Roles of the K101E Substitution in HIV-1 Reverse Transcriptase in Resistance to
Rilpivirine and other Non-Nucleoside Reverse Transcriptase Inhibitors

Hong-Tao Xu,1 Susan P. Colby-Germinario,1 Wei Huang,1 Maureen Oliveira,1 Yingshan Han,1
Yudong Quan,4 Christos J. Petropoulos,4 and Mark A. Wainberg1,2,3*

1 McGill University AIDS Centre, Lady Davis Institute for Medical Research, Jewish General
Hospital, Montreal, Quebec, Canada and Departments of Medicine and Microbiology and
Immunology, McGill University, Montreal, Quebec, Canada.

4 Monogram Biosciences, South San Francisco, California, USA

Running title: K101E and Rilpivirine Resistance

*Address correspondence to: Dr. Mark A. Wainberg, McGill University AIDS Centre, Lady
Davis Institute for Medical Research, Jewish General Hospital, 3755 Côte-St-Catherine Rd,
Montreal, Quebec H3T 1E2, Canada Tel: (514) 340-8260 Fax: (514) 340-7537 E-mail:
mark.wainberg@mcgill.ca
The abbreviations used are:

- 3TC, lamivudine; ABC, abacavir; ATCC, American Type Culture Collection; AZT/ZDV, zidovudine; BCO, biological cut-off; CBMCs, Cord blood mononuclear cells; d4T, stavudine;
- DAPY, di-aryl-pyrimidine; ddC, zalcitabine; ddI, didanosine; DLV, delavirdine; DPV, dapivirine;
- DMEM, Dulbecco's Modified Eagle Medium; ds DNA, double-stranded DNA; EC50, 50% drug effective concentration; EFV, efavirenz; ELISA, enzyme-linked immunosorbent assay; ETR, etravirine; FTC, emtricitabine; HAART, highly active antiretroviral therapy; HIV-1 RT, human immunodeficiency virus type 1 reverse transcriptase; IC50, 50% drug inhibitory concentration; IL-2: interleukin-2; M.O.I.: multiplicity of infection; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; NTA, Ni-nitrilotriacetic acid; NVP, nevirapine; PBS, primer binding site; PHA: phytohemagglutinin A; RAMs, resistance-associated mutations; RDDP, RNA-dependent DNA polymerase; RPV, rilpivirine; RTV: resistance test vector; SDM, site-directed mutagenesis; TDF, tenofovir disoproxilfumarate; T/P, template/primer.
ABSTRACT

Resistance against the recently approved non-nucleoside reverse transcriptase inhibitor (NNRTI) rilpivirine (RPV) commonly involves substitutions at positions E138K and K101E in HIV-1 reverse transcriptase together with a M184I substitution that is associated with resistance against co-utilized emtricitabine (FTC). Previous biochemical and virological studies have shown that compensatory interactions between substitutions E138K and M184I can restore enzyme processivity and viral replication capacity. Structural modeling studies have also shown that disruption of the salt bridge between K101 and E138 can affect RPV binding. The current study was designed to investigate the impact of K101E, alone or in combination with E138K and/or M184I on drug susceptibility, viral replication capacity, and enzyme function. We show here that K101E can be selected in cell culture by the NNRTIs etravirine (ETR), efavirenz (EFV), and dapivirine (DPV) as well as by RPV. Recombinant RT enzymes and viruses containing K101E, but not E138K were highly resistant to nevirapine (NVP) and delavirdine (DLV) as well as ETR and RPV but not EFV. The addition of K101E to E138K slightly enhanced ETR and RPV resistance, compared to E138K alone, but restored susceptibility to NVP and DLV. The K101E substitution can compensate for deficits in viral replication capacity and enzyme processivity associated with M184I, while M184I can compensate for the diminished efficiency of DNA polymerization associated with K101E. The coexistence of K101E/E138K does not impair either viral replication or enzyme fitness. We conclude that K101E can play a significant role in resistance to RPV.

Keywords
HIV-1, enzyme, mutations, enzyme inhibitors, mechanisms of resistance, reverse transcriptase, drug resistance, rilpivirine, etravirine, K101E, E138K.
Introduction

The reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) is crucial for HIV-1 replication and has been an important target of antiretroviral (ARV) therapy (1). Both nucleos(t)ide reverse transcriptase inhibitors (NtRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) are key components of ARV therapy (2), which has led to significant declines in HIV-associated morbidity and mortality (3, 4). N(t)RTIs that act as competitive inhibitors and cause chain termination of the growing viral DNA chain include zidovudine (AZT, ZDV), didanosine (ddI), stavudine (d4T), lamivudine (3TC), emtricitabine (FTC), abacavir (ABC), and tenofovir (TFV). In contrast, NNRTIs act allosterically by binding to the NNRTI binding pocket (BP) located 10 Å from the polymerase active site (3), and include earlier drugs such as nevirapine (NVP), delavirdine (DLV), and efavirenz (EFV), and newer products such as etravirine (ETR) and rilpivirine (RPV). However, the rapid replication rate of HIV-1 and the error-prone nature of its RT, can drive the development of drug resistance against all ARVs currently in use (5).

