Nonnucleoside Reverse Transcriptase Inhibitor Hypersusceptibility and Resistance by Mutation of Residue 181 in HIV-1 Reverse Transcriptase

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ABSTRACT 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 deoxynucleoside triphosphate (dNTP) more efficiently than the wild-type (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 hypersusceptibility 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.

KEYWORDS HIV, NNRTI, mechanisms of resistance, reverse transcriptase

Nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) bind to HIV-1 RT in the NNRTI-binding pocket (NNRTI-BP) and inhibit reverse transcription via an allosteric mechanism of action. Five NNRTIs—nevirapine (NVP), efavirenz (EFV), etravirine (ETR), rilpivirine (RPV), and doravirine—have been approved by the U.S. Food and Drug Administration and are routinely implemented in the clinical setting to prevent or treat HIV-1 infection. The NNRTI-BP resides in the DNA polymerase domain of HIV-1 RT and is located close to, but separate from, the active site (1, 2). Key residues involved in NNRTI binding include L100, K101, K103, V106, Y181, Y188, G190, F227, W229, and M230, and HIV-1 resistance to NNRTIs is typically associated with mutations at one or more of these residues (3). Substitutions at residue Y181, in particular Y181C/I/V, confer NNRTI cross-resistance. Y181C is typically selected by NVP, ETR, or RPV (4–6) and imparts >20-fold resistance to NVP and 2- to 5-fold resistance to EFV, RPV, and ETR. Y181I/V are 2-bp nonpolymorphic mutations that confer >50-fold resistance to NVP and 5- to 20-fold resistance to ETR and RPV (4, 7–9). Interestingly, in the Stanford University HIV Drug Resistance Database, Y181F is documented as a nonpolymorphic NNRTI resistance mutation (7). In this regard, it likely represents a transitional mutation between tyrosine and isoleucine/valine or a partial revertant mutation. In this study, we sought to gain insight into the mechanisms by which the cysteine, valine, isoleucine,

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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) ^a	Fold change ^b	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	>1,000	>714	7.7 ± 0.8	25.7
Y181V	>20	>250	>1,000	>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

^aThe concentrations of drug required to inhibit viral replication by 50% (EC₅₀). Data reported as a mean ± standard deviation from at least 3 independent experiments.

^bMean fold change in EC₅₀ of mutant versus WT virus. EC₅₀ values were compared for statistically significant differences (*P* value of <0.05) using a nonpaired, 2-sample equal variance (homoscedastic) test.

and phenylalanine substitutions at residue Y181 affect NVP, EFV, and RPV binding and inhibition on HIV-1 RT using both kinetic and thermodynamic approaches.

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 mutation 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- to 8-, 8- to 20-, and >20-fold changes in drug susceptibility compared to that of the wild-type (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 hypersusceptibility 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 hypersusceptible to both EFV (0.3-fold) and RPV (0.2-fold). We also noted hypersusceptibility to NVP (0.5-fold).

Determination of dissociation constants for NVP, EFV, and RPV binding to WT and Y181 mutant RT-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

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

RT	NVP		EFV		RPV	
	IC ₅₀ (μM) ^a	Fold change ^b	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	>5,000	>495	88 ± 3.5	41.9
Y181V	>20	>33	>5,000	>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

^aThe concentrations of drug required to inhibit RT activity by 50% (IC₅₀). Data reported as a mean ± standard deviation from at least 3 independent experiments.

^bMean fold change in IC₅₀ of mutant versus WT HIV-1 RT.

TABLE 3 Dissociation constants for NVP, EFV, or RPV binding to WT and mutant HIV-1 RT-T/P complexes determined by pre-steady-state kinetics

RT	K_d (nM) ^a		
	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

^aData reported as a mean ± standard deviation from at least 3 independent experiments.

(11–13) and represents the fraction of the RT-template/primer (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 dissociation constant (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 turnover conditions to determine the kinetic parameters of nucleotide incorporation facilitated by the WT, Y181C, Y181I, Y181V, and Y181F RT-T/P and NNRTI-RT-T/P complexes (Table 4). The saturating NNRTI concentration was defined as 20× the K_d determined in Table 3. Because K_d values

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} (s ⁻¹)	k_{pol}/K_d (μM ⁻¹ · s ⁻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 ^b	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

^aFold 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.^bnd, no data.

