The M230L Nonnucleoside Reverse Transcriptase Inhibitor Resistance Mutation in HIV-1 Reverse Transcriptase Impairs Enzymatic Function and Viral Replicative Capacity

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Highly active antiretroviral therapy (HAART) has been the standard of treatment for HIV infection since 1996 and has substantially increased the rates of survival of HIV-infected patients (20). Nonnucleoside reverse transcriptase inhibitors (NNRTIs), which include first-generation drugs such as nevirapine (NVP) and efavirenz (EFV), are important components of HAART, as is a newer agent, etravirine (ETR), which retains activity against HIV type 1 variants containing common drug resistance mutations, such as K103N, associated with the diminished activity of NVP and EFV. ETR seems to be able to adapt its orientation and, by so doing, overcome common NNRTI resistance-associated mutations (26, 39).

HIV-1 reverse transcriptases (RTs) are versatile DNA polymerases endowed with several properties essential for viral replication, i.e., RNA- and DNA-dependent DNA polymerases (RDDPs and DDDPs, respectively), RNase H, strand transfer, and strand displacement activities (37). NNRTIs inhibit RT by binding to a hydrophobic pocket adjacent to the active site of the enzyme (35). NNRTI resistance is due to mutations within the NNRTI binding pocket, often at amino acid positions 100 to 110, 180 to 190, and 220 to 240, that substantially decrease susceptibility to all first-generation NNRTIs (21), yet conflicting results on ETR have been reported. ETR seems to be able to confer resistance to the second-generation NNRTI etravirine (ETR) as well as to the first-generation NNRTIs efavirenz (EFV) and nevirapine (NVP). Phenotyping assays with TZM-bl cells confirmed that M230L conferred various degrees of resistance to each of the NNRTIs tested. Recombinant viruses containing M230L displayed an 8-fold decrease in RC compared to that of the parental wild-type (WT) virus. Recombinant HIV-1 WT and M230L mutant RT enzymes were purified; and both biochemical and cell-based phenotypic assays confirmed that M230L conferred resistance to each of ETV, NVP, and ETR. RT that contained M230L was also deficient in regard to each of minus-strand DNA synthesis, both DNA- and RNA-dependent polymerase activities, processivity, and RNase H activity, suggesting that this mutation contributes to diminished viral replication kinetics.

Therefore, we expressed and purified a recombinant HIV-1 RT enzyme containing M230L and performed both RNA- and DNA-dependent DNA polymerase assays to determine the impact of M230L on viral enzymatic capacity. As an additional control, we also studied dapivirine (DAP), a compound that has been licensed for possible development as a vaginal microbicide by Tibotec Pharmaceuticals to the International Partnership for Microbicides (IPM). Drug susceptibility was also determined in cell culture phenotyping assays with both wild-type (WT) viruses and recombinant viruses containing M230L.

MATERIALS AND METHODS

Chemicals, cells, and nucleic acids. ETR was a gift from Tibotec Inc. DAP was obtained from the International Partnership for Microbicides. EFV and NVP were obtained from Bristol-Myers Squibb Inc. and Boehringer Ingelheim Inc., respectively. The HEK293T cell line was obtained from the American Type Culture Collection. The following reagents and cells were obtained through the NIH AIDS Research and Reference Reagent Program: infectious molecular clone pNL4-3 from Malcolm Martin and TZM-bl (JC53-bl) cells from John C. Kappes, Xiaoyun Wu, and Tranzyme Inc.

The following oligonucleotides, which were synthesized by Integrated DNA Technologies Inc. and purified by 6% polyacrylamide–7 M urea gel electrophoresis, were used in this study: PPT17D (5'-TCTCCCATTCGCCGCAAAATAGCGCGC-3'), PPT19D (5'-CTGGGGGTGTGAAATTAGCCCTTCCAGTCCCCCCTTTTCTTTTAAAAAGTGGCTAA-3'), Kim32D (5'-CGGCCAATTCCCGGTAGCAATATCTTCTCAGACG-3'), Kappes, Xiaoyun Wu, and Tranzyme Inc.