The earlier NNRTIs, such as NVP and EFV, have a low genetic barrier for development of resistance, and cross-resistance among NNRTIs is common (6, 7). Recently, however, several newer NNRTIs that are di-aryl-pyrimidine (DAPY) compounds, ETR (8, 9) and RPV (10), have been developed and are active against viruses containing mutations associated with resistance to NVP and EFV (11, 12). However, two phase III clinical trials, ECHO and THRIVE, showed that treatment failure in HIV patients receiving co-formulated RPV/FTC/TDF was mostly frequently associated with a E138K substitution, or less often with a K101E substitution, both of which are known to cause NNRTI resistance, in most cases together with the M184I substitution known to cause resistance against 3TC and FTC (12). Until now, biochemical and virological studies to explain resistance to RPV have focused on interactions between E138K and M184I/V (13-17).
We and others have shown that the decreased viral replication capacity of M184I/V was restored in the presence of E138K (14). Furthermore, biochemical analyses showed that the addition of E138K to M184I in RT restored the processivity of DNA synthesis by enhancing dNTP usage (14, 15). It was also reported that the addition of M184I to E138K enhanced levels of resistance to both ETR and RPV compared to E138K alone (12, 16, 17). These data provide mechanistic insights into the favored emergence of E138K and M184I in patients failing RPV-containing regimens. However, the role of K101E and its interactions with other substitutions such as M184I and E138K, in resistance to RPV and other NNRTIs, has not been determined.

K101E is known to cause resistance to NVP and EFV (18, 19) and has been observed, usually in combination with other NNRTI resistance substitutions, in patients failing therapy (20, 21). K101E can also be a transmitted minority variant in acute HIV-1 infection (22) and may compromise the treatment efficacy of NNRTI-containing regimens. The addition of K101E to other NNRTI substitutions such as K103N or G190S can increase levels of resistance to EFV (19, 20). K101E, together with M184I, resulted in a diminution in susceptibility to RPV (12). Although the salt bridge between amino acid K101 of the p66 subunit of RT and E138 in the p51 subunit is involved in NNRTI binding (15, 16, 23), it is not known how the double mutant K101E/E138K might impact NNRTI susceptibility. The study of interactions between K101E and other resistance substitutions in RT is the topic of this manuscript.

MATERIALS AND METHODS

Chemicals, cells and nucleic acids

ETR and RPV were gifts of Janssen Pharmaceuticals (Titusville, NJ). FTC was kindly provided by Gilead Sciences (Foster City, CA). 3TC was a gift of Glaxo-SmithKline (Greenford, UK).
Cord blood mononuclear cells (CBMCs) were obtained through the Department of Obstetrics, Jewish General Hospital, Montreal, Canada. The HEK293T cell line was obtained from the American Type Culture Collection (ATCC). The following reagents and cells were obtained through the NIH AIDS Research and Reference Reagent Program: the infectious molecular clone pNL4-3 from Dr. Malcolm Martin; the TZM-bl (JC53-bl) cell from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.

The pNL4.3PFB plasmid DNA was a generous gift from Tomozumi Imamichi, National Institutes of Health, Bethesda, MD. The plasmid pRT6H-PROT was a generous gift from Stuart F. J. Le Grice, National Institutes of Health, Bethesda, Maryland, USA.

An HIV-1 RNA template, ~500 nucleotides (nt) in size, spanning the 5’ untranslated region (UTR) to the primer binding site (PBS), was transcribed in vitro from AccI-linearized pHIV-PBS DNA (24) by using an Ambion T7-MEGAshortscript kit (Invitrogen, Burlington, ON, Canada) as described previously (25). The oligonucleotides used in this study were synthesized by Integrated DNA Technologies Inc. (Coralville, IA) and purified by polyacrylamide-urea gel electrophoresis. For 5’-end labeling of oligonucleotides with \( {\gamma}^{32} \)P]ATP, the Ambion KinaseMax kit was used followed by purification through Ambion NucAway spin columns, according to protocols provided by the supplier (Invitrogen, Burlington, ON, Canada).

**Site-directed mutagenesis and preparation of virus stocks**

To construct HIV-1 RT expression plasmids and recombinant HIV-1 clonal variants harboring desired mutations in the RT gene, site-directed mutagenesis reactions (SDMs) were first carried out using a QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) on an HIV-1 RT expression plasmid pRT6H-PROT. Then fragments spanning RT amino acids (aa) 25-
314 were generated by PCR and were used to substitute for corresponding RT fragments in pNL4.3PFB plasmid DNA (26) as described previously (25), so as to generate recombinant HIV-1 viruses containing the desired RT mutations. DNA sequencing was performed to verify the absence of spurious mutations and the presence of any desired mutation. Recombinant HIV-1 wild-type and mutant viruses were generated by transfection of the corresponding proviral plasmid DNAs into HEK293T cells using Lipofectamine 2000 (Invitrogen, Burlington, ON) according to manufacturer’s instructions. Viral supernatants were harvested at 48 hours post-transfection, centrifuged for 5 minutes 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 by a Perkin-Elmer HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit. Virion-associated reverse transcriptase (RT) activity was verified by in vitro recombinant RT assay as described previously (27).

Selection of HIV-1 mutants in CBMCs under drug selection pressure.