FIG 1 Binding isotherms for WT and mutant HIV-1 RT to the T/P substrate measured by anisotropy. Data are shown as the mean $\pm$ standard deviation from 3 separate biological replicates.

could not be determined for NNRTI binding to the Y181I RT-T/P and Y181V RT-T/P complexes, we were unable to include these in these experiments. Our data revealed that all of the substitutions at position Y181 (i.e., Y181C, Y181I, Y181V, and Y181F) decreased the catalytic efficiency (k_{pol}/K_d) of RT and that these decreases were driven by both changes in K_d and k_{pol} (maximum rate of dNTP incorporation). This finding is consistent with prior reports that Y181C, Y181I, Y181V, and Y181F decrease the replicative capacity of HIV-1 (14, 15). NVP, EFV, and RPV binding exerted profound effects on both nucleotide affinity and the rate of nucleotide incorporation for both the WT and mutant RT-T/P complexes (Table 4). Specifically, we noted that the affinity of the Mg^{2+} -dTTP substrate was increased 130-fold compared with that of the RT-T/P complex, as reported previously, whereas the rate of Mg^{2+} -dTTP incorporation (k_{pol}) was significantly decreased (12, 13). Interestingly, the Y181C NNRTI-RT-T/P complexes exhibited improved catalytic efficiencies (k_{pol}/K_d)—compared to those of the respective WT complexes—which are driven entirely by changes in k_{pol} . In contrast, the catalytic efficiencies of the Y181F NNRTI-RT-T/P complexes were reduced compared to those of the WT RT (Table 4).

Anisotropy assay of RT binding to the T/P substrate. To evaluate whether the Y181C, Y181I, or Y181F substitution affected the interaction between RT and the T/P substrate, we used anisotropy (r) to assess the binding interactions. Fluorescence anisotropy measures the rotational mobility of the fluorophores that are excited with polarized 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 K_d values 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

FIG 2 EFV and RPV binding to the WT and mutant RT-T/P and RT-T/P-dNTP complexes measured by anisotropy. (A) EFV binding to the WT and mutant RT-T/P complex. (B) EFV binding to the WT and mutant RT-T/P-dNTP complex. (C) RPV binding to the WT and mutant RT-T/P complex. (D) RPV binding to the WT and mutant RT-T/P-dNTP complex. Data are shown as the mean $\pm$ standard deviation from 3 separate biological replicates.

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 primer, 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). Figure 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 the 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 that for 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 for 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 and D). 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.

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

Molecular models of Y181C, Y181F, Y181I, and Y181V HIV-1 RT in complex with RPV. We used molecular modeling 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. 3D) 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 reorientation of the phenylalanine ring relative to tyrosine that appears to enhance the π - π stacking interaction with RPV.

DISCUSSION

More than 100 crystal structures of WT and mutated HIV-1 RT in complex with different NNRTIs have been solved. This wealth of structural information has provided insight into the mechanisms by which mutations in the NNRTI-BP of RT impact inhibitor binding. Often, however, it is challenging to correlate the structural changes observed in the NNRTI-BP in crystal structures of NNRTI-bound mutant RTs with the fold changes in the resistance determined *in vitro*. For example, the Y181C substitution in RT confers high-level resistance to NVP (>100-fold) (Table 1). However, in the Y181C RT-NVP

crystal structure, the inhibitor is located in almost exactly the same position that it occupies in the WT RT-NVP structure, and there are only minor perturbations in the NNRTI-BP that could explain the observed resistance (21). Thus, kinetic and thermodynamic analyses of inhibitor interactions with WT and mutant RT, as conducted in this study, provide important complementary insights into the mechanisms of NNRTI resistance. In the current study, we focused on substitutions at residue Y181 in RT that confer broad NNRTI cross-resistance. While prior studies have focused on Y181C (11, 21), in contrast, there is little to no published data on Y181I, Y181V, and/or Y181F.

