Biochemical Mechanism of HIV-1 Resistance to Rilpivirine*

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Background: Reverse transcriptase mutations E138K and M184I emerged most frequently in HIV-1 patients who failed rilpivirine/emtricitabine/tenofovir combination therapy.

Results: M184I reduces polymerase efficiency, and E138K restores it. E138K also reduces rilpivirine binding affinity mainly by increasing its dissociation rate.

Conclusion: E138K abrogates the polymerase defect of M184I and increases rilpivirine dissociation.

Significance: Our results provide a biochemical explanation for the selection of E138K/M184I in patients who failed combination therapy.

Rilpivirine (RPV) is a second generation nonnucleoside reverse transcriptase (RT) inhibitor (NNRTI) that efficiently inhibits HIV-1 resistant to first generation NNRTIs. Virological failure during therapy with RPV and emtricitabine is associated with the appearance of E138K and M184I mutations in RT. Here we investigate the biochemical mechanism of RT inhibition and resistance to RPV. We used two transient kinetics approaches (quench-flow and stopped-flow) to determine how subunit-specific mutations in RT p66 or p51 affect association and dissociation of RPV to RT as well as their impact on binding of dNTP and DNA and the catalytic incorporation of nucleotide. We compared WT with four subunit-specific RT mutants, p66M184I/p51WT, p66E138K/p51E138K, p66E138K/M184I/p51E138K and p66E138Kp51E138K. Lys-138 in both subunits or in p51 alone abrogated the negative effect of p66E138K by restoring dNTP binding. Furthermore, p51E138K reduced RPV susceptibility by altering the ratio of RPV dissociation to RPV association, resulting in a net reduction in RPV equilibrium binding affinity (Kd,RPV = Kd,off,RPV/Kd,on,RPV). Quantum mechanics/molecular mechanics hybrid molecular modeling revealed that p51E138K affects access to the RPV binding site by disrupting the salt bridge between p51E138 and p66K101. P66E138K caused repositioning of the Tyr-183 active site residue and decreased the efficiency of RT, whereas the addition of p51E138K restored Tyr-183 to a WT-like conformation, thus abrogating the Ile-184-induced functional defects.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) has been the primary target of 13 anti-HIV drugs (1). First-line therapy for HIV infection generally comprises combinations of two nucleoside RT inhibitors (NRTIs)5 with a nonnucleoside RT inhibitor (NNRTI). Clinically used NRTIs act as chain terminators because they lack a 3′-OH group required for DNA synthesis (2–4), although some new highly potent experimental NRTIs retain a 3′-OH and inhibit RT by blocking translocation or by delayed chain termination (5, 6). Resistance to NRTIs can be imparted by either (a) reduced incorporation of the inhibitor into the nascent DNA (7–9) or (b) enhanced excision of the incorporated NRTIs (10–12). An example of the first mechanism is the high level resistance of M184V/I RT to emtricitabine (FTC) (13). However, this mutation also decreases HIV replication efficiency (14–16).

NRTIs are allosteric RT inhibitors that bind at the NNRTI binding pocket (NNIBP) located ~10 Å away from the polymerase active site and is formed by residues of both the p66 (Leu-100, Lys-101, Lys-103, Val-106, Thr-107, Val-108, Val-179, Tyr-181, Tyr-188, Val-189, Gly-190, Phe-227, Trp-229, Leu-235, Met-257, Ile-268, Thr-273, Ile-275, Val-281, Lys-284, Lys-285, Met-289, Thr-294, Val-295, Val-299, Leu-300, Val-303, Met-304, Thr-306, Val-308, and Lys-310) and p51 (His-107, Ile-110, Val-112, Lys-113, His-115, Arg-118, Lys-119, Thr-120, Asp-121, Lys-123, Thr-124, Gly-125, Val-126, Gly-127, Asp-128, Lys-138, Asp-145, and Thr-150) subunits. Recent studies have shown that both RT subunits contribute to RT resistance with NRTIs (16–19). For example, the addition of p51E138K restored Tyr-183 to a WT-like conformation, thus abrogating the Ile-184-induced functional defects.

5 The abbreviations used are: NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; NNI/H, NNRTI binding pocket; RPV, rilpivirine; ETV, etravirine; FTC, 4-amino-5-fluoro-1-((2-hydroxymethyl)-1,3-oxathiolan-5-yl)-1,2-dihydropyrimidin-2-one; TDF, 9-(4-((bis(((isopropoxycarbonyl)oxy)methoxy)phosphinyl)methoxy)phosphinyl)methoxy)propylladenine fumarate (1:1); NVP, nevirapine; ROF, rapid quench-flow; SF, stopped-flow; IFD, induced fit docking.
234, and Tyr-318), and the p51 (Glu-138) subunits (2, 3, 17, 18). First generation NNRTIs, such as nevirapine (NVP), are inflexible hydrophobic compounds. In contrast, second generation NNRTIs, such as etravirine (ETV, TMC125) and rilpivirine (RPV, TMC278) (19–21) are more flexible and can inhibit HIV strains resistant to first generation NNRTIs (22).

