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NNRTI Hyper-Susceptibility and Resistance: Mutational Analysis of Residue 181 in HIV-1 Reverse Transcriptase

Running Title: **Mutational analysis of Y181 in RT**

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Substitutions at residue Y181 in HIV-1 reverse transcriptase (RT), in particular Y181C, Y181I and Y181V, are associated with nonnucleoside RT inhibitor (NNRTI) cross-resistance. In this study, we used kinetic and thermodynamic approaches, in addition to molecular modeling, to gain insight into the mechanisms by which these substitutions confer resistance to nevirapine (NVP), efavirenz (EFV) and rilpivirine (RPV). Using pre-steady-state kinetics, we found that the dissociation constant (K_d) values for inhibitor binding to the Y181C and Y181I RT-template/primer (T/P) complexes were significantly reduced. In the presence of saturating concentrations of inhibitor, the Y181C RT-T/P complex incorporated the next correct dNTP more efficiently than the WT complex, and this phenotype correlated with decreased mobility of the RT on the T/P substrate. Interestingly, we found that the Y181F substitution in RT – which represents a transitional mutation between Y181 and Y181I/V, or a partial revertant – conferred hyper-susceptibility to EFV and RPV at both the virus and enzyme levels. EFV and RPV bound more tightly to Y181F RT-T/P. Furthermore, inhibitor-bound Y181F RT-T/P was less efficient than the WT complex in incorporating the next correct dNTP, and this could be attributed to increased mobility of Y181F RT on the T/P substrate. Collectively, our data highlight the key role that Y181 in RT plays in NNRTI binding.

47 **INTRODUCTION**

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49 Nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) bind to HIV-1 RT in the NNRTI-
50 binding pocket (NNRTI-BP) and inhibit reverse transcription via an allosteric mechanism of
51 action. Five NNRTIs - nevirapine (NVP), efavirenz (EFV), etravirine (ETR), rilpivirine (RPV)
52 and doravirine - have been approved by the United States Food and Drug Administration and are
53 routinely implemented in the clinical setting to prevent or treat HIV-1 infection. The NNRTI-BP
54 resides in the DNA polymerase domain of HIV-1 RT and is located close to, but separate from,
55 the active site [1,2]. Key residues involved in NNRTI binding include L100, K101, K103, V106,
56 Y181, Y188, G190, F227, W229 and M230, and HIV-1 resistance to NNRTIs is typically
57 associated with mutations at one or more of these residues [3]. Substitutions at residue Y181, in
58 particular Y181C/I/V, confer NNRTI cross-resistance. Y181C is typically selected by NVP, ETR
59 or RPV [4,5,6] and imparts > 20-fold resistance to NVP, and 2- to 5-fold resistance to EFV, RPV
60 and ETR. Y181I/V are 2-base pair nonpolymorphic mutations that confer >50-fold resistance to
61 NVP and 5- to 20-fold resistance to ETR and RPV [4, 7-9]. Interestingly, in the Stanford
62 University HIV Drug Resistance Database, Y181F is documented as a nonpolymorphic NNRTI
63 resistance mutation [7]. In this regard, it likely represents a transitional mutation between
64 tyrosine and isoleucine/valine, or a partial revertant mutation. In this study, we sought to gain
65 insight into the mechanisms by which the cysteine, valine, isoleucine and phenylalanine
66 substitutions at residues Y181 affect NVP, EFV and RPV binding and inhibition on HIV-1 RT
67 using both kinetic and thermodynamic approaches.

68

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RESULTS

Susceptibility of HIV-1 containing the Y181C, Y181I, Y181V and Y181F substitutions in RT to NVP, EFV and RPV

Using site-directed mutagenesis we constructed four subtype B HIV-1^{LAI} infectious viruses containing the Y181C, Y181I, Y181V or Y181F mutations in RT. HIV-1 susceptibility to NVP, EFV and RPV was assessed in a single cycle assay, as described previously [10]. Low-, intermediate- and high-level resistance was defined as 2→8, 8→20, and > 20-fold changes in drug susceptibility compared to the WT virus. Consistent with previously published studies [10], we found that the Y181C substitution in RT conferred low level resistance to EFV (2.1-fold) and RPV (4.7-fold), and high-level resistance to NVP (137.9-fold; **Table 1**). The Y181I and Y181V substitutions in RT conferred high-level resistance to all 3 of the drugs tested (**Table 1**). In contrast, the Y181F substitution conferred hyper-susceptibility to EFV (0.3-fold) and RPV (0.3-fold), but not NVP.