Whereas the Y181C substitution in RT conferred high-level resistance to NVP but only low-level resistance to EFV and RPV, the Y181I and Y181V mutations conferred high-level resistance across the NNRTI class at both the enzyme and virus levels (Tables 1 and 2). Notably, in pre-steady-state kinetic experiments, the Y181I and Y181V substitutions significantly decreased the binding affinity of NVP, EFV and RPV to RT (Table 3). While Y181I did not significantly affect the affinity of the RT and T/P substrate binding interaction (Fig. 1), the mutation appears to decrease the sliding of the enzyme on the nucleic acid substrate in the presence of EFV or RPV (Fig. 2). Molecular modeling studies suggest that the bulky side chains of Y181I and Y181V affect the binding orientation of RPV in the NNRTI-BP (Fig. 2C and D), thus providing a structural explanation for the observed resistance. In contrast to Y181I/V, Y181C has a modest impact on the binding affinity of EFV and RPV to RT (Table 3). Consistent with a previously published study (11), the NNRTI-bound Y181C RT-T/P complex has the capacity to incorporate the next correct dNTP more efficiently than the WT complex, as assessed by transient kinetic analyses (Table 4). To some extent, this is likely due to the mutation stabilizing the RT-T/P-dNTP ternary complex by decreasing enzyme sliding on the T/P substrate (Fig. 2C). One surprising finding from this study was that the Y181F substitution, which represents a transitional mutation between Y181 and Y181I or Y181V or possibly a partial revertant mutation, conferred hypersusceptibility to EFV and RPV at both the virus and enzyme levels (Tables 1 to 4). Of note, the EFV- or RPV-bound Y181F RT-T/P complex was significantly less efficient than the EFV- or RPV-bound WT complex in incorporating the next correct dNTP (Table 4), and this correlated with increased sliding of the mutant RT (Fig. 2). From a structural perspective, the hydroxyl group of tyrosine may weaken the extent of the π - π stacking interactions between the residue and RPV. Therefore, substitution of tyrosine with phenylalanine may enhance RPV binding by augmentation of aromaticity (Fig. 3).

In conclusion, in this study, we used kinetic and thermodynamic approaches to understand how cysteine, isoleucine, valine, and phenylalanine substitutions at residue 181 in RT affect NNRTI binding and enzyme inhibition. Interestingly, we found that the Y181F substitution conferred EFV and RPV hypersusceptibility. 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 (percentage mutant ranges from ~8 to 35%, depending on the NNRTI used). In contrast, the frequency of Y181I and Y181V is much less frequent (percentage mutant ranges from ~0.6 to 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 overexpressed and purified to homogeneity, as described previously (22). Enzyme concentration was determined spectrophotometrically at 280 nm using an extinction coefficient (ϵ_{280}) of $260,450 \text{ M}^{-1} \text{ cm}^{-1}$.

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 (nt) DNA primer (5'-GTCCCTGTTCGGGCGCCAC-3') annealed to a 45-nucleotide DNA template (5'-TAGTCAGAAATGAAAACTCTAGCAGTGGCGCCGAACAGGGACA-3'). The DNA primer was 5'-radiolabeled with [γ -³²P]ATP (Perkin Elmer) and T4 polynucleotide kinase (Fisher Scientific, Pittsburgh, PA, USA). The 5'-³²P-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 preincubated in 50 mM Tris-HCl, pH 7.5, and 50 mM KCl prior to mixing with an equivalent volume of nucleotide in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 15 mM MgCl₂. Reactions were quenched at different time points by addition of 0.5 M EDTA, pH 8.0. In reactions that included NNRTI, the inhibitor (dissolved in dimethyl sulfoxide [DMSO]) was preincubated 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, 10 mM EDTA, and 1 mg/ml each of bromophenol blue and xylene cyanol), denatured at 85°C for 5 min, and subjected to 7 M urea-14% polyacrylamide gel electrophoresis. Products were quantified using a Bio-Rad GS-525 Molecular Imager (Bio-Rad Laboratories, Inc., Hercules, CA).

Data analysis. Pre-steady-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: $[T/P_{+1}] = A(1 - e^{-k_{obs}t})$. 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 as follows: $k_{obs} = k_{pol}[dNTP]/(K_d + [dNTP])$, 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-nt DNA template (5'-GGGTTTGCTAAGCACCAGCGCCCGAACAGGGACTG-3'). Fluorescence anisotropy experiments were performed as previously described (19) using a JASCO FP-8500 fluorescence spectrophotometer. The excitation and emission wavelengths 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 μ l). Anisotropy (r) was calculated as follows: $r = (I_{VV} - G \cdot I_{VH}) / (I_{VV} + 2 \cdot G \cdot I_{VH})$, 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 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 coordinates of HIV-1 RT in complex with RPV (accession number 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 8 Å radius. Graphical representation of structures was performed using Chimera (<https://www.cgl.ucsf.edu/chimera/>).

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