NNRTIs have been proposed to block reverse transcription by (a) changing the mobility of the thumb subdomain of RT (23, 24), (b) distorting the catalytic triad (25), and (c) repositioning the primer grip (2, 3, 26). Crystallographic studies of RT in complex with NVP and nucleic acid substrate recently showed that NVP binding affects the positioning of the primer terminus at the polymerase active site (27). Based on pre-steady state kinetics, it was originally proposed that NVP blocks the chemical step of DNA synthesis without significantly affecting the nucleotide binding or the subsequent step (nucleotide-induced conformational change) (28, 29). In addition to approved NNRTIs, several other compounds (including DNA aptamers) have been biochemically studied for inhibition of HIV-1 RT (30–34).

Structural and biochemical transient kinetics studies have been used to study NNRTI resistance and have demonstrated that RT mutations confer resistance to first generation NNRTIs by decreasing their binding either through direct loss of interactions or through steric hindrance (2, 3, 35). Pre-steady state kinetics approaches have shown that mutations remote from the NNIBP reduce binding of the first generation NNRTI NVP primarily by decreasing the association rate of the inhibitor (36–41).

However, there are no published biochemical studies on RT inhibition by RPV or other second generation NNRTIs, and the molecular mechanism of their resistance has not been biochemically described. Recent phase III clinical trials (ECHO, TMC278-TiDP6-C209; THRIVE, TMC278-TiDP6-C215) (42–44) showed that clinical failure in HIV patients receiving combinations of RPV, FTC, and tenofovir disoproxil fumarate (TMC278-TiDP6-C209; THRIVE, TMC278-TiDP6-C215) (42–44) showed that clinical failure in HIV patients receiving combinations of RPV, FTC, and tenofovir disoproxil fumarate (TDF) was associated with mutations at RT codons 184 (primarily M184I), known to cause 3TC/FTC resistance (45–47), and 138 (E138K), known to cause NNRTI resistance (48, 49). Early reports have focused on the characterization of the effect of these mutations (50–52). Xu et al. (51) showed that the decreased viral replication capacity of M184V/I was restored in the presence of E138K. These findings were similar to those by Hu and Kuritzkes (52). However, Kulkarni et al. (50) presented data suggesting that HIV with single or double mutations (E138K, M184V/I, and E138K/M184I) had decreased replication fitness compared with wild-type (WT) HIV. Wainberg and colleagues (51, 53) carried out early biochemical experiments using homopolymeric substrates and reported that the addition of E138K to the M184I background increases the processivity of DNA synthesis (53). Their steady state kinetic constant determination showed that E138K mutation in both subunits is required for restoring \\n
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**Enzymes**—The p51 and p66 sequences of RT were amplified by PCR from pNL43-derived infectious clones and cloned into the pRSFduet-1 vector (Novagen) and pCDFDuet-1 vector (Novagen), respectively, using restriction sites Ncol and SalI for both subunits (53). Sequences coding for a hexahistidine tag were added at the N terminus of p51. There was no tag added to either terminus of the p66 subunit. WT and mutant RTs in heterodimeric forms were purified essentially as described previously (55). Briefly, RT was expressed in *E. coli* M15 (pREP4) (Qiagen, Mississauga, Canada) and induced with 1 mM isopropyl-β-d-thiogalactopyranoside at room temperature. The pelleted bacteria were lysed as described previously (5, 56). The supernatant was subjected to metal affinity chromatography (nickel-nitrioltriacetic acid) as described previously (5, 38). Hexahistidine-tagged RT was eluted with an imidazole gradient. RT-containing fractions were pooled, passed through a Mono Q anion exchange column (GE Healthcare), and further purified using a Superdex 200 gel filtration column (GE Healthcare). Purified RT fractions 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. Protein aliquots were stored at −80 °C.

Nucleic Acids, Nucleotide Triphosphates, and Nonnucleoside RT Inhibitors—Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Labeled primers were annealed to unlabeled templates at a 1:2.5 molar ratio. Deoxynucleotide and deoxyxynucleotide triphosphates were purchased from Fermentas (Glen Burnie, MD). The concentrations of nucleotides and nucleic acids were calculated spectrophotometrically using absorption at 260 nm. RPV was obtained...
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from the National Institutes of Health AIDS Research and Reference Reagent Program.

Active Site Titration and Determination of Template-Primer Binding Affinity (K_{d, DNA})—To determine polymerase-competent RT populations used in this study, we first carried out active site titration assays using pre-steady state experiments. A fixed concentration of RT (50 nM, determined by absorbance measurements) in RT buffer (50 mM NaCl and 50 mM Tris-HCl, pH 7.8) was incubated with increasing concentrations of template-primer (Td_{31}/Cy3-Pd_{18}), followed by rapid mixing with a solution containing 5 mM MgCl₂ and 50 μM dATP in the same RT buffer using a rapid quenched-flow instrument (RQF-3, KinTek Corp., Austin, TX) at 37 °C for 0.25 s. Unless otherwise mentioned, all values represent the final concentrations. The reaction mixtures were quenched by the addition of 150 mM EDTA. Reaction products were resolved in polyacrylamide-urea DNA gels and quantitated by phosphorimaging and densitometry using ImageQuant TL 1.2 (GE Healthcare). The amounts of extended primer (P) were plotted against concentration of template-primer, and the data were fit to the following quadratic equation (Equation 1) using GraphPad Prism version 4.0 (GraphPad, Inc.),

\[
P = 0.5(K_{d, DNA} + [RT] + [DNA]) - \sqrt{0.25(K_{d, DNA} + [RT] + [DNA])^2 - ([RT][DNA])} \quad (Eq. 1)
\]

where [RT] is the concentration of actively binding polymerase molecules, and K_{d, DNA} is the DNA binding constant. Subsequent transient biochemical experiments were performed using corrected active site concentrations.