Inhibition of HIV-1 RT containing the Y181C, Y181I, Y181V and Y181F substitutions in RT by NVP, EFV and RPV

We determined the *in vitro* inhibitory potency of NVP, EFV and RPV against the RNA-dependent DNA polymerase activity of recombinant purified WT, Y181C, Y181I, Y181V and Y181F HIV-1 RT (**Table 2**). Consistent with the antiviral data (**Table 1**), we found that Y181C conferred low-level resistance to EFV (3.3-fold) and RPV (4.0-fold) and high-level resistance (>20-fold) to NVP. The Y181I and Y181V substitutions in RT conferred high-level resistance to

each of the NNRTIs tested. Y181F RT was found to be hyper-susceptible to both EFV (0.3-fold) and RPV (0.2-fold). We also noted hyper-susceptibility to NVP (0.5-fold).

Determination of dissociation constants (K_d) for NVP, EFV and RPV binding to WT and Y181 mutant RT-template/primer (T/P) binary complexes using pre-steady-state burst reactions

Transient kinetic burst experiments provide insight into the rate of single nucleotide incorporation, the burst amplitude, and a steady-state turnover rate. The burst amplitude is decreased monotonically with increasing concentrations of NVP, EFV, or RPV [11-13], and represents the fraction of the RT-T/P complex that is not inhibited by drug and can therefore incorporate the next correct dNTP. By plotting the burst amplitude versus NNRTI concentration, one can estimate the affinity or K_d of an NNRTI for the RT-T/P binary complex. Using this method, we determined K_d values of 59.9±3.5 nM, 12.6±1.3 nM and 5.1±0.9 nM for NVP, EFV and RPV for the WT RT-T/P binary complex, respectively (**Table 3**). The Y181C, Y181V and Y181I substitutions in RT decreased the affinities of each of the NNRTIs to their respective RT-T/P binary complexes. Indeed, we could not determine the K_d values for binding of NVP or EFV to the Y181I RT-T/P binary complex, or of NVP, EFV or RPV to the Y181V RT-T/P complex. In contrast to the Y181C, Y181V and Y181I substitutions, Y181F increased the binding affinities of NVP, EFV and RPV to the RT-T/P complex (**Table 3**).

Nucleotide incorporation reactions carried out by WT and mutant RT-T/P and NNRTI-RT-T/P complexes

WT and mutant RT-T/P complexes with near saturating concentrations of NVP, EFV or RPV all exhibited slow but measurable DNA polymerization rates that enabled us to use single nucleotide

115 turnover conditions to determine the kinetic parameters of nucleotide incorporation facilitated by
116 the WT, Y181C, Y181I, Y181V and Y181F RT-T/P and NNRTI-RT-T/P complexes (**Table 4**).
117 The saturating NNRTI concentration was defined as 20 × the K_d determined in **Table 3**. Because
118 K_d values could not be determined for NNRTI binding to the Y181I RT-T/P and Y181V RT-T/P
119 complexes, we were unable to include these in these experiments. Our data revealed that all of
120 the substitutions at position Y181 (i.e., Y181C, Y181I, Y181V and Y181F) decreased the
121 catalytic efficiency (k_{pol}/K_d) of RT, and that these decreases in were driven by both changes in
122 K_d and k_{pol}. This finding is consistent with prior reports that Y181C, Y181I, Y181V and Y181F
123 decrease the replicative capacity of HIV-1 [14,15]. NVP, EFV and RPV binding exerted
124 profound effects on both nucleotide affinity and the rate of nucleotide incorporation for both the
125 WT and mutant RT-T/P complexes (**Table 4**). Specifically, we noted that the affinity of the
126 Mg^{2+}-dTTP substrate was increased 130-fold compared with the RT-T/P complex, as reported
127 previously, whereas the rate of Mg^{2+}-dTTP incorporation (k_{pol}) was significantly decreased
128 [12,13]. Interestingly, the Y181C NNRTI-RT-T/P complexes exhibited improved catalytic
129 efficiencies (k_{pol}/K_d) - compared to the respective WT complexes – which driven entirely driven
130 by changes in k_{pol}. In contrast, the catalytic efficiencies of the Y181F NNRTI-RT-T/P complexes
131 were reduced compared to those of the WT RT (**Table 4**).
132
133 **Anisotropy assay of RT binding to the T/P substrate**
134 To evaluate whether the Y181C, Y181I or Y181F substitutions affected the interaction between
135 RT and the T/P substrate, we used anisotropy (*r*) to assess the binding interactions. Fluorescence
136 anisotropy measures the rotational mobility of the fluorophores that are excited with polarized
137 light. In this instance, the fluorophore (fluorescein) is attached to the T/P substrate, and upon