CBMCs stimulated by Phytohemagglutinin A (PHA) were isolated and cultured as described (28) in 10% RPMI 1640 medium supplemented with 10% qualified FBS, 20 U of human interleukin-2 (IL-2)/ml, 5 µg of hydrocortisone/ml, 2 mM L-glutamine/ml, 100 U of penicillin/ml, and 100 µg of streptomycin/ml. Cells in 24-well tissue culture plates were infected with recombinant viral clones at a similar multiplicity of infection (moi). Selection of viral resistance mutations was performed using increasing concentrations of RT inhibitors at starting concentrations below the 50% effective concentration (EC50) as described previously (28, 29). Virus-containing culture media were harvested and kept at −80°C for subsequent standard genotypic analysis by population sequencing. Selections for resistance were performed over a period of 19 weeks.

Analysis of phenotypic drug susceptibility using the Monogram PhenoSense HIV RT assay
Resistance test vectors (RTVs) containing HIV-1 sequences containing protease and the first 305 amino acids of RT were constructed and used to evaluate NRTI and NNRTI susceptibility as previously described (19). Pseudotyped viruses were produced by co-transfecting HEK 293 cell cultures with RTV plasmid DNA, together with an expression plasmid encoding the Env protein of amphotropic murine leukemia virus. RTVs contain a luciferase reporter gene to monitor recombinant virus infection in cell culture following a single-cycle of replication. Results are expressed as the fold-change (FC) in the EC50 (50% effective concentration) for the mutant variants compared to a wild type reference virus (NL4-3). Assay techniques have been optimized to minimize variability and assay performance has been extensively validated. Several modifications have further enhanced assay performance (30).

Measurements of HIV-1 replication kinetics in CBMCs.

CBMCs were isolated and cultured as previously described (28). Recombinant wild type viruses and viruses containing desired mutations were normalized by p24 in order to minimize inter-inoculum effects as described (31). Briefly, 2 x 10^6 CBMCs were infected with viruses containing 8 x 10^6 pg of p24 for 2 hours. The cells were washed with medium and resuspended in 4 ml complete medium after centrifugation and split into 2 wells for each sample of a 12-well plate. The replication kinetics of mutant and WT viral stocks were assessed on the basis of p24 levels in culture supernatants sampled at days 1, 3, 4, 7, and 8 post-infection, viral growth kinetics were monitored by p24 levels as measured by ELISA as described above.

Recombinant reverse transcriptase expression and purification.

Recombinant RTs in heterodimeric form were expressed from plasmid pbRT6H-PROT (25) and
purified as described (32, 33) with minor modifications. In brief, RT expression in *Escherichia coli* M15 (pREP4) (Qiagen, Mississauga, ON) was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at room temperature. Pelleted bacteria were lysed under native conditions with BugBuster protein extraction reagent containing Benzonase (Novagen, Madison, WI) according to the manufacturer’s instructions. After clarification by high speed centrifugation, the clear supernatant was subjected to the batch method of Ni-nitrilotriacetic acid (NTA) metal affinity chromatography (QIAexpressionist; Qiagen, Mississauga, ON, Canada). All buffers contained complete protease inhibitor cocktail (Roche, Mississauga, ON, Canada). Hexahistidine-tagged RT was eluted using an imidazole gradient. RT-containing fractions were pooled, passed through DEAE–Sepharose (GE Healthcare, Mississauga, ON, Canada), and further purified using SP–Sepharose (GE Healthcare, Mississauga, ON). Fractions containing purified RT were pooled, dialyzed against storage buffer (50 mM Tris-HCl [pH 7.8], 50 mM NaCl and 50% glycerol), and concentrated to 4-8 mg/ml with Centricon Plus-20 MWC030 kDa (Millipore, Etobicoke, ON, Canada). Aliquots of proteins were stored at -80°C. Protein concentration was measured by a Bradford protein assay kit (Bio-Rad Laboratories, Saint-Laurent, QC, Canada), and the purity of the recombinant protein preparations was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The RNA-dependent DNA polymerase activity of each recombinant RT preparation was evaluated as described (34) using various concentrations of RT and a synthetic homopolymeric poly(rA)/p(dT)12-18 template/primer (T/P) (Midland Certified Reagent Company, Midland, TX).

**Enzyme processivity assays.**

The processivities of recombinant RT enzymes were analyzed as described previously using heteropolymeric HIV-1 PBS RNA template in the presence of a heparin enzyme trap to ensure a
single processive cycle, i.e., a single round of binding and of primer extension and dissociation (14). The template-primer (T/P) was prepared by annealing the HIV-1 PBS RNA with the $^{32}$P-5’ end-labeled 25-nt DNA primer D25 at a molar ratio of 1:1, denatured at 85 °C for 5 min, and then slowly cooled to room temperature to allow for specific annealing of primer to the template. RT enzymes with equal amounts of activity and 40 nM T/P were pre-incubated for 5 min at 37°C in a buffer containing 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 6 mM MgCl$_2$. Reactions were initiated by the addition of dNTPs and heparin trap (final concentration 3.2 mg/ml) and incubated at 37°C for 30 min; then, 2 volumes of stop solution (90% formamide, 10 mM EDTA, and 0.1% each of xylene cyanol and bromophenol blue) were added to stop the reaction. Reaction products were denatured by heating at 95 °C and analyzed using 6% denaturing polyacrylamide gel electrophoresis and phosphorimaging. The effectiveness of the heparin trap was verified in control reactions in which the trap was pre-incubated with substrate before the addition of RT enzymes and dNTP.

