HIV-1 recombinant reverse transcriptase enzymes containing the G190A and Y181C resistance mutations remain sensitive to etravirine

Hongtao Xu¹, Yudong Quan¹, Bluma G. Brenner¹, Tamara Bar-Magen¹, Maureen Oliveira¹, Susan M Schader¹,², and Mark A. Wainberg¹,²*

¹McGill University AIDS Centre, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada and ²Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada*

Running title: Etravirine drug resistance

*Correspondence to: Mark A. Wainberg, McGill University AIDS Centre, Jewish General Hospital, 3755 Cote Ste Catherine Rd., Montreal, Quebec Canada H3T 1E2. Tel: +1 514 340 8260; Fax: +1 514 340 7537 (E-mail: mark.wainberg@mcgill.ca)
ABSTRACT

Etravirine (ETR) is a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI) active against common human immunodeficiency virus type-1 (HIV-1) drug-resistant strains. This study was designed to determine the extent to which each of the Y181C or G190A mutations in reverse transcriptase (RT) might confer resistance against ETR and other members of the NNRTI family of drugs. Recombinant HIV-1 RT enzymes containing either the Y181C, G190A, or both of these mutations in tandem were purified. Both RNA- and DNA-dependent DNA polymerase assays were performed in order to determine the extent to which each of the above mutations might confer resistance in cell-free biochemical assays against each of ETR, efavirenz, and nevirapine. Both the biochemical as well as cell-based phenotypic assays confirmed the susceptibility of G190A-containing enzymes and viruses to ETR. The results of this study indicate that the G190A mutation is not associated with resistance against ETR.
INTRODUCTION

Etravirine (ETR), (formerly known as TMC-125), is a second generation non-nucleoside reverse transcriptase inhibitor (NNRTI) that retains activity against human immunodeficiency virus type-1 (HIV-1) variants containing common resistance mutations conferring resistance to nevirapine (NVP). In particular, ETR is a highly flexible diarylpyrimidine compound, able to adapt its orientation and overcome common NNRTI-associated mutations, including K103N that is present in 40-60% of patients failing NNRTI-containing treatment regimens (10, 17). Furthermore, ETR displays a high genetic barrier for resistance, requiring the accumulation of several NNRTI-associated mutations for high-level resistance to become manifest (10, 14, 17, 18).

The DUET-1 and DUET-2 clinical trials have identified an array of 17 resistance-associated mutations (RAMs) that confer diminished sensitivity to ETR, including V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190A/S and M230L (3,4). Many of these mutations may represent pre-existing resistance in NNRTI-experienced patients, with Y181C + G190A (27%) and K101E + G190A (12.5%) being the most prevalent RAM combinations (14, 17, 18).

Further studies are needed to determine the individual and interactive roles of RAMs in conferring diminished sensitivity to ETR, as is evident from poor concordance among different genotypic interpretative algorithms in regard to the role that is played by individual mutations (1, 5, 6, 15). It is also important to accurately describe the mutations tolerated by ETR, i.e. mutations that result in no effect, reduced efficacy, or a lack of virological response.
To determine the role of G190A in ETR resistance, we expressed purified recombinant RT enzymes containing this mutation alone or in tandem with Y181C. The latter mutation was chosen because of previous reports that it confers resistance against each of NVP and EFV, and to a much lesser extent against ETR (15). As an additional control, we also studied dapivirine (DAP) (formerly known as TMC-120), a compound that has been licensed for possible development as a vaginal microbicide by Tibotec Pharmaceuticals to the International Partnership for Microbicides (IPM).
MATERIALS AND METHODS

Site-directed mutagenesis

The G190A, Y181C and G190A plus Y181C mutations were introduced into the subtype B HIV-1 RT heterodimer expression plasmid pRT6H_PROT, kindly provided by Dr. S.F. LeGrice (12), using a QuickChange II XL site-directed mutagenesis kit (Stratagene). DNA sequencing was performed in both directions across the entire reverse transcriptase (RT) coding region to verify the absence of spurious mutations and the presence of the desired mutation.