interaction with RT there is a shift in the rotational mobility (or tumbling) of the complex that allows for K_d determination. The T/P substrate used in these experiments was identical in sequence to the substrate reported in a crystal structure of the RT-T/P-dNTP ternary complex [16], and was chain-terminated with 2',3'-dideoxycytosine-monophosphate. The fluorescein dye was attached to the 5'-end of the DNA primer. RT binding to the T/P resulted in an increase in *r*, which allowed us to calculate a K_d of 2.9 ± 0.2 nM for the WT RT-T/P complex, 2.2 ± 0.1 nM for the Y181C RT-T/P complex, 4.6 ± 0.3 nM for the Y181I RT-T/P complex and 2.0 ± 0.1 nM for the Y181F RT-T/P complex (**Fig. 1**). These data underscore that the substitutions at residue 181 did not affect the binding of RT to the T/P substrate.

Anisotropy assay of RT sliding on the T/P substrate

NNRTIs increase the sliding of RT on the T/P substrate, thus blocking the formation of catalytically competent RT-T/P or RT-T/P-dNTP complexes [17-19]. To evaluate how EFV and RPV affected the sliding of WT, Y181C, Y181I and Y181F RT on the T/P substrate, we used an anisotropy assay, previously developed in our laboratory, which serves as a proxy measurement for RT sliding on the T/P substrate. Briefly, in this assay, changes in anisotropy (*r*) are due to both rotation of the RT-T/P complex and the tumbling of fluorescein dye in solution (i.e., independent rotation due to flexibility of the linker). Interaction between RT and the fluorescein dye, as the enzyme shuttles to and from the DNA pimer, affects the tumbling of the fluorescent dye; and a larger *r* value indicates a broader distribution of RT on the T/P (or greater sliding of enzyme on the T/P substrate). **Fig. 2A** shows the binding of EFV to the WT, Y181C, Y181F or Y181I RT-T/P binary complex. The *r* values for each of the mutant RT-T/P complexes increased upon titration of EFV. Notably, the *r* values determined for the Y181C and Y181F RT-T/P

complexes were greater than that of WT RT-T/P complex, even in the absence of drug, suggesting increased sliding of these RTs on the nucleic acid substrate. In contrast, the Y181I RT-T/P complex exhibited lower *r* values, indicative of decreased sliding both in the absence and presence of NNRTI. When the next correct dNTP was added – to form the RT-T/P-dNTP ternary complex – we again noted that the *r* value for the Y181F RT complex was substantially greater than any of the other complexes (**Fig. 2B**). However, the *r* values for both the Y181C and Y181I RT-T/P-dNTP complexes were less than that of the WT complex (**Fig. 2B**). In general, a similar trend was observed for RPV binding to the RT-T/P binary and RT-T/P-dNTP ternary complexes, although subtle differences were noted (**Fig. 2C**, **2D**). Of note, K_d values for the binding of EFV or RPV to the RT-T/P or RT-T/P-dNTP complexes could be determined from the anisotropy isotherms, and these values are provided in **Fig. 2**. In general, we noted a larger K_d for the Y181I RT complexes suggesting decreased affinity for the inhibitor, and a smaller K_d value for the Y181F RT complexes suggesting increased affinity for the inhibitor.