Pre-steady State Kinetics of dNTP Incorporation—The optimal rate of polymerization (k_{pol}) for WT and mutant RTs was determined by pre-steady state kinetic analysis using single nucleotide incorporation assays. We used two methods: the rapid quenched-flow (RQF) and stopped-flow (SF) methods. For the RQF assay, a solution containing 50 mM WT or mutant RT enzymes and 50 mM Td_{31}/Cy3-Pd_{18} in RT buffer was rapidly mixed with a solution of MgCl₂ (5 mM) and dATP (1–50 mM) for reaction times ranging between 0.005 and 2 s, followed by quenching with EDTA (150 mM). The reaction products were resolved and quantitated as described above. The observed rates at each dNTP concentration were determined by fitting the data to Equation 2 and solving the equation by non-linear regression using GraphPad Prism version 4.0 (GraphPad, Inc.),

\[
P = A(1 - e^{-k_{st}t}) + k_{st}t \quad (Eq. 2)
\]

where A is the amplitude of the burst phase, which represents the concentration of E-DNA complex at the start of the reaction, k_{obs} is the observed burst rate for dNTP incorporation, k_{st} corresponds to the steady state rate constant, and t is the reaction time.

To determine k_{pol} and K_{d,dATP}, the observed burst rates (k_{obs}) were fit to the following hyperbolic equation.

\[
k_{obs} = (k_{pol}[dNTP])/(K_{d,dNTP} + [dNTP]) \quad (Eq. 3)
\]

Stopped-flow Nucleotide Incorporation Assays—The stopped-flow pre-steady state nucleotide incorporation assays were carried out using the KinTek stopped-flow apparatus SF-100 (KinTek Corp., Austin, TX). WT or mutant RTs (60 nM) were incubated on ice for 10 min with 60 mM template-primer that contained 2-aminopurine (2AP) at the +2-position of the 5’-overhang of the template strand. The mixture was equilibrated at 37 °C and then rapidly mixed with reaction mixture containing 5 mM MgCl₂, and varying concentrations of dATP in RT buffer. Changes in the fluorescence of 2AP during the course of a reaction have been previously used to determine kinetic parameters for DNA polymerase-catalyzed reactions, such as nucleotide binding and incorporation, as well as exonucleolytic cleavage (57–61). 2AP-based assays can also be used to probe polymerase-induced conformational changes in 2AP-containing DNA substrates, thus allowing the ability to monitor reaction steps that occur before product formation (57–59, 62). The excitation wavelength was 313 nm, and emission was monitored by using a 370-nm cut-off filter (model E370LP, Chroma Technology Corp., Rockingham, VT). At least four independent replicate fluorescence assays were carried out for each dNTP concentration. All reactions were carried out at 37 °C. The change in 2AP fluorescence as a function of time was monitored at different dNTP concentrations. The traces were averaged using the software provided by KinTek Corp. Relative fluorescence was plotted as a function of time and fit to a biphasic equation (Equation 2). All pre-steady state kinetics experiments were carried out independently at least three times. The S.D. values of all estimated parameters are reported in the respective tables.

Rilpivirine Susceptibility Assays—RPV susceptibility assays were carried out in 96-well plates by measuring the extension of Td_{100}/Pd_{18} template-primer using a PicoGreen-based spectrophotometric assay in the presence of increasing concentrations of RPV. Specifically, reactions containing 20 mM WT or mutant RTs, 50 nM Td_{100}/Pd_{18}, and 10 μM dNTPs in a buffer containing 50 mM Tris, pH 7.8, and 50 mM NaCl were initiated by the addition of 6 mM MgCl₂. DNA synthesis was carried out for 30 min at 37 °C. Reactions were arrested by the addition of 100 mM EDTA. Quant-iT™ PicoGreen reagent (Invitrogen) was added to quantify the amount of formed double-stranded DNA in TE buffer. The reaction mixtures were then excited at 480 nm, and fluorescence was monitored at 520 nm using a 96-well EnSpire Multilabel plate reader (PerkinElmer Life Sciences). Dose-response curves of triplicate samples were plotted using GraphPad Prism version 4.0 (GraphPad, Inc.) to determine IC_{50} values for RPV.