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Preparation of virus stocks. WT HIV-1 (HIV-1WT) and HIV-1 with the M230L mutation (HIV-1M230L) were generated by transfection of plasmids pNL4-3 and pNL4-3.MA210 into HEK293T cells by the use of Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The virus superna-
tants were harvested at 48 h posttransfection, centrifuged at 800 x g for 5 min to remove the cellular debris, filtered through a 0.45-μm-pore-size filter, aliquoted, and stored at −80°C. The levels of p24 in the virus supernatant were measured by an HIV-1 p24 antigen enzyme-linked immunosorbent assay (Perkin-Elmer). Virus stocks were quantified for RT activity prior to infections (14). Virion-associated RT activity was measured as described previously (17), but with the following modifications: 50 μl of the RT reaction mixture contained 10 μl of culture supernatants, 6.25 μg/ml of poly(A)-poly(dT)12-18 template-primer (T-P) in 50 mM Tris-HCl, pH 7.8-7.5, 5 mM dithiothreitol, 5 mM MgCl₂, 0.05% Triton X-100-2% ethylene glycol-0.3 mM reduced glutathione-5 μCi of [3H]dTTP (70 to 80 Ci/mm, 2.5 mM/μl). Following a 180-min incubation at 37°C, the reaction mixture was quenched by adding 0.2 ml of 10% cold trichloroacetic acid (TCA)-20 mM sodium pyrophosphate, and the mixture was incubated for at least 30 min on ice; the precipitated products were filtered onto 96-well MultiScreen high-throughput screening (HTS) FC filter plates (Millipore; Millipore) and sequentially washed with 200 μl of 10% TCA and 150 μl of 95% ethanol. The radioactivity of the incorporated products was analyzed by liquid scintillation spectrometry with a 1450 MicroBeta TriLux microplate scintillation and luminescence counter (Perkin-Elmer).

Determinative of relative replication capacity in TZM-bl cells. The replicative capacities of competent clonal wild-type and M230L HIV-1 were evaluated in a noncompetitive infectivity assay with TZM-bl cells in a manner similar to that described previously (40). In parallel experiments, recombinant viruses were serially diluted in DMSO stock suspensions, and a fixed amount of each dilution for RT activity, and added to 20,000 TZM-bl cells per well in a 96-well cell plate. The virus and cell cultures were cocultured for 48 h, after which the cells were washed with phosphate-buffered saline and lysed with 1× cell culture lysis reagent diluted from 5× cell culture lysis reagent (Promega, Madison, WI). This time point had been identified in previous experiments to be optimal for the performance of comparisons (data not shown), since earlier times did not show an indication of viral growth and later times resulted in reduced activity due to cell culture saturation. Luciferase activity was measured with a luciferase assay system (Promega), as recommended by the manufacturer. Luminescence was measured with a 1450 MicroBeta TriLux microplate scintillation and lucinescence counter (Perkin-Elmer). The viral replication level was expressed as a percentage of relative light units (RLU).

Phenotypic assay in TZM-bl cells. The antiviral activities of the NNRTIs against HIV-1WT and HIV-1M230L derived from pNL4-3 molecular clones were evaluated in TZM-bl cells, as described previously (43). Serial dilutions (1:2) of the test compounds were prepared in dimethyl sulfoxide (DMSO) and then in culture medium (Dulbecco modified Eagle medium) to yield a final DMSO concentration of 0.2% in the cultures. Serially diluted compounds were added to 10,000 wells (96-well plates), and the cells were then infected with either the M230L or WT virus. Standardization of the procedure was accomplished by always adding 200 50% tissue culture infective doses, as calculated in TZM-bl cells (i.e., ~180,000 ± 30,000 RLU) and as detected by luminescence. All cultures were maintained at 37°C under 5% CO₂ for 48 h. Luciferase activity was determined by quantifying the luciferase activity as a measure of viral replication by using the luciferase reagent (Promega). RLU were detected with a 1450 MicroBeta TriLux microplate scintillation and luminescence counter (Perkin-Elmer). The 50% effective concentrations (EC₅₀) were determined by nonlinear regression with GraphPad Prism (version 5.01) software.