Purification of recombinant HIV-1 RTs and activity determination

Recombinant wild-type (WT) and mutated RTs were expressed and purified as described (11). Protein concentration was measured by Bradford Protein Assay kit (Bio-Rad Laboratories) and the purity of the recombinant RT preparations was verified by SDS-PAGE. Quantification of RT DNA polymerase activity was performed as described previously (16). An active unit of RT was defined as the amount of enzyme that incorporates 1 pmol of dTTP in 10 min at 37°C.

NNRTI inhibition of RNA-dependent DNA polymerase (RDDP) activity

These reactions were performed as previously reported (13, 16, 19). Briefly, RT reaction buffer containing 50 mM Tris (pH 7.8), 5 mM MgCl$_2$, 60 mM KC1, 10 mM DTT, 10 μM of dTTP with 2.5 μCi of $[^3H]$ dTTP (70-90 mCi/mM), 5 U of template/primer poly (rA)/(oligo(dT)$_{12-18}$ (Amersham), 5 U of recombinant RTs and variable amounts of the RT inhibitors, NVP, EFV, ETR, and DAP were included in 50 μl reaction volumes that were incubated at 37 °C for 30 min. Reactions were terminated with 0.2 ml of 10% cold trichloracetic acid (TCA) and 20 mM sodium pyrophosphate. After 30 min on ice, the precipitated products were filtered onto a 96-
well plate with glass fiber filters (Millipore) and sequentially washed with 10% TCA and 95% ethanol. The radioactivity of incorporated products was analyzed by liquid scintillation spectrometry. The IC$_{50}$ of each NNRTI was determined by curve fit analysis using GraphPad Prism4.0 software, version 7.

**NNRTI inhibition of DNA-dependent DNA polymerase activity**

The primer-template (ppt18-ppt57) substrates (P/T) used to study the inhibition of DNA synthesis by NNRTIs were derived from the polypurine tract (PPT) of the HIV-1 genome. The ppt18 primer was radiolabeled at its 5' end with $\gamma^{32}$P ATP and annealed to ppt57 template as described (8). Catalysis by WT and mutant RT enzymes was determined by measuring the extension of the labeled ppt18 primer on the ppt57 template. Each of the NNRTI compounds to be evaluated, including NVP, EFV, ETR and DAP, was serially diluted in 50% dimethylsulfoxide (DMSO). Reactions contained 150 nM labeled primer-template (calculated as the primer concentration), 5 units of recombinant RTs, 50 mM Tris (pH 7.8), 5 mM MgC$_{12}$, 60 mM KC1, 10 mM DTT, and 5% DMSO in a total volume of 20 µl. Initiation of the reaction was performed by adding 100 µM of each of the dNTPs excluding dATP, so that primer extension would be restricted to 4 nucleotides for better resolution and quantification. After 15 min at 37 ºC, equal volume of formamide sample buffer was added and heat-denatured samples were resolved in a 6% polyacrylamide-7M urea gel followed by phosphorimaging. Band intensities were analyzed by ImageQuant software (GE Healthcare). The IC$_{50}$ of each NNRTI was determined using GraphPad Prism 4.0 software from plots of percentages of the level of primer extension relative to inhibitor concentration.
Phenotypic analysis of in vitro NNRTI drug susceptibility of G190A-containing viruses

Three wild-type viruses and three viruses harboring G190A and A98S, obtained from our primary HIV infection (PHI) cohort, were amplified as previously described (3, 5). Drug susceptibility was measured in cell culture-based phenotypic assays to determine the extent to which the NNRTI drugs inhibited HIV replication in vitro. Briefly, cord blood mononuclear cells (CBMCs) were infected with different viral isolates and the 50% drug effective concentrations (EC₅₀) were ascertained for each of NVP, EFV, ETR, and DAP by monitoring for production of p24 antigen as previously described (3).
RESULTS

Purification of recombinant HIV-1 RT

Recombinant WT heterodimeric (p66/p51) RT and RT enzymes containing G190A, Y181C, and both the G190A plus Y181C substitutions were purified to >95% homogeneity; the RT subunits p66 and p51 were processed to similar molar ratios based on SDS-PAGE analysis (Fig.1). The mutations introduced into the recombinant HIV-1 RT did not interfere with either heterodimer formation or enzyme purification.

