Differential impact of the HIV-1 non-nucleoside reverse transcriptase inhibitor mutations K103N and M230L on viral replication and enzyme function

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Objectives: We wished to study the resistance profile of etravirine, a novel non-nucleoside reverse transcriptase inhibitor (NNRTI) active against common human immunodeficiency virus type-1 (HIV-1) drug-resistant strains.

Methods: We compared the effects of K103N, the most prevalent NNRTI resistance mutation, and M230L on enzyme function, virus replication and extent of biochemical inhibition by etravirine, efavirenz and nevirapine.

Results: Growth kinetics analyses in cord blood mononuclear cells (CBMCs) demonstrated that K103N-containing virus replicated as well as wild-type (WT) virus and that the M230L-containing virus was severely impaired in replication ability in the absence of NNRTIs. K103N-containing viruses replicated well in the presence of efavirenz and nevirapine, while virus containing M230L displayed substantial replication in the presence of all NNRTIs tested. RNA-dependent DNA polymerase assays using a heterogeneous HIV-1 RNA template and purified recombinant WT or mutated reverse transcriptase enzymes revealed that the fold change (FC) for etravirine was 0.7 for K103N and 8 for M230L. K103N and M230L conferred high-level resistance to both efavirenz (FC = 39 and 15.3, respectively) and nevirapine (FC = 43.5 and 33), confirming that M230L confers cross-resistance to both drugs while K103N-containing viruses remain susceptible to etravirine. In enzymatic assays, the K103N mutation was associated with moderate reductions in the efficiency of 3′ DNA end-directed RNA template cleavage, while comparable efficiency to WT enzyme was observed with regard to minus-strand strong stop DNA synthesis and polymerase processivity.

Conclusions: These properties help to explain differences in the evolution and prevalence of these two NNRTI mutations.

Keywords: HIV-1 reverse transcriptase, NNRTI resistance, second-generation NNRTIs

Introduction

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are an important component of highly active antiretroviral therapy (HAART) for treatment of HIV infection.1,2 Regimens containing either efavirenz or nevirapine have become preferred therapy options for treatment-naive individuals and have gained increased popularity over protease inhibitor-based antiretroviral therapy. However, a low genetic barrier towards resistance of first-generation NNRTIs may limit their use.3 Second-generation NNRTIs such as etravirine can salvage patients who have failed first-line NNRTI-containing antiretroviral regimens.

Etravirine is an approved dianilinopyrimidine (DAPY) compound that can bind to HIV reverse transcriptase (RT) that contains mutations that confer resistance to first-generation NNRTIs.4–9 In vitro studies show that etravirine displays a high genetic barrier for resistance, requiring the accumulation of several (at least three) NNRTI-associated mutations for high-level resistance to become manifest.5–7 The DUET clinical trials have identified 17 resistance-associated mutations (RAMs), comprising V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190A/S and M230L, that confer diminished susceptibility to etravirine.8,9 A system for weighted scoring has been proposed, with the 17 etravirine RAMs having differential effects based on clinical response (3.0, Y181I/V; 2.5,
in independent study demonstrated that K103N was associated with susceptibility to either efavirenz or etravirine.19 A recent study showed that M230L alone did not confer loss of etravirine resistance, the six mutations with the highest NNRTI-BP, often at amino acid positions 100–110, 180–190 and 220–240, that substantially decrease susceptibility to all first-generation NNRTIs.13 Recently, mutations have been described in the connection domains that can also confer resistance to etravirine.19 Although it is generally accepted that NNRTIs block the HIV-1 replication by inhibiting the DNA polymerase active site of RT through allosteric interaction after binding to the NNRTI-BP, recent work has suggested that effects on RT RNase H activity and/or template/primer binding might also play a role.11,12