Purification of recombinant HIV-1 RTs and determination of enzyme activity. The polymerase activity of each recombinant RT preparation was evaluated in a noncompetitive infectivity assay with TZM-bl cells, as described previously (17), with the following modifications: 50 μl of the RT reaction mixture contained 10 μl of culture supernatants, 6.25 μg/ml of poly(A)-poly(dT)12-18 template-primer (T-P) in 50 mM Tris-HCl, pH 7.8-7.5, 5 mM dithiothreitol-5 mM MgCl₂, 0.05% Triton X-100-2% ethylene glycol-0.3 mM reduced glutathione-5 μCi of [3H]dTTP (70 to 80 Ci/mm, 2.5 mM/μl). Following a 180-min incubation at 37°C, the reaction mixture was quenched by adding 0.2 ml of 10% cold trichloroacetic acid (TCA)-20 mM sodium pyrophosphate, and the mixture was incubated for at least 30 min on ice; the precipitated products were filtered onto 96-well MultiScreen high-throughput screening (HTS) FC filter plates (Millipore; Millipore) and sequentially washed with 200 μl of 10% TCA and 150 μl of 95% ethanol. The radioactivity of the incorporated products was analyzed by liquid scintillation spectrometry with a 1450 MicroBeta TriLux microplate scintillation and luminescence counter (Perkin-Elmer). The viral replication level was expressed as a percentage of relative light units (RLU).

Efficiency of minus-strand ssDNA synthesis. Using a cell-free system, the efficiencies of synthesis of minus-strand strong-stop DNA (ssDNA) by WT and M230L mutant enzymes were monitored with human natural RNA viruses (Bio S&T, Lachine, Quebec, Canada) in an HIV primer binding site (PBS) RNA primer-template system (4). The PBS RNA was transcribed in vitro from BssHIII-linearized HIV-PBS DNA by using a T7-Megashortscript kit (Ambion, Austin, TX), as described previously (11). The tRNA primer was heat annealed to the RNA template prior to the initiation of the minus-strand ssDNA synthesis. The minus-strand ssDNA synthesis was performed in a reaction mixture containing 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 40 mM RNA templates, and 120 mM template PBS RNA. This mixture was incubated for 2 min at 95°C, followed by incubation for 20 min at 70°C and slow cooling to room temperature. The synthesis of minus-strand ssDNA was initiated by the addition of an equal amount of RT in the presence of 6 mM MgCl₂ and 10 μM deoxyadenosine triphosphates (dTTPs). Aliquots (4 μl) were removed at different times, and the reactions were stopped in 4 μl of formamide sample buffer (96% formamide, 0.05% each of bromophenol blue and xylene cyanol, 20 mM EDTA) and stored at −80°C. After incubation at 37°C for 15 min, the reactions were terminated by adding 0.2 ml of 10% cold TCA–20 mM sodium pyrophosphate, and the mixture was incubated for at least 30 min on ice. The precipitated products were filtered onto 96-well MultiScreen high-throughput screening (HTS) FC filter plates (Millipore). The filter plate was prewet with 150 μl assay buffer prior to use and sequentially washed with 200 μl of 10% TCA and 150 μl of 95% ethanol. The radioactivity of the incorporated products was analyzed by liquid scintillation spectrometry. The IC₅₀ of each NNRTI were determined by nonlinear regression analysis with GraphPad Prism (version 5.01) software.

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TABLE 1. Sensitivities of WT and M230L RTs to NNRTIs

<table>
<thead>
<tr>
<th>Polymerase and RT</th>
<th>ETR&lt;sup&gt;*&lt;/sup&gt; IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>DAP IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>EFV IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>NVP IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tr>
<td>DDDP WT</td>
<td>126 ± 28</td>
<td>131 ± 23</td>
<td>57 ± 7</td>
<td>3.2 ± 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>M230L WT</td>
<td>1,320 ± 31</td>
<td>1,290 ± 32</td>
<td>910 ± 26</td>
<td>156 ± 10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>Fold change</td>
<td>10.5 ± 1.6</td>
<td>9.9 ± 1.6</td>
<td>16 ± 1.6</td>
<td>48.8 ± 4.8</td>
</tr>
<tr>
<td>RDDP WT</td>
<td>68 ± 9</td>
<td>19 ± 3</td>
<td>30 ± 6</td>
<td>4.1 ± 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>M230L WT</td>
<td>696 ± 27</td>
<td>172 ± 31</td>
<td>340 ± 35</td>
<td>185 ± 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fold change</td>
<td>10.2 ± 2.2</td>
<td>9.1 ± 2.4</td>
<td>24.7 ± 2.4</td>
<td>45.1 ± 4.5</td>
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</table>

<sup>*</sup> Data represent the means ± standard deviations of three separate determinations.