Rilpivirine Binding Kinetics—Solutions of RT (30 nM) and Td_{31}/Cy3-Pd_{18} (30 nM) in RT buffer were incubated with various concentrations (0–75 nM) of RPV (with 2% final DMSO concentration) for 10 min at room temperature before initiating reactions with MgCl₂ (5 mM) and saturating dATP (50 μM). Reactions were allowed to proceed for times between 0.005 and 2 s before quenching with EDTA (150 mM). The reaction products were resolved on 16% polyacrylamide, 8 M urea gels and quantitated as described above. The amounts of extended primers were fit to the burst equation (Equation 2) to calculate burst amplitudes (A) at each concentration of RPV. Burst amplitudes were then fit to the following hyperbolic equation (31) to determine the dissociation constant for RPV (K_{d,RPV}).
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The apparent rate of RPV binding to RT ($k_{\text{app.RPV}}$) was obtained by preincubating a solution of RT (30 nM) and Td18/Cy3-Pd18 (30 nM) in RT buffer with saturating RPV (100 nM) for increasing amounts of time (0.005–2 s). Reactions were then initiated by the addition of MgCl2 (5 mM) and saturating dATP (50 μM), allowed to proceed for 0.2 s, and quenched with EDTA (150 mM). Reaction products were resolved and quantitated as described above. The product amounts were plotted, and the data points were fit with GraphPad Prism version 4 using an equation for single exponential decay,

$$P = A_0(e^{-k_{\text{off.RPV}}t}) + C \quad \text{(Eq. 5)}$$

where $P$ is the reaction product, $A_0$ is the amount of product in the absence of RPV, $t$ is the preincubation time with RPV before the addition of MgCl2 and dATP, and $C$ is a constant.

The $k_{\text{app.RPV}}$ values obtained next were used in the following equation to derive the RPV association constant ($k_{\text{on.RPV}}$) (28).

$$k_{\text{app.RPV}} = k_{\text{on.RPV}}([\text{RPV}] + K_{d_{\text{RPV}}}) \quad \text{(Eq. 6)}$$

The $k_{\text{on.RPV}}$ and $K_{d_{\text{RPV}}}$ values were then used to calculate the RPV dissociation rate ($k_{\text{off.RPV}}$) using the following equation.

$$k_{\text{off.RPV}} = k_{\text{on.RPV}}/K_{d_{\text{RPV}}} \quad \text{(Eq. 7)}$$

**RESULTS**

The subunit-specific mutations were generated to determine the exact role of residues from each subunit in the resistance mechanism of RPV. The crystal structures of RPV-bound HIV-1 RT (Protein Data Bank files 2ZD1 (63) and 3MEE (71)) showed that only p51E138 is part of NNIBP, whereas p66E138 is very distant from the NNIBP (part of the p66 finger subdomain). Therefore, we hypothesized that RPV resistance is due to the E138K change in p51. In addition, the co-emergence of M184I and E138K in THRIVE and ECHO clinical trials led us to generate M184I in p66 because (a) Met-184 of the p66, but not of p51, is close to polymerase active site residues Asp-185 and Asp-186, and (b) we have previously published that 3TC and FTC resistance is caused by mutations at M184I of p66 (46). We did not pursue the characterization of the p66E138K/M184I (p51E138K/M184I) variant of HIV-1 RT (M184I mutation in p51) because p51E138K did not show any change in activity profile (53).

**Effect of Subunit-specific Mutagenesis on Formation of Enzyme-Template-Primer Complex**—RFQ analysis of DNA/DNA binding to WT and mutant RTs showed that the mutations did not cause any significant change in DNA binding affinity of RT (Fig. 1). A small difference was observed between the $K_{d_{\text{DNA}}}$ of p66M184I/p51E138K (11.5 nM) and WT RT (16.3 nM) (Table 1). The t-test statistical analysis showed a probability of 0.08, suggesting that the difference was insignificant. These results were consistent with gel shift assays, which also showed no significant changes in the binding affinity of RTs to DNA/DNA template-primers (data not shown).

**Nucleotide Incorporation Rates and Nucleotide Binding Affinity**—Mutations at codon 184 have been shown to reduce the $K_{\text{m.dNTP}}$ of RT (32, 51, 65). Although steady state experiments provide some information on the efficiency of the reaction, they are less useful in determining detailed active site interactions because they cannot resolve reaction steps that are
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masked by the rate-limiting step of DNA synthesis (release of elongated DNA from RT) (66). This point is particularly important because RT follows an ordered reaction mechanism (67). Enzyme first binds template-primer to form E-DNA complex, which then binds dNTP substrate. This ternary complex (E-DNA-dNTP) undergoes a conformational change to assume a catalytically competent conformation followed by the chemistry of phosphodiester bond formation. The determination of these intermediate steps is critical because the mutation may affect one (or more) intermediate steps that are masked in steady state kinetic characterization. Some pre-steady state experiments have been previously carried out with M184V (46, 68) or RTs from clinical isolates that contained M184I together with other mutations (69), but there have not been such biochemical characterizations of E138K, E138K/M184I, or M184I RT, which are the clinically relevant mutations that appear during treatment failure of RPV-based regimens. Hence, we used pre-steady state experiments to determine the effect of E138K and M184I in either subunit of RT. To ensure that our findings are not biased by the sequence of the template-primer or by the type of kinetics experimentation used, we used two independent pre-steady state kinetics approaches: an RQF method (Fig. 2, A–D) that determines extension of 5’-labeled primers resolved by gel electrophoresis and an SF method that spectrophotometrically determines product formation by following changes in 2AP fluorescence (Fig. 2, E–H). To our knowledge, this is the first study where both methods were used to study the kinetics of RT inhibition and resistance. Fluorescence change was an expression of the reaction product and was fit to a biphasic equation (Equation 2), thus allowing us to determine $k_{\text{obs}}$ at each dATP concentration. The observed rates were then plotted as a function of dATP concentration and fit to Equation 3 to obtain $k_{\text{pol}}$ and $K_{\text{dATP}}$ (Fig. 3, A and B). Kinetic constants ($K_{\text{dATP}}$, $k_{\text{pol}}$, and nucleotide incorporation efficiencies $k_{\text{pol}}/K_{\text{dATP}}$) estimated using the RQF are presented in Table 1, whereas the same kinetic constants measured using the SF approach are presented in Table 2. Comparison of kinetic constants clearly demonstrates that the M184I mutation in p66 reduces the catalytic efficiency ($k_{\text{pol}}/K_{\text{dATP}}$) of RT by ~2.5-fold. This decrease in efficiency primarily emanates from reduced dNTP binding ($K_{\text{dATP}}$) (6.5 μM for WT versus 16.7 μM for p66M184I/p51WT using RQF and 3.4 μM for WT versus 7.9 μM for p66M184I/p51WT using SF).