NNRTI resistance is due to mutations clustered around the NNRTI-BP, often at amino acid positions 100–110, 180–190 and 220–240, that substantially decrease susceptibility to all first-generation NNRTIs.13 Recently, mutations have been described in the connection domains that can also confer NNRTI resistance.14 Among the 17 RAMs associated with etravirine resistance, the six mutations with the highest weight factor are Y181I/V/C, K101P, L100I and M230L.8,16,17 Introduction of M230L into an HIV-1/HXB2 backbone by site-directed mutagenesis (SDM) can yield etravirine resistance with a fold change (FC) varying between 3.4 and 13.8. In contrast, a recent study showed that M230L alone did not confer loss of susceptibility to either efavirenz or etravirine.19

K103N is present in 40%–60% of patients failing NNRTI-containing treatment regimens,5,16,20 but does not confer resistance to etravirine. In the DUET studies, it was shown that K103N had no effect on the virological response (VR) to etravirine. An independent study demonstrated that K103N was associated with a superior VR to etravirine. Therefore, it is important to determine whether K103N confers hypersusceptibility to etravirine.

Materials and methods

Chemicals, cells and nucleic acids

The HEK293T cell line was obtained from the ATCC. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program: etravirine; efavirenz; and nevirapine. pNL4.3PFB proviral stocks were obtained through the NIH AIDS Research and Reference Reagent Program: etravirine; efavirenz; and nevirapine. pNL4.3PFB proviral reagents were obtained through the NIH AIDS Research and Reference Reagent Program: etravirine; efavirenz; and nevirapine. The HEK293T cell line was obtained from the ATCC. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program: etravirine; efavirenz; and nevirapine. pNL4.3PFB proviral stocks were obtained through the NIH AIDS Research and Reference Reagent Program: etravirine; efavirenz; and nevirapine. pNL4.3PFB proviral reagents were obtained through the NIH AIDS Research and Reference Reagent Program: etravirine; efavirenz; and nevirapine. The HEK293T cell line was obtained from the ATCC. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program: etravirine; efavirenz; and nevirapine. pNL4.3PFB proviral stocks were obtained through the NIH AIDS Research and Reference Reagent Program: etravirine; efavirenz; and nevirapine.

Site-directed mutagenesis and preparation of virus stocks

To construct HIV-1 variants containing desired mutations in the RT gene, site-directed mutagenesis reactions were first carried out using a QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) on HIV-1 RT expression plasmid pRT6H-PROT22 containing the wild-type (WT) RT gene from the HIV HXB2 strain. Fragments spanning RT amino acids 25–314 from MscI and PflM1 digestion from the pRT6H-PROT WT and mutant DNAs were used to replace the corresponding fragment of pNL4.3PFB proviral DNA.23 DNA sequencing was performed in both directions across the entire RT coding region to verify the absence of spurious mutations and the presence of any desired mutation. HIV-1 WT, HIV-1 K103N and HIV-1 M230L viruses were generated by transfection of corresponding proviral plasmid DNAs into HEK293T cells using Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer’s instructions. Viral supernatants were harvested at 48 h post-transfection, centrifuged for 5 min at 800 g to remove cellular debris, filtered through a 0.45 μm pore size filter, aliquoted and stored at −80°C. Levels of p24 in viral supernatant were measured with a Perkin Elmer HIV-1 p24 antigen ELISA kit. Virion-associated RT activity was measured as described previously,24 with modifications as follows: 50 μL of RT reaction mixture contained 10 μL of culture supernatants, 0.5 U/mL poly(rA)/poly(dT)12–18 template/primer (Midland Certified Reagent Company, Midland, TX, USA) in 50 mM Tris-HCl, pH 7.8, 75 mM KCl, 5 mM dithiothreitol, 5 mM MgCl2, 0.05% Triton X-100, 2% ethylene glycol, 0.3 mM reduced glutathione and 5 μCi of [3H]dTTP (70–80 Ci/mmol, 2.5 mCi/μL). Following a 180 min incubation at 37°C, the reaction mixture was mixed with 0.2 mL of cold trichloroacetic acid (TCA) and 20 mM sodium pyrophosphate and incubated for at least 30 min on ice; the precipitated products were filtered onto Millipore 96-well MultiScreen HTS FC filter plates (MFSN68) and sequentially washed with 200 μL of 10% TCA and 150 μL of 95% ethanol. The radioactivity of incorporated products was analysed by liquid scintillation spectrometry using a Perkin Elmer MicroBeta Trilux Microplate Scintillation and Luminescence Counter.