RESULTS

Purification of recombinant HIV-1 RT enzymes. Recombinant WT heterodimeric RT (p66 and p51 subunits) and the RT enzyme containing M230L were purified by single-step nickel chelate chromatography to >95% homogeneity; the RT p66 and p51 subunits were processed to similar molar ratios, on the basis of SDS-PAGE analysis (data not shown). The M230L mutation introduced into the recombinant HIV-1 RT did not interfere with either heterodimer formation or enzyme purification.

Inhibitory effects of NNRTIs determined by RDDP and DDDP assays. The inhibitory effects of various NNRTIs on RDDP activity were measured by a filter-based filtration RT assay. The sensitivities of the WT and mutant RTs to each of NVP, EFV, ETR, and DAP were determined. The results show that the mutated RT displayed high-level resistance to all these NNRTIs. The IC<sub>50</sub> for each drug tested with each of the RTs are shown in Table 1. The fold change (FC) of the IC<sub>50</sub> ranged from approximately 10 for ETR and DAP to 16 for EFV and 45 for NVP.

The inhibitory effects of the NNRTIs on DDDP activity were measured by a gel-based primer extension assay (Fig. 1). Figure 1 presents representative gels showing the dose-dependent inhibition of DNA polymerase activity by NNRTIs. The IC<sub>50</sub> were determined and are summarized in Table 1. For ETR and DAP, the difference in the IC<sub>50</sub> between the WT and mutant RT was approximately 10-fold. The IC<sub>50</sub> of EFV for the mutant RT was increased by 16-fold relative to that for the WT RT. The highest level of resistance was observed with NVP, the IC<sub>50</sub> of which was approximately 50-fold greater for the mutant RT than for the WT RT (Table 1). These results suggest that the M230L mutation confers high-level resistance to both first- and second-generation NNRTIs.

Inhibitory activities of NNRTIs determined by phenotypic assay in TZM-bl cells. TZM-bl cells were infected with HIV-1<sub>WT</sub> and HIV-1<sub>M230L</sub> in the presence of various concentrations of NNRTIs (ETR, DAP, EFV, and NVP). Relative luciferase activity was used as a measure of inhibition of viral growth, and the EC<sub>50</sub> for each inhibitor was calculated (Table 2). The EC<sub>50</sub> of ETR, DAP, EFV, and NVP were 1.25 nM, 0.91 nM, 1.87 mM, and 1.48 mM, respectively.

FIG. 1. Inhibition of DNA-dependent DNA polymerase RT activity by NNRTIs determined by a fixed-time gel-based RT assay. (A) Graphic representation of the primer-template system (ppt17D-ppt57D) used to monitor the inhibition of HIV-1 RT DNA polymerase activity by NNRTIs. The 17-mer DNA primer ppt17D was labeled with <sup>32</sup>P at the 5′ terminus and annealed to the 57-mer DNA template ppt57D. +1 and +4, positions of the first and the last nucleotides incorporated, respectively; *, position of the incorporated dGTP. (B) Dose-dependent inhibition of DNA polymerase activity by NNRTIs. All reactions were resolved by denaturing 6% polyacrylamide gel electrophoresis, visualized by phosphorimaging, and quantified with ImageQuant software (GE Healthcare). The positions of the labeled primer (P) and the full-length extension product (+) are indicated on the left. The concentrations of the NNRTIs used are as follows: for ETR, 0, 0.6, 1.7, 5, 15.2, 45.6, 137, and 411 nM; and 1.23, 3.7, and 11.1 μM; for DAP, 0, 5, 15, 45.7, 137, and 410 nM; 1.23, 3.7, 11.1, 100, and 500 μM; and 1 nM; for EFV, 0, 0.56, 1.69, 5, 15, 45.7, 137, and 410 nM; and 1.23, 3.7, 11.1, 33.3, and 100 μM; and for NVP, 0, 10, 31, 95, 285, 857 nM; 2.5, 7.7, 23, 69, 208, and 625 μM; and 1.87 mM.