RNA-dependent DNA polymerase activity assay.

The same HIV-1 PBS RNA template and 5’end $^{32}$P-labeled D25 primer described above were used to assess primer extension efficiency in processive DNA synthesis by recombinant RT enzymes in gel-based time-course experiments (13, 14). Final reaction mixtures contained 20 nM T/P, 400 nM RT enzyme, 50 mM Tris-HCl (pH 7.8), and 50 mM NaCl. Reactions were initiated by adding 6mM MgCl$_2$ and dNTP at 200 μM, and mixed with 2 volumes of stop solution at various time points. Reaction products were separated by 6% denaturing polyacrylamide gel electrophoresis and analyzed by phosphorimaging.

RT-catalyzed RNase H Activity
RNase H activity was assayed using a 5'-end $^{32}$P-labeled 40-mer RNA template kim40R annealed to a complementary 32-mer DNA primer kim32D(35) at a 1:4 molar ratio. Reactions were conducted as described(13, 14, 36) 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, 6 mM MgCl$_2$ in the absence or presence of 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 time points 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 by 6% polyacrylamide-7M urea gels. The dried gels were exposed to phosphoscreens and analyzed by PhosphorImager (GE Healthcare, Mississauga, ON) using 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 $^{32}$P-RNA/DNA hybrid duplex substrate and magnesium in the same assay buffer as described above.

RESULTS

Selection of K101E by NNRTIs in CBMCs

K101E has been observed as a minority NNRTI substitution, generally in combination with other known NNRTI resistance substitutions, in patients failing NVP and EFV containing regimens (20, 21). Recently, K101E was also reported to be the second most frequent NNRTI substitution, together with E138K (usually accompanied by M184I), in patients with virologic failure during treatment with RPV, although combinations of K101E/M184I and K101E/M184V/ and other
substitutions were also observed (12). To verify whether K101E can be independently selected by NNRTIs, we performed tissue culture selection experiments using CBMCs in the presence of two DAPY compounds, ETR and DPV, as well as EFV. Table 1 shows that K101E emerged following selection with all three compounds, alone or in combination with NRTIs. In some instances, K101E emerged after 13 weeks of ETR/FTC pressure but was eventually outgrown by viruses containing E138K by week 19, suggesting that these two substitutions might be exclusive or antagonistic. In the ECHO and THRIVE trials, K101E and 138K were observed together only in the presence of M184I/V and other substitutions. Since exclusion/antagonism between these two substitutions may be related to viral replication capacity, and/or enhanced drug susceptibility, we performed biochemical and virological analyses to evaluate this relationship.

Phenotypic drug susceptibilities determined by the Monogram PhenoSense HIV assay

The drug susceptibility of recombinant HIV-1 viruses containing specific mutations were determined and compared to those of a wild type reference virus using the Monogram PhenoSense HIV assay (19, 30). Susceptibility to five NNRTIs and 7 NRTIs was evaluated (Table 2). The K101E substitution conferred large reductions in susceptibility to NVP and DLV while more modest reductions in susceptibility to EFV, ETR or RPV were observed. In contrast, the E138K substitution did not confer large reductions in susceptibility to any of the NNRTIs and only small reductions in susceptibility to ETR and RPV.

The addition of E138K to K101E did not further reduce susceptibility to RPV, ETR, or EFV susceptibility compared to the reductions conferred by K101E alone, yet notably restored susceptibility to NVP and DLV. Combinations of E138K/M184I or K101E/E138K/M184I comparably confer very modest and comparable reductions in NNRTI susceptibility. These
findings suggest that the salt bridge between residues K101 and E138 (15, 16) can play a different role in regard to resistance against NVP/DLV and ETR/RPV.

In addition, we verified that the double mutant E138K/M184I exhibited slightly larger reductions in ETR and RPV susceptibility compared to the E138K single mutant, in agreement with previous findings that M184I enhances ETR/RPV resistance in the background of E138K (16, 17). It was recently reported that K101E in the presence of M184I conferred a further reduction in RPV susceptibility compared to K101E alone (12) and in the present study, we found that the susceptibility to RPV, ETR, EFV and all NRTTIs of the K101E/E138K/M184I triple mutant was comparable to the E138K/M184I double mutant.

The K101E substitution compensates for the impaired viral replication capacity of HIV-1 containing M184I

We previously showed that the E138K substitution can restore the replication capacity of HIV-1 that harbors the M184I substitution in a short-term viral replication assay in TZM-bl cells (14). Now, we wished to investigate the impact of interactions between M184I and K101E and between E138K and K101E on viral replication over multiple cycles in CBMCs that are more physiologically relevant in regard to HIV-1 infection than cell lines. Therefore, we infected CBMCs with viruses that were normalized in terms of inoculum on the basis of amount of p24 (37). Quantification of virus production at various time points was carried out by measurement of p24 antigen, and some samples were also tested for RT activity (37, 38). As shown in Figure 1, the relative replication ability of viruses containing M184I alone was severely impaired compared to WT while the replication capacity of the E138K/M184I double mutant virus was restored to WT levels, in agreement with previously published data (14), (17). An even more pronounced
A compensatory effect on viral replication capacity was observed with K101E/M184I doubly mutated viruses, indicating that K101E can function together with M184I to restore viral fitness. The K101E/E138K double mutant virus replicated faster than either WT or mutated K101E/M184I viruses. In contrast, viruses containing K101E alone replicated less efficiently than WT virus as assessed by p24 antigen but attained a peak of replication at the same time as did WT virus, in agreement with the observation that K101E impaired replication fitness (39). These findings are also in agreement with the enzymatic data described below which show that K101E, like E138K, can function as a compensatory substitution for M184I in regard to RT enzymatic fitness.