Measurements of HIV replication kinetics

Cord blood mononuclear cells (CBMCs) were isolated and cultured as previously described.25 pNL4.3-derived WT viruses and viruses containing the K103N and M230L mutations were normalized by RT enzyme assays in order to minimize inter-inoculum effects as described previously.26 Virus inocula were added to wells of culture plates containing CBMCs in the absence or presence of various concentrations of NNRTIs.25 The replication kinetics of mutant and WT viral stocks were assessed on the basis of RT enzymatic activity in culture supernatants at 2 or 3 day intervals post-infection for a period of 2 weeks and viral growth kinetics were monitored by RT assay as described above.

Expression and purification of HIV-1 recombinant RT enzymes

The subtype B HIV-1 RT expression plasmid pRT6H-PROT22 was used to create mutant pRT6H-PROT DNA constructs containing K103N and M230L. The presence of mutations and accuracy of the RT coding sequence were verified by DNA sequencing. Recombinant WT and mutated RTs were expressed and purified as described with modifications.22,23 In brief, RT expression in Escherichia coli M15 (pREP4) (Qiagen, Mississauga, Ontario, Canada) was induced with 1 mM IPTG at room temperature. The pellets bacteria were lysed under native conditions with BugBuster Protein Extraction Reagent (EMD Chemicals, Gibbstown, NJ, USA) and clarified by high-speed centrifugation, and the supernatant was subjected to the batch method of Ni-NTA metal affinity chromatography using QIAexpressionist (Qiagen), according to the manufacturer’s specifications. All buffers contained complete protease inhibitor cocktail (Roche). Histidine-tagged RT was eluted with an imidazole gradient. RT-containing fractions were pooled, passed through DEAE–Sephrose (GE Healthcare, Mississauga, Ontario, Canada) and further purified using SP–Sephrose (GE Healthcare). Fractions containing purified RT heterodimers were pooled, dialysed against storage buffer (50 mM Tris, pH
7.8, 25 mM NaCl and 50% glycerol) and concentrated to 4–8 mg/mL with Centricron Plus-20 MWCO 30 kDa (Millipore, Etobicoke, Ontario, Canada). Protein concentration was measured using a Bradford Protein Assay Kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and the purity of the recombinant RT preparations was verified by SDS-PAGE. The polymerase activity of each recombinant RT preparation was evaluated using varying amounts of RTs and a synthetic homopolymeric poly(rA)/poly(dT) template/primer (Midland Certified Reagent Company), as described previously.28