Inhibitory effects of NNRTIs determined by RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP) assays

The inhibitory effects of various NNRTIs on RDDP activity of HIV-1 WT and mutant RTs were measured by a filtration RT assay. Sensitivities of WT and mutant RTs were determined for each of NVP, EFV, ETR, and DAP. The results of Fig 2 show that each of the mutated RTs displayed high level resistance against NVP but that all of the enzymes remained fully susceptible to each of EFV, DAP, and ETR. The IC\textsubscript{50} values for each drug tested with each of the RTs are shown in Table 1.

The inhibitory effects of the NNRTIs on DDDP activity were measured using a gel-based primer extension assay (Fig 3A). Fig 3B presents representative gels showing dose-dependent inhibition of DNA polymerase activity by NNRTIs. In the case of NVP, only the results of the G190A and WT analyses are shown, since the other two mutant RTs were highly resistant to NVP. IC\textsubscript{50} values were determined and are summarized in Table 1. For ETR and DAP, differences in IC\textsubscript{50s} between WT and mutant RTs were less than 3-fold, indicating that these two drugs remained...
potent inhibitors of the mutated RTs. The IC$_{50}$ of NVP for all mutant RTs was increased by more than 100-fold relative to WT. Each of the Y181C and the Y181C/G190A double mutant RT were highly resistant to NVP. Y181C conferred only slight resistance (≈2-fold) against EFV while G190A RT and G190A plus Y181C RT were ≈12- and 21-fold resistant to EFV, respectively.

**Drug susceptibility of G190A and Y181C viruses determined by cell-based phenotypic assays**

Previous studies have shown that G190A is present in approximately 3% of treatment-naïve newly-infected persons (13). Three treatment-naïve viruses containing G190A were isolated and compared to WT viruses for susceptibility to NVP, EFV, ETR and DAP. Table 2 shows that viruses harboring G190A showed high-level resistance to NVP and low-level resistance to EFV, while remaining susceptible to ETR and DAP. Moreover, both moderate and extensive hypersensitivity of the A98S/G190A mutants to EFV and DAP, respectively, were observed in the cell-based phenotypic assay (Table 2).

The relative susceptibility of the Y181C mutation was also determined using recombinant single cycle assays using TZM-bl cells. As demonstrated in Fig. 4, Y181C viruses showed low-level ≈3-fold phenotypic resistance to ETR and ≈ 5-fold resistance to EFV.
DISCUSSION

ETR is a recently approved NNRTI that can overcome single point mutations such as K103N that confer cross-resistance to both NVP and EFV. In general the high genetic barrier of ETR requires the accumulation of more than three resistance-associated mutations in order for diminished drug efficacy to result, and the relative roles of each of these mutations in the development of such resistance are unclear. The present study was initiated to determine the extent to which the G190A and Y181C mutations might affect viral susceptibility to ETR and other NNRTIs. Our findings indicate that neither G190A nor Y181C can, on its own confer high-level resistance to ETR. We believe that these two mutations might currently be weighted inappropriately in drug resistance interpretation algorithms and that this might lead to ETR being potentially excluded from use in certain therapeutic situations.