Molecular models of Y181C, Y181F, Y181I and Y181V HIV-1 RT in complex with RPV

We used molecular modelling to gain structural insight into how the Y181F, Y181I and Y181V substitutions in RT affect RPV binding. We observed that Y181C had minimal impact on the binding orientation of RPV or spatial arrangement of amino acid residues in the NNRTI-BP (**Fig. 3A**). This observation is consistent with prior studies [20]. In contrast, the bulky side-chains of both the valine (**Fig. 3C**) and isoleucine (**Fig. 4D**) substitutions significantly affected the placement of RPV in the binding pocket, which likely impacts on the inhibitor's binding affinity. For the Y181F substitution in RT (**Fig. 3D**), we noted a subtle re-orientation of the phenylalanine ring relative to tyrosine that appears to enhance the π-π stacking interaction with RPV.

185 **DISCUSSION**

186

187 More than one hundred crystal structures of WT and mutated HIV-1 RT in complex with
188 different NNRTIs have been solved. This wealth of structural information has provided insight
189 into the mechanisms by which mutations in the NNRTI-BP of RT impact inhibitor binding.
190 Often times, however, it is challenging to correlate the structural changes observed in the
191 NNRTI-BP in crystal structures of NNRTI-bound mutant RTs with the fold-changes in the
192 resistance determined *in vitro*. For example, the Y181C substitution in RT confers high-level
193 resistance to NVP (> 100-fold) (**Table 1**). However, in the Y181C RT-NVP crystal structure the
194 inhibitor is located in almost exactly the same position that it occupies in the WT RT-NVP
195 structure, and there are only minor perturbations in the NNRTI-BP that could explain the
196 observed resistance [21]. Thus, kinetic and thermodynamic analyses of inhibitor interactions
197 with WT and mutant RT – as conducted in this study – provide important complementary
198 insights into the mechanisms of NNRTI resistance. In the current study, we focused on
199 substitutions at residue Y181 in RT that confer broad NNRTI cross-resistance. While prior
200 studies have focused on Y181C [11, 21], in contrast, there is little to no published data on Y181I,
201 Y181V and/or Y181F.

202

203 Whereas the Y181C substitution in RT conferred high-level resistance to NVP but only low-
204 level resistance to EFV and RPV, the Y181I and Y181V mutations conferred high-level
205 resistance across the NNRTI class, at both the enzyme and virus levels (**Tables 1** and **2**).
206 Notably, in pre-steady-state kinetic experiments, the Y181I and Y181V substitutions
207 significantly decreased the binding affinity of NVP, EFV and RPV to RT (**Table 3**). While

208 Y181I did not significantly affect the affinity of the RT and T/P substrate binding interaction
209 (**Fig. 1**), the mutation appears to decrease the sliding of the enzyme on the nucleic acid substrate
210 in the presence of EFV or RPV (**Fig. 2**). Molecular modeling studies suggest that the bulky side-
211 chains of Y181I and Y181V affects the binding orientation of RPV in the NNRTI-BP (**Fig. 2C**,
212 **2D**), thus providing a structural explanation for the observed resistance. In contrast to Y181I/V,
213 the Y181C has a modest impact on the binding affinity of EFV and RPV to RT (**Table 3**).
214 Consistent with a previously published study [11], the NNRTI-bound Y181C RT-T/P complex
215 has the capacity to incorporate the next correct dNTP more efficiently than the WT complex, as
216 assessed by transient kinetic analyses (**Table 4**). To some extent, this is likely due to the
217 mutation stabilizing the RT-T/P-dNTP ternary complex by decreasing enzyme sliding on the T/P
218 substrate (**Fig. 2C**). One surprising finding from this study was that the Y181F substitution –
219 which represents a transitional mutation between Y181 and Y181I or Y181V, or possibly a
220 partial revertant mutation – conferred hyper-susceptibility to EFV and RPV at both the virus and
221 enzyme levels (**Tables 1**, **2**, **3**, **4**). Of note, the EFV- or RPV- bound Y181F RT-T/P complex
222 was significantly less efficient than the EFV- or RPV-bound WT complex in incorporating the
223 next correct dNTP (**Table 4**), and this correlated with increased sliding of the mutant RT (**Fig.**
224 **2**). From a structural perspective, the hydroxyl group of tyrosine may weaken the extent of the π-
225 π stacking interactions between the residue and RPV. Therefore, substitution of tyrosine with
226 phenylalanine may enhance RPV binding by augmentation of aromaticity (**Fig. 3**).
227
228 In conclusion, in this study we used kinetic and thermodynamic approaches to understand how
229 cysteine, isoleucine, valine and phenylalanine substitutions at residue 181 in RT affect NNRTI
230 binding and enzyme inhibition. Interestingly, we found that the Y181F substitution conferred