**Activity of recombinant HIV-1 RT enzymes**

Amino acid residues K101 in the p66 subunit of HIV-1 RT and E138K in the p51 subunit constitute the floor of the NNRTI binding pocket (40-42) at the p66/p51 interface. It was previously shown that E138K alone and E138K/M184I in tandem did not interfere with either heterodimer formation or enzyme purification (14, 43, 44). However, it is possible that the addition of K101E to E138 alone or with M184I in tandem might have negative effects. Therefore, WT enzymes as well as four recombinant heterodimeric (p66/p51) RT enzymes containing K101E substitutions in both RT subunits, i.e., K101E, K101E/M184I, K101E/E138K, and K101E/E138K/M184I, and E138K/M184I were purified to >95% homogeneity as demonstrated by Coomassie blue staining in SDS-PAGE gels (data not shown). We also purified enzymes that contained these substitutions together with M184I or E138K/M184I. The RT p66 and p51 subunits were processed to similar molar ratios in each case, verifying that the substitutions did not affect proteolytic cleavage, p66/p51 heterodimer formation, or RT enzyme purification.
The RT preparations were titrated by standard RNA-dependent DNA polymerase activity assay (25, 34) and all of the mutant RTs showed activities similar to that of WT RT (data not shown). These results validate the quality of the recombinant RT preparations used for biochemical analyses.

**The K101E substitution restores the enzyme processivity of RT containing M184I**

HIV-1 RT drug resistance substitutions, especially those conferring resistance to NRTIs, can often affect RT enzymatic fitness by decreasing enzyme processivity (45-47) (i.e. the number of nucleotides incorporated in a single round of enzyme binding, elongation, and dissociation). Earlier studies showed that diminished HIV-1 RT processivity is a major determinant of the impaired viral replication capacity that is associated with the M184I/V substitutions, especially at low dNTP concentrations (45, 48). Previous reports also showed that E138K can compensate for the replication capacity deficit of viruses containing either M184I or M184V by restoring RT enzyme processivity (13, 14) but that the Y181C substitution in RT can diminish the processivity of E138K-containing RT. Indeed, interactions among different drug resistance substitutions, whether compensatory or antagonistic, may constitute the molecular basis for the occurrence of certain patterns of drug resistance. Now, we wished to determine how the K101E substitution, alone or in combination with M184I and/or E138K, might affect RT processivity. Therefore, we performed single-cycle processivity assays using recombinant RT enzymes at both low (0.5 μM) and high (200 μM) dNTP concentrations as previously described (13, 14). The results of these assays at low dNTP concentration showed that K101E-containing mutant enzymes, in combination with M184I or E138K/M184I, had higher processivity than M184I alone, indicating that K101E, like E138K, can compensate for the diminished enzyme processivity associated with M184I (Figure 2). Furthermore, the K101E substitution, alone or in combination with E138K, did not display diminished processivity compared to WT. At high dNTP concentrations, all enzymes...
displayed similar processivities based on the production of full-length DNA products (Figure 2). This is in agreement with previous studies that the processivity defect of the M184I RT mutant is minimized at high dNTP concentrations (14, 45, 49). These results imply that K101E is able to function as a compensatory substitution for M184I, and are confirmed in the cell-based replication capacity assays presented below.

**Impact of K101E on the efficiency of processive DNA synthesis**

Cell culture passage experiments have shown that the K101E and E138K substitutions are antagonistic, unless other substitutions such as M184I are simultaneously present and, in the ECHO and THRIVE clinical trials, K101E and M184I were often observed together. Previously, it was shown that E138K can impair the efficiency of DNA polymerization in a dNTP concentration-dependent manner (14). At high dNTP concentrations, E138K-containing RT possessed diminished efficiency of DNA polymerization, while M184I was able to compensate for the E138K deficit in polymerization. At low dNTP concentrations, E138K was able to restore the efficiency of polymerization of M184I. These mutually compensatory effects constitute the molecular basis for competent replication of doubly mutated E138K/M184I viruses at variable dNTP concentrations, i.e. in cells with both large and low dNTP pools. Now, we wished to determine how the K101E substitution, in a background of WT, E138K and/or M184I, might impact the efficiency of processive DNA synthesis.