**NNRTI Inhibition of RNA-dependent DNA polymerase (RDDP) activity**

Inhibition of RDDP activity by NNRTIs was assayed as previously reported.28 A heterogeneous HIV primer-binding site (PBS) RNA template, containing a 497 bp HIV-1 sequence spanning the RT region of the HIV-1 long terminal repeat (LTR) and a portion of gag, was prepared in vitro with a MEGAscript transcription kit (Ambion, Austin, TX, USA) from plasmid pHIVPBS.29 The 18 nt DNA primer dPR is complementary to the HIV-1 PBS. The template and primer were mixed at a molar ratio of 1:3, denatured at 85°C for 5 min and then sequentially cooled to 55°C for 8 min and to 37°C for 5 min to allow the specific annealing of primer to the template. The reaction mixture (50 μL) contained buffer, 50 mM Tris-HCl (pH 7.8), 5 mM MgCl2, 60 mM KCl and 5 μM of dNTPs with 2.5 μCi of [3H]dTMP (70–80 Ci/mmol, Perkin Elmer), 100 nM heterogeneous HIV-1 PBS RNA template/dPR primer and recombinant RTs at equal activities and variable concentrations of the NNRTIs. After an incubation of 30 min at 37°C, the reactions were terminated by adding 0.2 mL of 10% cold TCA and 20 mM sodium pyrophosphate and incubated for at least 30 min on ice. The precipitated products were filtered onto a 96-well Multiscreen HTS FC filter plate (Millipore). The filter plate was pre-wetted with 150 μL of assay buffer prior to use and sequentially washed with 200 μL of 10% TCA and 150 μL of 95% ethanol. The radioactivity of incorporated products was analysed by liquid scintillation spectrometry. The IC50 of each NNRTI were determined by non-linear regression analysis using GraphPad Prism 5.01 software.

**Efficiency of synthesis of minus-strand (−) strong stop (ss) DNA**

The efficiency of (−) ssDNA synthesis was determined using well-established assays as described previously.30–33 Briefly, 20 nmol/L HPLC-purified human tRNA32P was heat-annealed to 80 nmol/L HIV-1 PBS RNA. PBS RNA was transcribed from PvuII-linearized pHIVPBS DNA using a T7-MegaShortscript Kit (Ambion) as previously described.33 Then, WT or mutated RT enzymes and 6 nmol/L MgCl2 were added. Reactions were initiated by addition of a mixture of dNTPs to a final concentration of 10 μM of each of dATP, dGTP and dTTP and 1 μM dCTP. The efficiency of (−) ssDNA synthesis was monitored by incorporation of [α-32P]dCMP in time-course experiments. Aliquots of reaction mixtures were removed at various time intervals after initiation and quenched with formamide loading buffer (96% formamide, 20 mM EDTA, 0.05% xylene cyanol FF and 0.05% bromophenol blue). Samples were heat-denatured and resolved using 6% polyacrylamide/7 M urea gel electrophoresis, and analysed using a Molecular Dynamics Typhoon Phosphorimager (GE Healthcare) and ImageQuant software.

**Processivity assay**

The processivity of recombinant RT enzymes was studied using a heparin enzyme trap to ensure a single round of binding, primer extension and dissociation. The template/primer was prepared by annealing a 5′-end 32P-labelled oligo(dT) (GE Healthcare) to an equal molar ratio of poly(rA) (GE Healthcare) homopolymeric RNA template prior to the initiation of reactions as described previously.18 RT with an equal amount of activity and template/primer were preincubated for 5 min at 37°C. Reactions were initiated by the addition of dTTP and heparin trap (final concentration 2 mg/mL) and incubated at 37°C for 3 min; 2 μL of reaction mixture was removed and mixed with 8 μL of stop solution (90% formamide, 10 mM EDTA and 0.1% each of xylene cyanol FF and bromophenol blue). The effectiveness of the heparin trap to limit polymerization on the RNA template was verified in control reactions in which the heparin trap was preincubated with substrate before the addition of RT and dTTP. Reaction products were analysed by 6% denaturing PAGE and phosphorimaging.

**RT-catalysed RNase H activity**

DNA 3′-end-directed RNase H activity was assayed using a 5′-end 32P-labelled 40-mer RNA template, kim40R, annealed to a complementary 32-mer DNA primer, kim32D, at a 1:4 molar ratio. Reactions were conducted as described previously34 at 37°C in mixtures containing RNA/DNA hybrid duplex substrate with purified RT enzymes normalized by activity in assay buffer, 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 0.1 mM EDTA, pH 8.0, and 6 mM MgCl2, in the absence or presence of a heparin trap at a final concentration of 2 mg/mL. RNase H cleavage was monitored in time-course experiments, with aliquots of samples removed at different timepoints after initiation of reactions and quenched by adding 3 volumes of formamide loading buffer (96% formamide, 20 mM EDTA, 0.05% xylene cyanol FF and 0.05% bromophenol blue). The samples were heated to 90°C for 3 min, cooled on ice and resolved using 6% polyacrylamide/7 M urea gels. The dried gels were exposed to phosphoscreens and analysed using a PhosphorImager (GE Healthcare) and ImageQuant software. The efficacy of the heparin trap was verified by pre-incubation experiments performed through a 10 min pre-incubation of RT enzymes with various concentrations of heparin trap followed by initiation with the [32P]RNA/DNA hybrid duplex substrate and magnesium in the same assay buffer as described above.