HIV resistance to EFV and NVP is responsible for a high proportion of treatment failures, since single point mutations, such as K103N can confer very high-level resistance against these drugs. ETR has the distinct advantage of showing virological activity in patients who have failed NVP- and EFV-based regimens, despite the fact that their viruses commonly harbour such mutations as K103N. Although Y181C and G190A have been included as ETR mutations in genotypic resistance algorithms, our findings and those of others are of importance in showing that these two mutations confer only low-level resistance that border on clinical cutoff values (14). The potential of mutated viruses to remain susceptible to ETR is of clinical relevance given the high frequency of transmitted G190A resistance in drug-naïve subjects as well as the high frequency of Y181C in NVP-experienced individuals.
Previous data have shown that the Y181C mutation in RT is associated with partial resistance against ETR but is insufficient, on its own, to eliminate the antiviral activity of this compound (15). This result is confirmed in the present study on the basis of recombinant RT assays performed with RNA and DNA templates and through use of both filter- and gel-based assays. Our results also demonstrate that the G190A substitution in RT, while associated with resistance against each of NVP and EFV, does not confer resistance against either ETR or a related NNRTI termed DAP, that is currently undergoing consideration for possible development as an anti-HIV microbicide. Our results also demonstrate that the presence of both the Y181C and G190A substitutions in tandem resulted in higher level resistance against both NVP and EFV than either mutation acting alone. However, the simultaneous presence of both mutations did not significantly add to levels of resistance observed in regard to ETR and DAP obtained with either mutation as a single substitution.

It is also instructive that high-level Y181C-mediated resistance to ETR against NVP was documented with each of filter-based and gel-based reverse transcriptase assays that were performed with RNA and DNA templates, respectively. The use of DNA template also revealed resistance against EFV, but this result was not obtained with the RNA-dependent DNA polymerase assay. The fold-change of Y181C-mediated resistance to ETR in the DNA- and RNA-dependent polymerase assays was 2-fold and 1.35-fold respectively. This is consistent with our findings of ≈3-fold phenotypic resistance on the part of Y181C to ETR and ≈ 5-fold resistance to EFV, similar to data reported by others (14). It is also instructive that high-level Y181C-mediated resistance against NVP was documented with each of filter-based and gel-based reverse transcriptase assays that were performed with RNA and DNA templates, respectively. The use of DNA template also revealed resistance against EFV, but this result was
not obtained with the RNA-dependent DNA polymerase assay. This illustrates that RNA-based and DNA-based reverse transcriptase assays can sometimes yield qualitatively different results (2). Further studies should be performed with G190A together with the ETR-associated major mutation G190S that confers $\approx$8-23 fold resistance to ETR in association with Y181C (14).

Many of the clinical data sets regarding ETR resistance are based on the DUET studies, that involved highly treatment-experienced patients, including 14% who possessed $\approx$3- pre-existing NNRTI mutations (5). The frequency of NNRTI resistance polymorphisms at baseline may be different in treatment-naïve versus treatment-experienced individuals (1). ETR may demonstrate antiviral activity in treatment-naive individuals who harbor transmitted NNRTI resistance mutations, including K103N and G190A. As shown here in enzymatic and cell-based phenotypic assays, viruses harboring the G190A substitution retained susceptibility to both ETR and DAP.

Others have reported 3- and 3.3-fold resistance against ETR for Y181C and Y181C+G190A, respectively (14). Our cell culture phenotypic data show that Y181C and G190A show $\approx$3- and $\approx$5-fold resistance to ETR and EFV, respectively, as compared to >100-fold resistance to NVP. Data from the Stanford HIV-1 resistance database (http://hivdb.stanford.edu) show 21.5% and 3.6% comparative frequencies of Y181C in NVP- and EFV-experienced persons, respectively. This suggests that Y181C is a major NVP mutation but not for EFV.