We first performed RNA-dependent DNA polymerase reactions using all of the single and combination mutated enzymes in comparison with WT RT in time-course experiments at high dNTP concentrations (200 μM) (Figure 3A). RT molecules were used at ~20-fold excess over substrate, so that any RTs that dissociated from the primer terminus during synthesis would be rapidly replaced. In this case, the rate-limiting step would be nt addition (49). The efficiency of
DNA polymerization was assessed and compared in terms of length of the longest extension products (indicated by arrows) that were synthesized after 1 min (Figure 3A). E138K RT was included as a control in regard to diminished efficiency of DNA polymerization. The data of Table 3A show that K101E exhibited diminished efficiency of DNA polymerization as did E138K at high dNTP concentrations, while the doubly mutated RT enzymes K101E/E138K and K101E/M184I showed the same efficiency of DNA polymerization as did the WT enzyme. These results indicate that the diminished efficiency of DNA polymerization associated with K101E contributes to the impaired replication capacity of mutated HIV-1 in CBMCs and that E138K like M184I can rescue the efficiency of DNA polymerization.

In addition, E138K can compensate, at low dNTP concentrations, for the diminished polymerization efficiency of M184I RT (25), which is defective in regard to dNTP usage (45, 49), even though E138K, on its own, possesses diminished catalytic efficiency compared to WT at high dNTP concentrations. Now, we evaluated how K101E, alone or in combination with M184I and/or E138K, might impact the efficiency of DNA polymerization at low dNTP concentrations. The data of Figure 4B show that K101E RT displayed similar efficiency of DNA polymerization under these conditions as did WT enzyme, and that the addition of E138K to K101E or E138K/M184I to K101E enhanced DNA polymerization, as demonstrated by the presence of longer extension products after 4 min (Figure 3B).

RNase H activity

As stated, E138K in HIV RT impairs RNase H activity and contributes, in combination with diminished efficiency in processive DNA synthesis, to reductions in viral replication capacity (13, 14). Now we wished to test the impact of the K101E substitution alone or in combination with M184I on RNase H activity. Therefore, we performed an RNase H time course study in the
presence of 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 (Figure 4). The presence of the heparin trap permitted the analysis of cleaved products from a single binding event of RT to the substrate. The results show that the RNase H activity of the K101E mutant RT was comparable to that of WT RT and that the addition of the M184I or E138K substitutions did not impair the RNase H activity of K101E RT (Figure 4). Thus, the K101E substitution does not impair RNase H activity, despite the fact that it results in a deficit in efficiency of polymerization as shown above.

**DISCUSSION**

RPV and ETR are compounds that can bind efficiently to the NNRTI-BP, and possess activity against HIV-1 variants that are resistant to earlier NNRTIs such as NVP, DLV and EFV(10, 50, 51). The once-daily fixed-dose combination of RPV/FTC/TFV has provided an additional option for management of HIV-1 infection in treatment-naive patients (52, 53). Most data sets regarding RPV resistance are based on the ECHO and THRIVE clinical studies, and recent work has focused on the RT enzymatic properties and replication capacity of viruses resistant to RPV (12, 50) as well as interactions between the E138K and M184I/V substitutions (13-17).

The ECHO and THRIVE clinical trials also showed that K101E was the second most frequent substitution in patients who failed RPV/FTC/TDF(12). Here, we have shown that K101E can be selected in cell culture by each of ETR, DPV and EFV and that K101E can restore the enzyme processivity of RT and the viral replication capacity of viruses containing the M184I substitution. Thus, doubly mutated K101E/M184I viruses are competent in regard to replication capacity while displaying resistance to each of FTC, 3TC, RPV and ETR.
Both K101E and E138K emerged in cell culture selection experiments with RPV and, interestingly, K101E was frequently observed together with Y181C (50). In contrast antagonism seems to exist between E138K and Y181C and the mechanisms involved have been reported (54, 55). In the present study, we have compared the effects of the K101E and E138K substitutions on drug susceptibility using the Monogram PhenoSense HIV RT assay. Our results confirm that K101E and E138K possess different NNRTI resistance profiles; K101E confers high-level resistance to the earlier NNRTIs NVP and DLV but only low-level resistance to EFV; K101E also confers higher-level resistance to ETR and RPV than EFV. In contrast, E138K-containing virus is susceptible to NVP, modestly resistant to DLV and EFV, and confers resistance against the newer NNRTIs ETR and RPV. We have shown that a K101E/E138K double mutant can restore susceptibility to NVP and DLV, compared to K101E alone, without affecting resistance against RPV and ETR. This is the first demonstration that resistance to a NNRTI can be reversed by the presence of a different NNRTI resistance mutation.

The salt bridge between K101 of the p66 subunit and E138 of the p51 subunit of RT has been shown to be important for NNRTI binding by structural (23) and molecular modelling studies (15, 16). Recently, it was shown that E138K, which disrupts the salt bridge between p51E138 and p66K101, can change the dynamics of the entrance of the RPV-binding pocket due to the proximity of these two positive residues. Quantum mechanics and as well as biochemical analysis molecular modeling have shown that E138K in p51 affects access to the RPV binding site by disrupting this salt bridge (15).

This raises the question whether the salt bridge has the potential to reform within RT when both the K101E and E138K substitutions are present, even though such doubly mutated viruses are still drug resistant, as shown here.
Ideally, this issue could be further explored through molecular modelling analysis, that is beyond the scope of this manuscript. However, it is worth pointing out that two groups have published different versions of the RT-RPV complex (56, 57), which may make it difficult to probe the impact of changes within the salt bridge on RPV binding affinity. Transit kinetic analysis has demonstrated that the mechanism of RPV resistance is largely due to an increase in the dissociation rate of RPV and RT rather than a direct decrease in binding affinity (15). The use of such methodology may provide further insights into mechanisms of resistance associated with the K101E and E138K substitutions.