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3.57 nM, and 163 nM, respectively, for the WT virus. The HIV-1 variant with the M230L mutation showed reduced sensitivities to each of these NNRTIs, i.e., approximately 6-fold higher EC\textsubscript{50}s for ETR and DAP, 18-fold higher EC\textsubscript{50}s for EFV, and 16-fold higher EC\textsubscript{50}s for NVP. Thus, NVP was the least potent of the NNRTIs tested against both WT virus and viruses containing M230L.

**Evaluation of relative replication capacity.** To determine the effect of the M230L mutation on replication capacity, we employed an infectivity assay using TZM-bl cells and serially diluted clonal virus stocks. The relative infectivities of the WT and M230L viruses were determined by measuring the luciferase activity at 48 h postinfection. The results presented in Fig. 2 show that HIV-1\textsubscript{M230L} had an 8-fold replication disadvantage compared with the replication capacity for HIV-1\textsubscript{WT}.

**Effect of the M230L mutation on efficiency of minus-strand ssDNA synthesis from the natural tRNA\textsubscript{3Lys} primer.** The first step in the reverse transcription of HIV-1 requires tRNA\textsubscript{3Lys} as the primer, which is annealed to a region near the 5' end of the viral RNA termed the PBS. This annealing primes the synthesis of minus-strand ssDNA catalyzed by reverse transcriptase, and this step can sometimes be impeded by the presence of drug resistance mutations (11, 13, 42, 44). We therefore investigated whether the M230L mutation affected the efficiency of minus-strand ssDNA synthesis by using HIV-1 PBS RNA template and a tRNA\textsubscript{3Lys}-specific primer. Reactions involving M230L RT yielded much less product than those performed with WT RT, and the decrease in the level of formation of full-length DNA products was pronounced at all points in these time course experiments (Fig. 3). Furthermore, a pause at position +5, was significantly increased with the use of the mutated RT. These results suggest that the M230L substitution in HIV-1 RT can lead to diminished levels of tRNA-primed synthesis of minus-strand ssDNA.

**Decreased rate of processive polymerization by RT containing M230L in regard to both RNA and DNA templates.** To investigate the effect of M230L on the rates of processive polymerization, we tested both WT and M230L RTs by using heteropolymeric DNA (57-mer) and an RNA template (40-mer) annealed to 5' P\textsuperscript{32}P-labeled oligodeoxynucleotide primers (19-mer and 17-mer, respectively) in a time course experiment. Figure 4 shows that the M230L mutation caused decreased polymerization in both the DDDP and the RDDP assays; thus, RT containing M230L is less active than the WT enzyme.

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**TABLE 2. EC\textsubscript{50} values for WT viruses and viruses containing M230L**

<table>
<thead>
<tr>
<th>Virus</th>
<th>ETR (nM)</th>
<th>DAP (nM)</th>
<th>EFV (nM)</th>
<th>NVP (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.25</td>
<td>4.1</td>
<td>5.25</td>
<td>8.75</td>
</tr>
<tr>
<td>M230L</td>
<td>4.1</td>
<td>5.25</td>
<td>8.75</td>
<td>1.63</td>
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</tbody>
</table>