EFV and RPV hyper-susceptibility. As noted above, Y181F likely represents an intermediate between Y181 and Y181I/V. In the Stanford University HIV Drug Resistance Database, Y181C is frequently associated with individuals failing NNRTI-based therapies (% mutant ranges from ~8-35 % depending on the NNRTI used). In contrast, the frequency of Y181I and Y181V is much less frequent (% mutant ranges from ~ 0.6-1.2%). This may be explained in part by the fact that these mutations require a two nucleotide change and that the intermediate – Y181F – increases NNRTI susceptibility.

MATERIALS AND METHODS

Reagents

DNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coralville, IA, USA). NVP, EFV and RPV were purchased from Selleckchem (Houston, TX, USA).

Construction and Purification of HIV-1 RTs

The Y181C, Y181I, Y181V and Y181F mutations were introduced into the p6HRT-Prot vector by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene La Jolla, CA), and confirmed by full-length sequencing of HIV-1 RT. The WT and mutant HIV-1 RTs were over-expressed and purified to homogeneity, as described previously [22]. Enzyme concentration was determined spectrophotometrically at 280nm using an extinction co-efficient (ε_{280}) of 260 450 M^{-1} cm^{-1}.

Activity of RT constructs

The RNA-dependent DNA polymerase activities of the purified and labeled RTs were assessed as described previously [23]. The concentration of NNRTI required to inhibit 50% of the RT DNA polymerase activity (IC_{50}) was determined as described previously [19].

HIV-1 drug susceptibility assays

The genes for Y181C, Y181I, Y181V and Y181F were cloned into HIV-1_{LAI}. NNRTI susceptibility was determined in TZM-bl cells, as described previously [10].

Pre-Steady State Kinetics of Single Nucleotide Incorporation

Reactions were carried out using a 19 nucleotide DNA primer (5'-GTCCCTGTTCGGGCGCCAC-3') annealed to a 45 nucleotide DNA template (5'-TAGTCAGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACA-3'). The DNA primer was 5'-radiolabeled with [γ-^{32}P]-ATP (Perkin Elmer) and T4 polynucleotide kinase (Fisher Scientific, Pittsburgh, PA, USA). The 5'-^{32}P-labeled primer was annealed to the template by adding a 1:1.5 molar ratio of primer to template at 90˚C and allowing the mixture to slowly cool to ambient room temperature. Rapid quench experiments were carried out using a Kintek RQF-3 instrument (Kintek Corporation, Clarence, PA, USA). In all experiments described, RT and T/P were pre-incubated in 50mM Tris-HCl pH7.5, 50mM KCl prior to mixing with an equivalent volume of nucleotide in 50mM Tris-HCl pH7.5, 50mM KCl, 15 mM $MgCl_2$. Reactions were quenched at different time points by addition of 0.5M EDTA, pH 8.0. In reactions that included NNRTI, the inhibitor (dissolved in DMSO) was pre-incubated with RT-T/P. The final concentration of DMSO in the experiment was < 1 %. Quenched samples were mixed with an equal volume of gel loading buffer (98% deionized formamide, 10mM EDTA and 1mg/ml each of bromophenol blue and xylene cyanol), denatured at 85˚C for 5min, and subjected to 7M urea-14% polyacrylamide gel electrophoresis. Products were quantified using a Bio-Rad GS525 Molecular Imager (Bio-Rad Laboratories, Inc., Hercules, CA).

Data Analysis

Pre-stead-state data were fitted by nonlinear regression using Sigma Plot software (Jandel Scientific). For the burst experiments, the following equation was used: $[T/P_{+1}] = A[1 - \exp(-k_1 t) + mt)$, where A represents the burst amplitude, k_1 the burst rate, and m the slope. For single

turnover experiments, the following equation was used: $[T/P_{+1}] = A(1-e^{-kobst})$. K_d and k_{pol} values were calculated by fitting the observed single rate constants (k_{obs}) obtained at different concentrations of dNTP to the hyperbolic expression: $k_{obs} = k_{pol}[dNTP]/(K_d + [dNTP])$, where K_d is the equilibrium dissociation constant for the interaction of dNTP with the RT-T/P complex and k_{pol} is the maximum first order rate constant for dNTP incorporation.