**Results**

**Effects of the K103N and M230L RT mutations on viral replication kinetics in CBMCs**

It was recently shown that the M230L mutation in an HXB2 genetic background did not confer either a loss of susceptibility to etravirine or a significant decrease in viral replication capacity.19 To address this inconsistency with our own published work,18 we constructed a series of NL4-3-derived viruses in which these mutations were introduced into the gag gene. In vitro replication kinetics of WT and mutant viruses were measured and compared in CBMC cultures,36 in which quantification of virus production at various timepoints was carried out, i.e. p24 CA antigen or RT activity.36,37 The results in Figure 1 show that K103N viruses replicated as well as did WT viruses in the absence of NNRTIs, while the M230L viruses replicated much more poorly. In the presence of drug (i.e. 10 nM etravirine and efavirenz, and 1 μM nevirapine), M230L virus displayed substantial replication capacity while WT virus was fully inhibited. At the highest concentrations of NNRTIs tested, only the K103N virus showed substantial replication ability in the presence of efavirenz and nevirapine, while only M230L virus could grow in the presence of 10 nM etravirine. These results confirm that M230L confers both resistance to etravirine as well as lower replication capacity compared with...
Purification of recombinant HIV-1 heterodimeric RT enzyme

Recombinant WT RT heterodimer (p66/p51) and RT enzymes containing either the K103N or M230L substitutions were purified to >95% homogeneity; the RT p66 and p51 subunits were processed to similar molar ratios based on SDS-PAGE analysis (Figure 2). The mutations introduced did not interfere with either heterodimer formation or enzyme purification.

Inhibitory effects of NNRTIs in RDDP RT filter-binding assays

The inhibitory effects of various NNRTIs on RDDP activity were measured by a filter-binding RT assay using HIV-1 PBS RNA template. This assay reflects physiologically relevant conditions compared with the use of commonly used homopolymeric
Susceptibilities of WT and mutant RTs were determined for each of etravirine, efavirenz, and nevirapine (Table 1). The results show that both K103N and M230L RTs displayed high-level resistance to efavirenz and nevirapine. The fold change (FC) for efavirenz ranged from approximately 15 for M230L RT to 39 for K103N RT, while for nevirapine the FCs ranged from 33 for M230L to 43 for K103N RT. The M230L RT conferred 8-fold resistance to etravirine, consistent with results obtained using homogeneous poly(rA) template/oligo(dT) primer, while the K103N mutant RT showed an FC of 0.7 compared with WT enzyme. Thus, recombinant RT harbouring K103N is as susceptible as WT enzyme to etravirine. We also observed an FC of 0.7 in susceptibility to etravirine using K103N RT in the CBMC phenotyping assay (H.-T. Xu and M. A. Wainberg, unpublished results). It is known that K103N is associated with a 0.5 FC for etravirine.