Fold change: 5.8

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**Fig. 3. Efficiency of tRNA\textsubscript{3Lys}-primed minus-strand ssDNA synthesis in cell-free assays.** The efficiencies of synthesis of full-length DNA with WT and M230L mutant RTs were compared in time course experiments. (A) Graphic representation of the cell-free system used for the synthesis of full-length minus-strand ssDNA. The PBS RNA template used in this system consists of 258 nt at the 5' end of the HIV-1 genome, which contains the R, U5, and PBS regions. (B) Synthesis of full-length (FL) DNA by recombinant WT and mutant enzymes monitored in time course experiments. The reactions were initiated by the addition of 10 \mu M dNTPs and were monitored by measurement of the incorporation of [\alpha-\textsuperscript{32}P]dCMP into the extending DNA strand. The reactions were stopped at different time points during a period of 45 min. Time point zero indicates samples taken at 20 s after initiation. The full-length DNA product and the +1, +3, and +5 pausing sites are shown on the right.
Processivity defect of RT containing the M230L mutation. The processivity of a polymerase is defined as the number of nucleotides incorporated in a round of binding, elongation, and dissociation. Earlier studies have shown that HIV replication efficiency is related to RT processivity (3, 8), and some drug resistance mutations, such as M184V, have been shown to decrease RT processivity in *in vitro* processivity assays (6, 7, 41). To investigate the effect of M230L on enzyme processivity, we tested WT and M230L RTs by using a homopolymeric poly(rA) RNA template (average length, 500 nt) annealed to 5′/32P-labeled oligo(dT) primers in a fixed-time experiment in the presence of a heparin trap to ensure that each synthesized DNA molecule resulted from a single processive cycle. Figure 5 shows that the M230L mutation caused decreased processivity within a size range of the longest products at 75 nt to 150 nt, whereas the corresponding product lengths for the WT enzyme were 100 nt to 225 nt. Thus, the RT containing M230L is less processive than the wild-type enzyme, consistent with the diminished replication capacity of viruses containing this mutation.

**RNase H activity.** Previous studies have shown that substitutions at a number of residues in or adjacent to the primer grip region impair RNase H activity and contribute to reductions in HIV-1 replication fitness (15, 31). We performed an RNase H time course analysis in the absence and the presence of a heparin trap by using a recessed 32-mer DNA primer hybridized to a 5′-end-labeled 40-mer RNA to monitor 3′-DNA-directed RNase H activity (Fig. 6). The presence of the heparin trap permits the analysis of cleaved products from a single event of binding of RT to the substrate. Under both conditions, the RNase H activity of the M230L mutant RT was lower than that of the WT RT (Fig. 6). We also performed RNase H cleavage analysis in the absence of the heparin trap by using a 75-mer DNA oligonucleotide (75D) annealed to 5′-end-labeled RNA substrate kim40R to monitor 5′ RNA-directed RNase H activity. The results demonstrated that M230L RT results in a decreased cleavage efficiency compared to that of WT RT, as observed in the 3′-DNA-directed RNase H activity assay (data not shown). Thus, the M230L mutation also impairs RNase H activity.

**DISCUSSION**

The present study was designed to determine the impact of the M230L mutation in the HIV RT on the antiviral activity of the second-generation NNRTIs ETR and DAP, as well as the first-generation NNRTIs EFV and NVP. M230L was first identified by *in vitro* passage of HIV in the presence of the NNRTI delavirdine (DLV) (24, 30, 34). This mutation has also been detected in clinical isolates from patients and has been
were visualized by phosphorimaging. The processivities of the recombinant WT and M230L RT proteins were assessed with a homopolymeric RNA template poly(rA)-oligo (dT)$_{12-18}$ DNA primer. The DNA primer was labeled with $^{32}$P at the 5′ terminus and was annealed to the poly(rA) RNA template at an equimolar ratio. Processivities were analyzed by monitoring the size distribution of the DNA products in fixed-time experiments in the presence of a heparin trap. Parallel reactions were run in the absence of the heparin trap to ensure that similar amounts of enzyme activities were present in the reactions. Lane C, control run in the absence of the heparin trap to ensure that similar amounts of reaction products were used as a size standard. The sizes (in kilobases) of some fragments of the standard are indicated on the left side. All reactions were resolved by denaturing 6% polyacrylamide gel electrophoresis and were visualized by phosphorimaging.

shown to reduce the levels of susceptibility to each of NVP, DLV, and EFV by 23- to 58-fold (22). M230 is located within the highly conserved primer grip region of RT, and this position is highly conserved among all HIV isolates. Therefore, the effects of the M230L mutation on virus replication and on the underlying properties of RT should not depend on the particular RT or virus strain that is used in the assays. In particular, defects in replication capacity associated with M230L have previously been documented by other groups (23) and confirmed here. Moreover, a previous paper by our group has revealed the absence of important differences in enzymatic function between the RT enzymes of HIV-1 subtype B and HIV-1 subtype C (47).