The subunit-specific mutants p66E138K/p51E138K and p66E138K/M184I/p51E138K displayed catalytic efficiency comparable with that of WT RT (Tables 1 and 2). These data also suggested that E138K restored the dNTP binding affinity of RT that was reduced by M184I. To determine if this effect is solely due to the E138K mutation in p51, we generated and characterized p66M184I/p51E138K. We found that the dNTP binding affinity of p66M184I/p51E138K is very similar to that of WT ($K_{\text{dATP}}$ values of 5.6 versus 6.5 μM measured by RQF or 3.4 versus 3.4 μM measured by SF), clearly demonstrating that E138K in p51 alone is sufficient to compensate for the defect imparted by M184I.

Susceptibility of WT and Mutant RTs to RPV—We used a PicoGreen dsDNA quantitation assay to determine the susceptibility of WT and mutant RTs to RPV under steady state conditions. PicoGreen only forms a fluorescent complex when it specifically binds to the minor groove of dsDNA (70). We exploited this property and used PicoGreen to measure DNA synthesis by RT in the presence of various concentrations of RPV and to obtain IC$_{50}$ values for WT and mutant enzymes. Dose-response curves are shown in Fig. 4, and the IC$_{50}$ values estimated from these curves are listed in Table 3. The IC$_{50}$ values for WT and p66M184I/p51WT were ~14 nM (Table 3 and Fig. 4). However, the IC$_{50}$ for p66E138K/p51E138K (57.7 nM) was ~4-fold higher than the WT enzyme, suggesting that E138K alone is responsible for RPV resistance. To assess the contribution of E138K to the resistance to RPV in a subunit-specific manner, we also determined the IC$_{50}$ for p66WTP/p51E138K which was 3.3-fold higher than for WT (data not shown), suggesting that E138K in p51 is sufficient for RPV resistance. p66M184I/p51E138K and p66E138K/p51E138K had comparable RPV resistance (IC$_{50}$ = 54.3 versus 57.7 nM), which was ~2-fold greater than p66E138K/M184I/p51E138K (IC$_{50}$ = 29.2 nM).

![FIGURE 1. Active site titration and DNA binding affinity determination.](image-url)

**TABLE 1**

| Enzyme                  | $k_{\text{DNA}}$ | $k_{\text{pol}}$ | $k_{\text{dATP}}$ | $k_{\text{pol}}/K_{\text{dATP}}$ | Ratio* (mutant/WT) |
|-------------------------|------------------|------------------|------------------|----------------------------------|--------------------|
| WT                      | 16.3 ± 3.1       | 17.6 ± 1.2       | 6.5 ± 1.6        | 2.7 ± 0.8                        | 1                  |
| p66M184I/p51WT          | 16.8 ± 3.3       | 17.7 ± 1.0       | 16.7 ± 3.1       | 1.1 ± 0.4                        | 0.60               |
| p66E138K/p51E138K       | 11.9 ± 3.3       | 28.1 ± 2.0       | 7.9 ± 1.8        | 2.9 ± 0.8                        | 1.07               |
| p66E138K/M184I/p51E138K | 18.6 ± 2.6       | 19.8 ± 1.4       | 7.9 ± 1.6        | 3.2 ± 1.1                        | 1.18               |
| p66M184I/p51E138K       | 11.5 ± 3.1       | 17.3 ± 1.0       | 5.6 ± 1.1        | 3.1 ± 1.1                        | 1.15               |

*The ratio represents -fold change in incorporation efficiencies ($k_{\text{pol}}/K_{\text{dATP}}$) by mutant enzyme compared with WT.
Kinetics of RPV Binding and Determination of $K_{d,RPV}$, $k_{on,RPV}$, and $k_{off,RPV}$—The kinetic models for NNRTI inhibition of HIV-1 RT are complex and may involve binding of the drug to three different catalytic forms of HIV-1 RT (28, 32). The inhibitor may bind to free enzyme, to RT-TP binary complex, and to RT-TP-dNTP ternary complex. When the mechanism of inhibition (competitive or noncompetitive) is not known, the resulting rate equations of inhibition mechanism are very complex and impractical to use (28, 32). Therefore, simplified RT inhibition models have been employed to understand the inhibition mechanism of NNRTIs (28, 29, 32). In these models, the inhibitor binds to $E$-DNA complex reversibly, and the observed
nucleotide incorporation in transient state assays can only be carried out by uninhibited RT, due to the slow release of inhibitor followed by rapid binding of dNTP to the uninhibited enzyme (28, 29). Therefore, we used transient state kinetics similar to that used previously to probe the interactions of HIV RT with an NNRTI (28, 29, 32).

