations elicit NNRTI hypersusceptibility (9, 22), the reverse situation (i.e., NNRTI resistance mutations conferring NRTI hypersusceptibility) is rare. The Y181C mutation has been shown previously to confer modest hypersus-
ceptibility (~0.5- to 0.8-fold-greater susceptibility) to AZT when present with AZT resistance-conferring mutations (11), but it may decrease susceptibility to other NRTIs, such as stavudine (2, 7). Furthermore, the mechanisms by which I132M and Y181C confer NRTI hypersusceptibility are different. Whereas Y181C diminishes the capacity of RT to excise AZT-monophosphate from chain-terminated primers (20), our study shows that I132M directly affects the ability of the enzyme to discriminate between the natural nucleotide and the nucleoside analog. To our knowledge, this is the first study that has identified a mutation outside of the polymerase active site and within the p51 subunit of HIV-1 RT that can significantly influence nucleotide selectivity.

Pre-steady-state kinetic experiments demonstrated that, in comparison with the WT enzyme, I132M HIV-1 RT bound the natural dCTP substrate with decreased affinity but 3TC-TP with increased affinity. While this finding provides a kinetic explanation for the observed NRTI hypersusceptibility, it does not address a structural mechanism. In the p51 subunit of RT, I132M is situated at the base of the β7-β8 loop, which contributes to the dimer interface and also the formation of the base of the NNRTI-binding pocket. In this regard, the results of our studies do not suggest a link between RT dimerization and nucleotide selectivity at the DNA polymerase active site.

However, previous studies have demonstrated communication between the NNRTI-binding pocket and the DNA polymerase active sites of RT that has an impact on nucleotide binding (23, 28), although the precise nature of the conformational changes responsible for the observed communication between the NNRTI-binding pocket and the polymerase active site have not been identified (28). In this regard, preliminary modeling experiments have also failed to provide a plausible structural explanation for the I132M-induced NRTI hypersusceptivity (data not shown).

HIV-1 containing the I132M mutation in RT was also found to replicate significantly less efficiently than the WT virus. This decrease in replication efficiency can be explained, in part, by the decreased DNA polymerase activity of the mutant RT observed in both the pre-steady-state and steady-state kinetic analyses. Characterization of A62V/I132M HIV-1 indicates that A62V partially eliminates the growth defect conferred by the I132M mutation. The A62V mutation has also been reported to eliminate the replication defect associated with the K65R and Q151M mutations in RT (10, 24). Of interest, Oli vares et al. reported that the F130W substitution significantly diminishes viral replication efficiency but that a compensatory change at codon 58 (T58S) can mitigate this effect (19). Taken together, the results of that study and ours may suggest a functional link between the β7-β8 loop (which contains residues 130 and 132) and the β3-β4 loop (which contains residues 58 and 62). In addition, we selected a second-site compensatory mutation, L214I, in RT. According to the Stanford HIV Drug Resistance Database, L214I is a polymorphism (i.e., it exists in both treatment-naïve and -experienced individuals) and is not associated with drug resistance. Nevertheless, the results of our study show that this substitution almost completely compensated for the replication defect of I132M HIV-1, as well as the decrease in RT activity. This finding highlights the notion that the genetic backbone of a given virus may significantly affect the selection of drug resistance mutations or the interpretation of the effects of a given drug resistance mutation on viral fitness.

In conclusion, our data show that the I132M mutation in the p51 subunit of HIV-1 RT confers NRTI hypersusceptibility and also decreases viral RC. Taken together, these findings help to explain why I132M is infrequently selected by treatment regimens containing either nevirapine or delavirdine, which most likely also include NRTIs, and further demonstrate that a single mutation outside of the polymerase active site and within the p51 subunit of HIV-1 RT can significantly influence nucleotide selectivity.

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