Determination of equilibrium constants for NNRTI for RT-T/P

Burst phase amplitudes were plotted versus the concentration of NNRTI, and were fitted to the following equation: $y = E_0 - 0.5\{(K_d + E_0 + I_0) - [(K_d + E_0 + I_0)^2 - 4E_0I_0]^{1/2}\}$, where y represents the RT-T/P complex, E_0 is the total enzyme concentration, and K_d is the equilibrium dissociation constant for the NNRTI.

Fluorescence Anisotropy

Assays were carried out using a fluorescein labeled T/P substrate. We used a 19 nt DNA primer (5'-Fluorescein-dT-CAGTCCCTGTTCGGGCGC-ddC-3') that was annealed to the 35 nucleotide DNA template (5'-GGGTTTGCTAAGCACCGGCGCCCGAACAGGGACTG-3'). Fluorescence anisotropy experiments were performed as previously described [19], using a JASCO FP-8500 fluorescence spectrophotometer. The excitation and emission wavelength were set at 545 and 560 nm, respectively, and the excitation and emission slit widths were set at 5 and 2.5 nm respectively. The concentration of T/P in all experiments was 10 nM (in a total volume of 600 µL). Anisotropy (*r*) was calculated as: $r = (I_{VV}-G\cdot I_{VH})/(I_{VV}+2\cdot G\cdot I_{VH})$; where I_{VV} is the fluorescence intensity with vertically oriented excitation and emission polarizers and I_{VH} is the fluorescence intensity with a vertically oriented excitation polarizer and a horizontally oriented

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emission polarizer. The G-factor, defined as $G=I_{HV}/I_{HH}$, was measured before each experiment to ensure a value of ~2.3. Anisotropy values were collected in triplicate using an integration time of 5.0 s.

Structural analyses of RPV-RT complexes

X-ray co-ordinates of HIV-1 RT in complex with RPV (4G1Q [24]) were downloaded from the Protein Data Bank. PyMol 2.3 (https://pymol.org/2/) was used to introduce mutations at residue Y181 in RT, and to perform energy minimization of the mutation and all residues within an 8Å radius. Graphical representation of structures was performed using Chimera (https://www.cgl.ucsf.edu/chimera/).

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Table 1. Susceptibility of HIV-1 containing mutations at residue Y181 in RT to NVP, EFV and RPV

Virus	NVP		EFV		RPV	
	EC_{50} (μM)[1]	Fold-Change[2]	EC_{50} (nM)	Fold-Change	EC_{50} (nM)	Fold-Change
WT	0.08±0.01	-	1.4±0.3	-	0.3±0.03	-
Y181C	11.03±0.87	137.9	3.1±0.7	2.1	1.4±0.1	4.7
Y181I	>20	>250	>1000	>714	7.7±0.8	25.7
Y181V	>20	>250	>1000	>714	7.4±1.2	24.7
Y181F	0.10±0.02	1.3	0.4±0.1	0.3	0.1±0.01	0.3

[1] The concentrations of drug required to inhibit viral replication by 50% (EC_{50}). Data reported as a mean ± standard deviation from at least 3 independent experiments.

[2] Mean fold change in EC_{50} of mutant versus WT virus. EC_{50} values were compared for statistically significant differences (p-value < 0.05) using a non-paired, 2 sample equal variance (homoscedastic) test.

Table 2. Inhibition of the RNA-dependent DNA polymerase activity of WT and mutant HIV-1 RT

RT	NVP		EFV		RPV	
	IC_{50} (μM)[1]	Fold-Change[2]	IC_{50} (nM)	Fold-Change	IC_{50} (nM)	Fold-Change
WT	0.6±0.1	-	10.1±0.8	-	2.1±0.1	-
Y181C	>20	>33	33.3±3.3	3.3	8.4±0.3	4.0
Y181I	>20	>33	>5000	>495	88±3.5	41.9
Y181V	>20	>33	>5000	>495	81±5.1	38.6
Y181F	0.3±0. 2	0.5	2.9±0.8	0.3	0.5±0.1	0.2

[1] The concentrations of drug required to inhibit RT activity by 50% (IC_{50}). Data reported as a mean ± standard deviation from at least 3 independent experiments.