First, we carried out the time course of dNTP incorporation by WT and subunit-specific mutants with increasing concentration of RPV. The amplitudes of the burst phase were fit to a hyperbolic function (Equation 4) to obtain $K_{d,\text{RPV}}$ (Fig. 5 and Table 4). The data shown in Table 4 show that the $K_{d,\text{RPV}}$ of p66/E138K/M184I/p51E138K was ~2-fold greater than that of WT (36.8 versus 17.9 nM) (Fig. 5 and Table 4), indicating a decreased affinity of p66/E138K/M184I/p51E138K for RPV. However, M184I alone in p66 did not alter the binding affinity of RT to RPV ($K_{d,\text{RPV}}$ of p66/M184I/p51WT and WT were comparable at 21.1 and 17.9 nM, respectively) (Table 4). Importantly, mutation E138K in p51 was sufficient to reduce the binding affinity for RPV because both p66/E138K/p51E138K (31.9 nM) and p66/M184I/p51E138K (28.4 nM) had higher $K_{d,\text{RPV}}$ than WT RT (17.9 nM) (Table 4).

$K_{d,\text{RPV}}$ is the end point and does not necessarily reflect whether E138K primarily affects the association or the dissociation of RPV. Hence, we determined $k_{\text{on,RPV}}$ and $k_{\text{off,RPV}}$ as described under “Experimental Procedures.” It is important to note that $k_{\text{on,RPV}}$ and $k_{\text{off,RPV}}$ in this study correspond to the association and dissociation rates of RPV to and from the $E$-$\text{DNA}$ binary complex. Because there is no direct method to determine either $k_{\text{on,RPV}}$ or $k_{\text{off,RPV}}$, we determined the apparent binding rate ($k_{\text{app,RPV}}$) by mixing the $E$-$\text{DNA}$ complex with a saturating concentration of RPV for specified times, followed by the addition of MgCl2-dNTP for 200 ms. The amounts of DNA product were then fit to Equation 5 to determine $k_{\text{app,RPV}}$. The $k_{\text{app,RPV}}$ was then used in Equation 6 to estimate $k_{\text{on,RPV}}$. Subsequently, using the relationship $K_{d,\text{RPV}} = k_{\text{off,RPV}}/k_{\text{on,RPV}}$, the $k_{\text{off,RPV}}$ values for WT and mutant enzymes were determined.

We and others have shown that M184I in both subunits does not affect NNRTI resistance (51–53). Individual curve fitting of our data in Table 4 shows that the $k_{\text{on,RPV}}$ for WT and p66/M184I/p51WT were comparable (0.07 and 0.06 nM $-1$ s$^{-1}$, respectively) (Fig. 6 and Table 4). However, $k_{\text{on,RPV}}$ values for p66/E138K/p51E138K, p66/E138K/M184I/p51E138K, and p66/E138K/p51E138K were 4.7-, 2.1-, and 4.4-fold greater than $k_{\text{on,RPV}}$ for p66WT/p51WT, suggesting that E138K can increase the association rate of the inhibitor. However, E138K increased the dissociation rate to an even larger extent. Hence, the $k_{\text{off,RPV}}$ values for p66/E138K/p51E138K, p66/E138K/M184I/p51E138K, and p66/E138K/p51E138K were increased 7.2-, 4.2-, and 7.1-fold with respect to the $k_{\text{off,RPV}}$ of WT RT (Table 4). Taken together, our data demonstrate that E138K confers RPV resistance by changing both the association and dissociation rates of the inhibitor, with a net effect of an overall decrease in binding affinity.

We also used KinTek Explorer (64) to simulate the binding of RPV ($K_{d,\text{RPV}}$) and globally fit our quench-flow kinetics data to estimate $k_{\text{on,RPV}}$ and $k_{\text{off,RPV}}$ for various enzymes (Table 4).
global fitting and conventional fitting analyses were generally in agreement, and changes in calculated $k_{on,RPV}$ and $k_{off,RPV}$ values did not appear as significant changes in $K_d,RPV$ values.

Interestingly, conventional data analysis did not show a very significant increase in RPV resistance due to E138K in p51 ($K_d,RPV$ values of p66M184I/p51WT and p66M184I/p51E138K were 21 and 28 nM, respectively. This increase was considerably smaller than the observed changes in IC$_{50}$ for p66M184I/p51WT and p66M184I/p51E138K (Table 3; 15 versus 54 nM, respectively). However, global fitting of the same data clearly showed more significant changes that were in line with the data in Table 3. Hence, the binding affinity of RPV to RT measured by global fitting of the data was changed by 3-fold ($K_d,RPV$ of 10.5 versus 32.9 nM for p66M184I/p51WT and p66M184I/p51E138K, respectively). The global fitting analysis of the data shows that the $K_d,RPV$ was indeed smaller for p66E138K/M184I/p51E138K than for p66E138K/p51E138K and p66M184I/p51E138K, which was consistent with the IC$_{50}$ data in Table 3.