Recently, the DUET-1 and DUET-2 clinical trials have identified an array of 17 resistance-associated mutations (RAMs) that confer diminished sensitivity to the second-generation NNRTI ETR; these mutations include V90I, A98G, L100I, K101E/H/P, V167I, E138A, V179D/F/T, Y181C/I/V, G190A/S, and M230L (38). Our data confirm that M230L confers resistance to all currently available NNRTIs in both phenotypic and biochemical assays. In the former, recombinant viruses harboring M230L displayed similarly moderate levels (5- to 6-fold) of resistance to ETR and DAP and higher-level (>10-fold) resistance to EFV and NVP. The data that we obtained with ETR are consistent with those presented in previous reports, which showed that M230L-containing viruses were 3- to 5-fold more resistant to ETR than WT RT-containing viruses (38, 39). However, a different study reported that M230L was not associated with resistance to either ETR or EFV (36). Our RDDP and DDDP enzyme assays further confirm that M230L causes similar 10-fold higher levels of resistance to each of the second-generation NNRTIs ETR and DAP and over 16-fold higher levels of resistance to EFV and NVP. Interestingly, a recent study showed that a newer NNRTI termed rilpivirine (formerly TMC278) was not affected by the presence of most single NNRTI RAMs, including those at positions 100, 103, 106, 138, 179, 188, 190, 221, 236, and 230 (5).

The prevalence of the M230L mutation in clinical isolates obtained from patients failing NNRTI-based therapy is low (2, 28). The emergence of resistance mutations can also depend on the effect of such mutations on viral replicative fitness (10), with mutations that confer high-level resistance with minimal effects on enzyme activity theoretically developing first. The use of a TZM-bl cell infectivity assay has confirmed that M230L-containing virus replicates less efficiently than WT virus, in agreement with the findings presented previously (23). The impaired replication capacity of M230L-containing virus might explain the rarity of this mutation in clinical isolates. However, we cannot exclude the possibility that hypersusceptibility to nucleoside and/or nucleotide RT inhibitors might result from the presence of the M230L mutation and that this resensitization to NNRTIs might also take place in the presence of other NRTI resistance mutations, including the thymidine analogue mutations (TAMs) K65R and M184V. As a consequence, hypersusceptibility/resensitization effects might contribute to the rarity of M230L in clinical samples. We are investigating the interaction of the M230L mutation with other NRTI resistance mutations. It remains to be determined...
whether the emergence of this mutation may require other compensatory mutations that might enhance NNRTI resistance and restore viral replicative fitness.

Our biochemical characterization has shown that M230L severely impaired each of minus-strand ssDNA synthesis, both DNA- and RNA-dependent polymerase activity, processivity, and RNase H activity. Previous studies by us and others have documented that the efficiency of each of these reactions can be correlated with viral replication efficiency (11–13, 42, 44, 47).

M230L is located at the tip of the β12-β13 hairpin comprising amino acids 227 to 235 (FLWGMGYELH), which defines the so-called primer grip, a highly conserved motif of retroviral RT. The primer grip is involved in maintenance of the primer terminus in an orientation appropriate for nuclease attack on an incoming dNTP (25). Mutational analysis of primer grip residues has shown their influence on various RT functions, including dNTP binding (46), polypurine tract removal (32), RNase H activity (31), template-primer utilization (16, 25), and the fidelity of DNA synthesis (9, 19, 45). The biochemical data presented here show that the described impairment in enzyme function associated with M230L contributes to reduced viral replication.

Further research is needed to determine how the M230L mutation might affect the binding kinetics of NNRTIs. We are performing a biochemical investigation of the interplay of M230L with other NNRTI and/or NRTI resistance mutations. We are also studying whether other resistance-related mutations that may appear in association with M230L may be able to help restore viral replication capacity while also potentially increasing the overall levels of drug resistance.

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The authors’ contributions are as follows. M.A.W. provided research resources for the project, supervised the project, and corrected the manuscript. H.-T.X. and Y.Q. performed the biochemical experiments and drafted the manuscript. S.M.S. and M.O. performed the phenotypic analyses. T.B.-M. performed some of the site-directed DNA mutagenesis work.

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