Table 1. IC₅₀ values (nM) of recombinant RTs containing K103N and M230L for various NNRTIs as determined by RDDP filter-binding assay

<table>
<thead>
<tr>
<th></th>
<th>etravirine</th>
<th>efavirenz</th>
<th>nevirapine</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.4 ± 0.6 (1)</td>
<td>6.9 ± 0.4 (1)</td>
<td>8.7 × 10³ ± 4.8 × 10² (1)</td>
</tr>
<tr>
<td>K103N</td>
<td>3.8 ± 0.3 (0.7)</td>
<td>269.1 ± 11 (39)</td>
<td>378.5 ± 12 × 10² (43.5)</td>
</tr>
<tr>
<td>M230L</td>
<td>43.2 ± 6 (8)</td>
<td>105.6 ± 8.3 (15.3)</td>
<td>287 ± 9 × 10² (33)</td>
</tr>
</tbody>
</table>

Data represent means ± SD of two independent experiments in triplicate.

Effect of K103N and M230L on efficiency of (−)ssDNA synthesis

We next used human tRNA³Lys as primer in reactions in which the latter was annealed to the PBS near the 5′-end of viral RNA to study synthesis of (−)ssDNA. Previous results showed that the G190S and G190A mutations impaired efficiency of tRNA-primed (−)ssDNA synthesis. Now, time-course reactions involving M230L RT and K103N RT yielded less product than those performed with WT RT and the decrease in formation of full-length DNA products was pronounced at all timepoints in these experiments (Figure 3). Thus, M230L leads to both diminished levels of tRNA-primed synthesis of (−)ssDNA as well as a loss of viral replication efficiency.

Processivity of RTs containing K103N and M230L

The processivity of a polymerase is defined as the number of nucleotides incorporated in a single round of binding, elongation and dissociation. To compare the impact of the K103N and M230L mutations on enzyme processivity, we tested the WT and mutant RTs using homopolymeric poly(rA) RNA template annealed to 5′-end P-labelled 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 4 shows that the M230L mutation caused decreased processivity, the longest products made having a size of ~100 nt, while the WT enzyme yielded products of ~200 nt, as did the K103N enzyme. Thus, RT containing M230L...
cleavage in time-course experiments using a 3′- recessed DNA primer hybridized to a 5′- end-labelled RNA to study DNA 3′- end-directed RNase H activity in the presence or absence of a heparin trap. In the absence of the heparin trap, both the K103N and M230L RTs produced the same cleavage profiles as WT enzyme (Figure 5). The first cleavage at the –18 position relative to the 3′-end of the DNA primer indicates the distance of the active sites of polymerase and RNase H, showing that the RNA template is cleaved 18 nt upstream of the 3′-end of the primer terminus. The second cleavage at the –15 position appears very soon after the first cleavage and also appears in the trap reaction, indicating that the RT enzyme does not dissociate from the substrate, but slides 3 nt toward the 3′-end of the primer for this cut. The third cleavage product at the –7 position does not appear in the trap reaction, indicating that the RT enzymes need to rebind to the substrate after dissociation for this cleavage. These results show that the K103N and M230L substitutions introduced into RT did not substantially change the spatial and temporal relationships between the active sites of polymerase and RNase H components of RT (Figure 5).

The RNase H activity of M230L RT was much lower than that of WT RT and K103N RT, both in the absence and presence of trap, as indicated by accumulation of the –7 and –15 cleavage products, respectively. M230L resulted in decreased cleavage efficiency compared with WT RT, indicating that this mutation impairs RNase H activity. Compared with M230L RT, K103N showed much higher cleavage efficiency under both conditions, but slightly lower than WT enzyme, consistent with previous reports showing that K103N slows DNA 3′- end-directed RNase H cleavage.

Discussion

This study compared the K103N and M230L mutations with regard to RT enzyme function and biochemical inhibitory activities with regard to nevirapine, efavirenz and etravirine. In addition, the effects of these mutations on viral growth properties and the presence of NNRTIs were studied in CEM cultures.