**Molecular Modeling Studies with RT-RPV Complexes**—The crystal structure of WT HIV-1 RT in complex with RPV was initially solved at 1.8 Å resolution by Arnold and colleagues (63) (Protein Data Bank entry 2ZD1). A 2.4 Å resolution RT-RPV structure with differences in crystallographic parameters, including unit cell dimensions and crystal contacts, was also published later by Lansdon et al. (71) (Protein Data Bank entry 3MEE). Although all molecular modeling studies were carried out independently using coordinates from both structures and resulted in the same conclusions, we present and discuss here only the data generated from the higher resolution structure (Protein Data Bank entry 2ZD1) (63).

The crystal structures of the RT-RPV complexes revealed the molecular details of RPV binding to WT HIV-1 RT and offered insights into the mechanism of RT inhibition by second generation NNRTIs. However, the structural basis of the mechanism of RPV resistance is still not well understood because the RPV interactions with RPV-resistant RTs and the orientation of K138 in p51 remain unknown. To gain insights into the role of mutations at positions 138 and 184 in RPV resistance and efficiency of DNA polymerization, we used molecular dynamics simulations and generated structural models of p66WT/p51WT, p66E138K/p51E138K, and p66M184I/p51WT. The resulting models were highly similar to the starting crystallographic coordinates of WT-RPV (root mean square deviation of C$_\alpha$, <1 Å). In the WT-RPV crystal structures, there is an important salt bridge between the side chains of Glu-138 in p51 and of Lys-138 in p66. This salt bridge was also present in the almost identical molecular model that resulted from our quantum mechanical/molecular mechanical protocol. Interestingly, the presence of Lys-138 in p66E138K and p66WT/p51E138K and p66M184I/p51E138K not only eliminates this salt bridge but also results in a significantly larger gap at the bottom of the NNIBP, leading to a repositioning of the inhibitor at the NNRTI-binding site (Fig. 7C). Moreover, although RPV binding is identical in the modeled and crystal structures of WT RT, it appears to change significantly in the modeled structure of p66E138K/p51E138K (Fig. 7A). This repositioning is accompanied by adjustments in the orientation of NNIBP residue Tyr-181. The molecular model of p66M184I/p51WT did not affect the salt bridge between Glu-138 and Lys-138. Also, no significant change in the position of RPV compared with WT was noted in p66M184I/p51E138K and p66M184I/p51E138K (Fig. 7, A and B), suggesting that the mutation at position 184 does not significantly affect the affinity for RPV, which is consistent with the biochemical data shown in Table 4. The conformation of p66101K in the modeled structures of p66WT/p51E138K (Fig. 8A) and p66E138K/p51E138K (Fig. 7) is significantly different. To assess the effect of these two conformations on the opening of the RPV binding pocket, we measured the distances between atoms of key residues (p51E138 or p51_138K and p66K101) of the pocket. The distance between OE atoms of p51E138 from NZ of p66K101 (i.e. the salt bridge) is ~3 Å, whereas the distance between NZ atoms p51_138K (E138K

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**TABLE 3**

| Enzyme | IC$_{50}$ | Resistance |
|--------|----------|------------|
| WT     | 14.1 ± 1.6 | 1.0 |
| p66M184I/p51WT | 14.7 ± 2.1 | 1.0 |
| p66E138K/p51E138K | 57.7 ± 2.8 | 4.1 |
| p66E138K/p51E138K | 29.2 ± 2.1 | 2.1 |
| p66M184I/p51E138K | 54.3 ± 2.6 | 3.9 |
mutation) and p66E101 is 5.2 Å, suggesting a larger opening in RPV binding site in p66E138K/M184I/p51E138K compared with that in WT enzyme. The distance between the NZ atom of p51138K and p66K101 is nearly twice the corresponding distance in p66 E138K/M184I/p51E138K. These differences in atom positions appear to be consistent with RPV IC50 (58 nM for p66E138K/p51E138K versus 29 nM for p66E138K/M184I/p51E138K) and koff.RPV (11.5 s^-1 for p66E138K/p51E138K versus 7.5 s^-1 for p66E138K/M184I/p51E138K) for two enzymes. In addition, we would like to point out here that the substitution of Glu-138 by Lys-138, which disrupts the salt bridge between p51E138 and p66K101 changes the dynamics of RPV-binding pocket entrance due to proximity of two positive residues (Lys-101 and Lys-138). The breaking of the salt bridge upon E138K mutation was also noted by Kulkarni et al. (50). The other notable changes were in the position of Tyr-183 in p66M184I/p51WT and Tyr-181 in p66E138K/M184I/p51E138K (Fig. 8B).