Mechanisms of resistance to NNRTIs are often not as clear as those against NRTIs(58-63). The current data show that the disrupted salt bridge involving K101E and E138K might impact differentially on drug binding, based on the NNRTI involved. Further detailed structural and biochemical analysis of this salt bridge in NNRTI binding and resistance is warranted.

However, our biochemical and virological data do not explain why K101E occurs less often than E138K in patients who have failed RPV/TDF/FTC, especially since the replication capacity of doubly mutated K101E/M184I viruses was higher than that of E138K/M184I viruses and compensation in regard to enzyme processivity and efficiency of DNA polymerization was observed in both cases. The drug susceptibilities of these two doubly mutated viruses were also similar. One possibility might be that K101E derives from an A to G substitution (AAA to GAA), while E138K results from the G to A hypermutation (GAG to AAG), which also explains the prevalence of K138 over other substitutions at position E138 (64). Recently, deep sequencing analysis of patients who suffered from RPV virologic failure in the Phase III studies ECHO and THRIVE showed that K101E and E138K rarely occurred on the same genome (65). However, no natural antagonism seems to exist between K101E and E138K; thus, the occurrence of other mutational pairs associated with NNRTI resistance such as Y181C and E138K may be in part a
matter of chance in the context of RT enzymes that are functional and commensurate with viral replication capacity (55). In conclusion, enzymatic and cell-based assays show that K101E can play a significant role in resistance to RPV.

Acknowledgments

This work was supported by research grants from the Canadian Institutes of Health Research (CIHR).

We thank Stuart Le Grice for providing pRT6H-PROT DNA, and Tomozumi Imamichi for pNL4.3PFB plasmid DNA.
References


FIGURE LEGENDS

FIGURE 1. Replication kinetics in CBMCs of wild-type (WT) and mutant viruses. Viruses were harvested from transfection of HEK293T cells and were used to infect CBMCs through normalization of the p24 antigen level of the inoculum. Virus production was monitored at the indicated time points by measuring p24 levels in cell-free supernatants by ELISA. Each experiment was performed in triplicate. The Figure is representative of two independent experiments.

FIGURE 2. Comparative analysis of enzyme processivity of wild-type (WT) RT and RT enzymes containing the indicated substitutions. The processivity of purified recombinant RT enzymes was analyzed using 5'-end labelled DNA primer (D25) annealed to a 471-nt HIV-1 PBS RNA template as substrate; the resulting full-length DNA (FL DNA) is 471 nt in length. Processivities were determined by the size distribution of DNA products in fixed-time experiments at low (0.5 µM) and high (200µM) concentrations of dNTPs in the presence of heparin trap. All reaction products were resolved by denaturing 6% polyacrylamide gel electrophoresis and visualized by phosphor imaging. Positions of 32P-labeled D25 primer (32P-D25) and the 471-nt full-length extension DNA product are indicated on the right. Lanes: 1, WT; 2, M184I; 3, K101E/M184I; 4, E138K/M184I; 5, K101E/E138K/M184I; 6, K101E; 7, K101E/E138K. A control reaction to verify the efficiency of the heparin trap by preincubation with substrate prior to addition of WT RT is also shown (Lane: C). Each experiment was repeated at least twice and yielded similar results on each occasion. The Figure shows a gel from a representative experiment.
FIGURE 3. Analysis of efficiency of processive DNA synthesis.

A) Comparative analysis of enzyme efficiency of processive DNA synthesis of HIV-1 wild-type (WT) RT and mutant RT enzymes at high dNTP concentrations (200 μM). The 5’-end ³²P-labeled D25 primer (³²P-D25) was annealed to the 471-nt HIV-1 PBS RNA template, and primer extension assays were performed at an excess of recombinant RT enzymes at high dNTP concentrations (200 μM). Reactions were stopped at 0.1 min, 1 min, and 6 min, respectively. The longest extension products generated at 1 min were identified by arrows and indicate differences in efficiency of polymerization. Each experiment was repeated at least twice and yielded similar results on each occasion. The Figure shows a gel from a representative experiment.

B) Comparative analysis of enzyme efficiency of processive DNA synthesis of HIV-1 wild-type (WT) RT and mutant RT enzymes at low dNTP concentrations (0.5 μM). Reactions were stopped at 2 min and 4 min, respectively. The longest extension products generated at 4 min were identified by arrows and indicate differences in efficiency of polymerization. Each experiment was repeated at least twice and yielded similar results on each occasion. The Figure shows a gel from a representative experiment.