On the basis of these data, we conclude that G190A and Y181C are not directly associated with resistance against ETR. Our data are also supported by separate analyses of ETR resistance involving Y181C and G190A in the DUET clinical study (Vingerhoets J, Clotet B, Peeters M, Picchio G, Tambuyzer L, Cao-Van K, De Smedt G, Woodfall B, de Béthune MP. Impact of
baseline NNRTI mutations on the virological response to TMC125 (etravirine; ETR) in the DUET-1 and DUET-2 Phase III clinical trials. 11th European AIDS Conference, Madrid, Spain, 24-27 October 2007. Abstract P7.3/05). This is not unexpected given the conformational flexibility of ETR and its ability to reorient when single mutations are present in the RT binding pocket (7). ETR with its high genetic barrier and low-level (≈3-fold) resistance may remain an NNRTI option for patients harbouring Y181C and G190A mutations, in the absence of other ETR-associated NNRTI mutations.

The relative weighting of resistance mutations has led to discordance among a variety of interpretative algorithms including those of ANRS, IAS-USA, Monogram Diagnostics and Virco Diagnostics, and, in some cases, has led to the exclusion of ETR as a potentially useful drug (1, 5, 9). Additional clinical data will be necessary to re-evaluate the role of certain mutations in regard to the durability of ETR-based therapy in NNRTI-experienced patients. In all likelihood, additional studies based on cell culture selections, enzymatic analysis, and site-directed mutagenesis performed with additional mutations may further define the drug resistance profile of ETR.
Acknowledgements

We thank Dr. Stuart Le Grice for providing the pRT6H-PROT DNA construct. This research was supported by grants from the Canadian Institutes of Health Research (CIHR), the Fonds de recherche en santé du Québec (FRSQ)-Réseau SIDA and the International Partnership for Microbicides.
References


Table 1. IC₅₀s of recombinant RTs for NNRTIs by RNA-dependant DNA polymerase (RDDP) filter-based assay and DNA-dependent DNA polymerase (DDDP) gel-based assay

<table>
<thead>
<tr>
<th>RTs</th>
<th>ETR (nM)</th>
<th>DAP (nM)</th>
<th>EFV(nM)</th>
<th>NVP(µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RDDP</td>
<td>DDDP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>175 ± 32</td>
<td>97 ± 13</td>
<td>18 ± 3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>G190A</td>
<td>191 ± 25</td>
<td>129 ± 22</td>
<td>216 ± 31</td>
<td>&gt;67</td>
</tr>
<tr>
<td>Y181C</td>
<td>257 ± 35</td>
<td>107 ± 16</td>
<td>36 ± 5</td>
<td>&gt;67</td>
</tr>
<tr>
<td>G190A/ Y181C</td>
<td>162 ± 10</td>
<td>279 ± 25</td>
<td>380 ± 43</td>
<td>&gt;67</td>
</tr>
</tbody>
</table>

Data represent the means ± SD of three separate determinations.

ETR, etravirine; DAP, daprivine; EFV, efavirenz; NVP, nevirapine.
Table 2. Susceptibilities of viruses harboring G190A to non-nucleoside reverse transcriptase inhibitors in cell-based phenotypic assays.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Drug Susceptibility (EC$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NVP</td>
</tr>
<tr>
<td>5269 (WT)</td>
<td>0.007</td>
</tr>
<tr>
<td>5331 (WT)</td>
<td>0.010</td>
</tr>
<tr>
<td>5512 (WT)</td>
<td>0.020</td>
</tr>
<tr>
<td>Mean</td>
<td>0.012 ± 0.003</td>
</tr>
<tr>
<td>8116 (A98S, G190A)</td>
<td>0.852 (71x)</td>
</tr>
<tr>
<td>8117 (A98S, G190A)</td>
<td>0.703 (58x)</td>
</tr>
<tr>
<td>9225 (A98S, G190A)</td>
<td>0.997 (83x)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.850 ± 0.084</td>
</tr>
</tbody>
</table>

Drug susceptibilities of wild-type viruses and resistant viruses harboring A98S and G190A were determined in two to three separate experiments. Overall susceptibilities of WT and G190A RT are expressed as mean ± SEM.