[2] Mean fold change in IC_{50} of mutant versus WT HIV-1 RT.

476 **Table 3. Dissociation constants for NVP, EFV or RPV binding to WT and mutant HIV-1**

477 **RT-T/P complexes determined by pre-steady-state kinetics**

RT	K_d (nM)[1]		
	NVP	EFV	RPV
WT	59.9±3.5	12.6±1.3	5.1±0.9
Y181C	1,253±225	36.1±8.8	19.8±3.4
Y181I	> 20,000	> 2,000	420.6±76.6
Y181V	> 20,000	> 2,000	> 500
Y181F	48.8±4.4	3.7±0.9	1.2±1.0

478 [1] Data reported as a mean ± standard deviation from at least 3 independent experiments.

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Table 4. Pre-steady-state kinetic parameters determined for incorporation of TTP by WT and mutant RT-T/P and NNRTI-RT-T/P complexes

NNRTI	RT-T/P	K_d (μM)	k_{pol} (sec^{-1})	k_{pol}/K_d ($\mu M^{-1}.sec^{-1}$)	[1]Fold change in		
					K_d	k_{pol}	k_{pol}/K_d
	WT	2.2±0.3	26.25±3.3	11.9	-	-	-
	Y181C	7.6±1.1	12.34±1.9	1.63	3.5	0.5	0.14
	Y181I	20.7±1.5	1.84±0.6	0.08	9.4	0.07	0.006
	Y181V	24.1±3.3	0.7±0.6	0.03	11.0	0.03	0.003
	Y181F	8.3±1.2	8.66±4.1	1.04	3.8	0.33	0.088
NVP	WT	0.02±0.01	0.039±0.01	1.95	-	-	-
	Y181C	0.02±0.01	0.389±0.04	19.45	1	9.97	10
	Y181I	nd	nd	nd	-	-	-
	Y181V	nd	nd	nd	-	-	-
	Y181F	0.02±0.01	0.012±0.01	0.6	1	0.31	0.10
EFV	WT	0.02±0.01	0.032±0.01	1.6	-	-	-
	Y181C	0.02±0.01	0.098±0.03	4.9	1	3.06	3.95
	Y181I	nd	nd	nd	-	-	-
	Y181V	nd	nd	nd	-	-	-
	Y181F	0.02±0.01	0.003±0.01	0.15	1	0.094	0.3
RPV	WT	0.02±0.01	0.028±0.006	1.4	-	-	-
	Y181C	0.02±0.01	0.067±0.008	3.35	1	2.4	2.4
	Y181I	nd	nd	nd	-	-	-
	Y181V	nd	nd	nd	-	-	-
	Y181F	0.02±0.01	0.001±0.001	0.05	1	0.04	0.04

[1]Fold change in kinetic parameter as determined by: [RT-T/P]/[NNRTI-RT-T/P]

Data reported as a mean ± standard deviation from at least 3 independent experiments

513 **FIGURE LEGENDS**

514

515 **Fig.1. Binding isotherms for WT and mutant HIV-1 RT to the T/P substrate measured by**
516 **anisotropy.** Data are shown as the mean ± standard deviation from 3 separate biological
517 replicates.

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519 **Fig.2. EFV and RPV binding to the WT and mutant RT-T/P and RT-T/P-dNTP complexes**
520 **measured by anisotropy.**
521 **a**) EFV binding to the WT and mutant RT-T/P complex; b) EFV binding to the WT and mutant
522 RT-T/P-dNTP complex; c) RPV binding to the WT and mutant RT-T/P complex; d) RPV
523 binding to the WT and mutant the RT-T/P-dNTP complex. Data are shown as the mean ±
524 standard deviation from 3 separate biological replicates.

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526 **Fig.3. Molecular models of Y181C, Y181F, Y181I and Y181V HIV-1 RT in complex with**
527 **RPV.**
528 **A**) Overlay of the WT and Y181C RT; **B**), Overlay of WT and Y181F RT; **C**) Overlay of WT
529 and Y181V RT; **D**) Overlay of WT and Y181I RT. Only key residues in the NNRTI-BP are
530 shown. The mutant RT is always colored in cyan.

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