FIGURE 4. RNase H activity of wild-type (WT) and mutant recombinant RT enzymes. (A) Graphic representation of the RNA/DNA (kim40R/kim32D) substrate duplex used to monitor the cleavage efficiency of mutant and WT RTs. The 40-mer RNA kim40R was labelled at its 5’-terminus by ³²P and annealed to 32-mer DNA oligo kim32D. The positions of RNase H cleavage relative to the 3’-terminus of the DNA primer are indicated by arrows. (B) RNase H activity was analyzed by monitoring substrate cleavage in time-course experiments. The time points were 0.2, 0.5, 1, 2, 6, 8, 10, and 16 min, respectively. The time point 0 shows the uncleaved substrate in a control reaction without RT enzyme. The positions of cleaved products relative to the 3’-terminus...
of the DNA primer are indicated on the left. All reactions were resolved by denaturing 6% polyacrylamide gel electrophoresis. Each experiment was repeated at least twice and yielded similar results on each occasion. The Figure shows a gel from a representative experiment.
TABLE 1. Summary of emergent substitutions in selection experiments using CBMCs

<table>
<thead>
<tr>
<th>Virus⁵</th>
<th>Subtype</th>
<th>Backbone Mutations</th>
<th>Drug(s)</th>
<th>Selected Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>8336</td>
<td>B</td>
<td>A98S, G190A</td>
<td>EFV</td>
<td>K101E</td>
</tr>
<tr>
<td>NL₄³ WT</td>
<td>B</td>
<td>None</td>
<td>EFV</td>
<td>K101E, V108I</td>
</tr>
<tr>
<td>10583</td>
<td>C</td>
<td>Y181C</td>
<td>EFV</td>
<td>K101E/K, V106M, Y181C</td>
</tr>
<tr>
<td>8116</td>
<td>B</td>
<td>A98S, G190A</td>
<td>DPV</td>
<td>K101E, Y181C</td>
</tr>
<tr>
<td>NL₄³-Y181C</td>
<td>B</td>
<td>Y181C</td>
<td>DPV</td>
<td>K101E, V179I, Y181C</td>
</tr>
<tr>
<td>10583</td>
<td>C</td>
<td>Y181C</td>
<td>DPV/TDF</td>
<td>K101K/E, V108I/V, V179I/V, Y181C, H221Y</td>
</tr>
<tr>
<td>Mole 03⁶</td>
<td>C</td>
<td>None</td>
<td>ETR/FTC</td>
<td>K101E/K, E138E/K</td>
</tr>
<tr>
<td>Mole 03⁶</td>
<td>C</td>
<td>None</td>
<td>ETR/FTC</td>
<td>E138K, M184I.</td>
</tr>
<tr>
<td>Mole 03</td>
<td>C</td>
<td>None</td>
<td>ETR/3TC</td>
<td>K101E, M184I</td>
</tr>
</tbody>
</table>

⁵Selection experiments were performed over 19 weeks. Abbreviations: 3TC, lamivudine; DPV, dapivirine; EFV, efavirenz; ETR, etravirine; FTC, emtricitabine;

⁶Viruses other than NL₄³ clones are clinical isolates from treatment-naive patients undergoing acute HIV-1 infection.
Viruses were genotyped at week 13.

Viruses were genotyped at week 19.
TABLE 2. Changes in drug susceptibilities for recombinant HIV-1 wild type and site-directed mutants containing various substitutions compared to wt NL4.3 as assessed by the Monogram PhenoSense RT assay\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>RPV</th>
<th>ETR</th>
<th>ETV</th>
<th>NVP</th>
<th>DLV</th>
<th>ZDV</th>
<th>D4T</th>
<th>3TC</th>
<th>FTC</th>
<th>DDI</th>
<th>ABC</th>
<th>TDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>K101E</td>
<td>3.0</td>
<td>4.9</td>
<td>2.1</td>
<td>170</td>
<td>50.8</td>
<td>0.4</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>E138K</td>
<td>2.3</td>
<td>2.5</td>
<td>1.3</td>
<td>0.9</td>
<td>1.6</td>
<td>3.8</td>
<td>1.4</td>
<td>1.5</td>
<td>1.4</td>
<td>1.2</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>K101E/E138K</td>
<td>3.2</td>
<td>3.1</td>
<td>2.1</td>
<td>3.3</td>
<td>3.0</td>
<td>1.2</td>
<td>1.4</td>
<td>1.2</td>
<td>1.4</td>
<td>1.1</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>E138K/M184I</td>
<td>3.2</td>
<td>3.3</td>
<td>1.6</td>
<td>0.8</td>
<td>1.8</td>
<td>0.75</td>
<td>1.6</td>
<td>&gt;103.8</td>
<td>&gt;75.0</td>
<td>2.4</td>
<td>3.2</td>
<td>0.8</td>
</tr>
<tr>
<td>K101E/E138K/M184I</td>
<td>3.3</td>
<td>3.2</td>
<td>1.9</td>
<td>2.0</td>
<td>2.6</td>
<td>0.5</td>
<td>1.4</td>
<td>&gt;105.8</td>
<td>&gt;75.0</td>
<td>2.2</td>
<td>2.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

\(^a\)Shown are fold-change (FC) EC\(_{50}\) (50% drug effective concentrations) values (nM) normalized to HIV-1 wild type.
A

Kim40R 5' -AAGCUUGGCUGCAGAAUAUUGCAGCGGGAAUUCGCGC-3'
Kim32D 3' -GACGTCTTATAACGATCGCCCTTAAGCCGCGC-5'

B

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>WT</th>
<th>K101E</th>
<th>K101E/M184I</th>
<th>K101E/E138K</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>