NVP, nevirapine; EFV, efavirenz; ETR, etravirine; DAP, daprivine.
**Figure Legends**

Fig. 1. Coomassie-Brilliant Blue staining of purified heterodimer RTs after purification on 8% SDS-PAGE. MW: molecular mass standards in kilo daltons are shown on the left. The positions of purified recombinant RT heterodimers (WT, G190A, Y181C, or both) are indicated on the right.

Fig. 2. Inhibition of RNA-dependent DNA polymerase RT activity by NNRTIs determined by filtration RT assay. The inhibition of wt and mutant RT activity was determined in the absence and presence of variable amounts of NVP, EFV, DAP, or ETV.

Fig. 3. Inhibition of DNA-dependent DNA polymerase RT activity by NNRTIs determined by gel-based RT assay. (A) Graphic representation of the primer-template system (ppt18-ppt57) used to monitor inhibition of HIV-1 RT DNA polymerase activity by NNRTIs. +1 and +4 indicate the positions of the first and last nucleotide incorporated respectively. (B) Dose-dependent inhibition of DNA polymerase activity by NNRTIs. Reactions were performed with increasing concentrations of NNRTIs. The positions of labelled primer (P) and the full-length extension product (+4) are indicated on the left. The concentrations of the NNRTIs used are as follows:

- ETR (0, 17, 26, 39, 58.5, 87.8, 131, 197.5, 296, 444, 666nM, 1µM, 10µM).
- DAP (0, 23, 34, 52, 78, 117, 175, 260, 395, 590, 888, 1330, 2000nM).
- EFV (0, 4, 8, 16, 32, 64, 128, 180, 250, 352, 493nM, 10µM, 100µM).
Fig. 4. The reverse transcriptase mutation Y181C confers ≈ 3-4 fold resistance to ETR in short-term replication assays. NNRTIs were diluted two-fold on TZM-bl cells 1 hour prior to infection with wild type (●) or Y181C (◊) HIV-1 derived from molecular clones. After 48 hours, cells were rinsed, lysed and luciferase activity was quantified. Data points depict the means and standard deviations of 2 independent experiments wherein each drug dilution was performed and analyzed in duplicate. Percent inhibition of wild type and Y181C virus replication is shown on the y-axis while the x axis denotes the dose of ETR (A) or EFV (B) tested. ETR concentrations required for half maximal inhibition (EC50) of wild-type and Y181C HIV-1 replication were determined as 8.53 (+/- 0.0134) nM and 33.5 (+/- 1.68) nM, respectively. EFV IC50 values were 106 (+/- 1.27) pM and 910 (+/- 2.62) pM for wild type and Y181C HIV-1, respectively. These values translate to approximately 4-fold resistance toward ETR and 9-fold resistance for EFV.
Fig. 1

<table>
<thead>
<tr>
<th>MW</th>
<th>WT</th>
<th>Y181C</th>
<th>G190A</th>
<th>181C/190A</th>
</tr>
</thead>
<tbody>
<tr>
<td>32.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>47.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>83</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>p66-His6</th>
</tr>
</thead>
<tbody>
<tr>
<td>p51</td>
</tr>
</tbody>
</table>

MW, WT, Y181C, G190A, 181C/190A, p66-His6, p51
Fig. 3

A

\[ \text{ppt18 5'} \quad TTAAAAGAAAAGGGGGG\text{Actgg} \quad 3' \]

\[ \text{ppt57 3'} \quad AGAATCGGTGAAAAATTTTCTTTTCCCCCTGACC TTCCCGATTAAGTGAGGGTTGC \quad 5' \]

B

<table>
<thead>
<tr>
<th>DRUG</th>
<th>WT</th>
<th>G190A</th>
<th>Y181C</th>
<th>G190A/Y181C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETR</td>
<td>+4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DAP</td>
<td>+4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EFV</td>
<td>+4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NVP</td>
<td>+4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 3
Figure 4.

A. B. 

% inhibition
0 25 50 75 100

ETR [nM]

wild type
Y181C

% inhibition
0 25 50 75 100

EFV [nM]

wild type
Y181C

ETR EFV