K103N is the most common mutation that emerges in patients who fail the first-generation NNRTIs efavirenz and nevirapine, while M230L is less prevalent in clinical settings. We have shown that M230L, introduced into chimeric HIV-1NL43 virus, caused a significant reduction in viral replication capacity, unlike K103N virus, and that the latter virus has similar replication kinetics to WT virus, consistent with previous reports. M230L RT enzyme was also deficient with regard to each of (−)DNA synthesis, processivity and RNase H activity, while K103N enzyme behaved similarly to WT, consistent with prevous reports. Although both residues are located around the NNRTI-BP, the K103N and M230L mutations have differential NNRTI resistance profiles. Our data provide further evidence confirming that M230L confers resistance to all currently available NNRTIs, consistent with previous reports.

Structural studies of HIV-1 RT have shed light on mechanisms of NNRTI resistance. K103 is located in the β5–β6 connecting loop near the entrance of the NNRTI-BP. Structural studies of K103N suggest that an extra hydrogen bond within the collapsed NNRTI-BP between N103 and Y188 side chains is created, making
it difficult for rigid first-generation NNRTIs to enter the hydrophobic NNRTI-BP, thus conferring cross-resistance. Recent co-crystal structures of K103N mutant RT in complex with etravirine have shown that etravirine binds and adapts to changes in the NNRTI-BP. Although hypersusceptibility of K103N RT to etravirine was not demonstrated based on the fact that hypersusceptibility to NNRTI was generally defined as an FC in IC₅₀ of <0.4, we observed an FC of 0.7 in IC₅₀ for K103N against etravirine using an RDDP RT enzyme assay performed on an HIV-1 PBS RNA template with a PBS DNA primer, compared with that of the WT enzyme, confirming that K103N is fully susceptible to etravirine. These data are in support of recent studies showing that K103N was associated with a better VR to etravirine.

The M230 residue is located at the loop of the β₁₂–β₁₃ hairpin, comprising amino acids 227–235 (FLWMGYELH), which defines the so-called primer grip, playing important roles in both RT enzyme function and formation of the NNRTI binding pocket. The primer grip helps to maintain the primer terminus in an orientation appropriate for nucleophilic attack on an incoming dNTP. Recently, an NMR study of HIV-1 RT both in the presence and absence of nevirapine showed that M230 in the p66 subunit is more mobile and solvent-exposed than suggested by crystal structures of the apo enzyme, which have a ‘closed’ fingers–thumb conformation. This mobility of the primer grip is presumably important for binding of NNRTIs, since the NNRTI-BP is not observed in the absence of the inhibitors, requiring instead that the BP be dynamically accessible. The M230L substitution might affect this mobility and influence the formation of the NNRTI-BP. By doing so, it might interfere with NNRTI binding and cause drug resistance. Mutational analysis of primer grip residues has shown their influence on various RT functions, including dNTP binding, polyuridine tract removal, RNase H activity, template/primer utilization and fidelity of DNA synthesis. Virus containing an M230A substitution was shown to be non-infectious. The biochemical data presented here show that the impairment in enzyme function associated with M230L contributes to reduced viral replication. However, an M230I mutation was able to compensate for a Y115W substitution, restoring viral replication capacity, whereas Y115W impaired RT activity by decreasing the dNTP binding affinity of the polymerase. Thus, different substitutions at M230 may have different effects on viral replication and RT function. Biochemical investigation of the interplay of M230L with other NNRTI and/or NRTI mutations is under way in our laboratory.

In summary, the differential impacts associated with K103N and M230L help to explain differences in the evolution and prevalence of these two NNRTI mutations. Although the prevalence of the M230L mutation in the clinic is still low, future studies may well reveal that M230L is a key mutation with regard to etravirine and possibly other second-generation NNRTIs as well. While other NNRTI mutations may also impact on viral susceptibility to etravirine and other second-generation NNRTIs, as observed in the DUET trial, it is also possible that the relatively low prevalence of M230L may be attributable to its ability to hypersensitize to certain nucleoside drugs, such as zidovudine and lamivudine, and the resultant enhanced activity of such compounds against M230L-containing viruses.
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Transparency declarations

We have no conflicts of interest to declare.

References

HIV-1 RT mutations K103N and M230L