**TABLE 4**

| Enzyme                          | Kd,RPV | kon.RPV | koff.RPV |
|---------------------------------|--------|---------|----------|
| WT                              |        |         |          |
| p66M184I/p51WT                  |        |         |          |
| p66E138K/p51E138K               |        |         |          |
| p66E138K/M184I/p51E138K         |        |         |          |
| p66M184I/p51E138K               |        |         |          |

* Values obtained by fitting data to Equations 4–6.
* Values obtained by globally fitting the data using KinTek Explorer.

**FIGURE 6.** RPV binding kinetics, determination of apparent RPV binding rates (kapp.RPV). To determine kapp.RPV, 30 nm WT or mutant RTs were first incubated with 30 nM Td31/Cy3-Pd18. This reaction mixture was rapidly mixed with an equal volume of 100 nm RPV for the indicated times before then being mixed with MgCl2 (5 mM) and saturating dATP (50 μM). The reactions were quenched with 150 mM EDTA, pH 8.0. Reaction products were fit to a single exponential decay equation to obtain the observed binding rates for two enzymes. In addition, we would like to point out here that the substitution of Glu-138 by Lys-138, which disrupts the salt bridge between p51E138 and p66K101 changes the dynamics of RPV-binding pocket entrance due to proximity of two positive residues (Lys-101 and Lys-138). The breaking of the salt bridge upon E138K mutation was also noted by Kulkarni et al. (50). The other notable changes were in the position of Tyr-183 in p66M184I/p51WT and Tyr-181 in p66E138K/M184I/p51E138K (Fig. 8B). The addition of mutation M184I to p66E138K/p51E138K (i.e., p66E138K/M184I/p51E138K) appeared to abrogate
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FIGURE 7. Binding of RPV with WT and mutant RTs. A, superposition of RPV-bound structures of p66<sub>wt</sub>/p51<sub>wt</sub> (magenta) and p66<sub>wt</sub>/p51<sub>E138K</sub> (cyan). The dotted lines represent the salt bridge between p51<sub>E138K</sub> and p66<sub>E138K</sub>. Mutation of Glu-138 (magenta) to Lys-138 (cyan) rearranges the side chain of Lys-101, and the ion pair that existed in the WT (dotted lines) enzyme is broken. E138K mutation also causes a slightly altered binding position of RPV (magenta versus cyan). A conformational change in the Tyr-181 side chain is also shown. B, surface representation of binding pocket and position of Glu-138 and M184I in WT and E138K (C) showing the breaking of the Glu-138–Lys-101 salt bridge, which enhances access to the NNIBP.

the repositioning of Tyr-183 (Fig. 8B), suggesting a possible structural mechanism by which the concomitant presence of E138K and M184I mutations might restore the structural integrity of the region next to the polymerase active site. Similarly, Das et al. (27) have predicted that β7-β8 loop mutations facilitate the exit of NNRTIs from NNIBP.

DISCUSSION

Rilpivirine and ETV are second generation NNRTIs that belong to the diarylpiperidine family of compounds. Both are potent inhibitors of HIV-1 RTs that are resistant to first generation NNRTIs (nevirapine, delavirdine, and efavirenz). RPV and ETV demonstrate “strategic flexibility” that allows efficient binding to a high plasticity NNIBP (20, 21, 26, 72, 73). These two drugs were discovered by a multidisciplinary approach that involved x-ray crystallography, structure-based drug design, and in vivo testing (21, 74–80). RPV has considerably better potency and turnover constants. Hence, similar to data on M184V or other RTs that also included mutation at codon 184, we found that M184I decreased the efficiency of RT polymerization (K<sub>pol</sub>/K<sub>d, dNTP</sub> (92), primarily by decreasing the binding affinity for the dNTP substrate (K<sub>d, dNTP</sub>) without affecting the turnover of the reaction (k<sub>pol</sub>).

To determine the structural basis of the changes in fitness, we carried out molecular modeling studies and compared crystal structures previously solved by us and by others. Our molecular model of the M184I RT–RPV complex suggests that M184I affects the position of Tyr-183 (Fig. 8A). We hypothesize that this change affects the positioning of DNA and interactions at the dNTP binding pocket. This is supported by our previously solved crystal structure of M184I RT-DNA (47), where we dem-
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FIGURE 8. Effect of M184I and E138K mutations on neighboring residues. A, effect of M184I on Tyr-183 position. Superposition of modeled RPV-bound structures of WT (magenta) and p66E138K/p51WT (orange) RTs. Nitrogen and oxygen atoms are colored blue and red, respectively. The change in the position of Tyr-183 by the M184I mutation is marked by an arrow. The RPV in WT structure is shown in color by atom (carbon (gray) and nitrogen (blue)) and in p66E138K/p51E138K, in orange. The salt bridge between p51E138 and p66K101 is observed in both WT and M184I RTs. B, mutation E138K in the M184I background (p66E138K/M184I/p51E138K, dark green) repositions Tyr-183 to a position similar to that in WT (magenta) RT. RPV (in a ball-and-stick representation) is shown in gray carbon atoms in WT and dark green carbon atoms in mutant enzyme. The other atoms are colored as carbons, pink for WT and dark green for p66E138K/M184I/p51E138K. The oxygen and nitrogen atoms are red and blue, respectively, in both enzymes.

E138K appears to enhance the efficiency of M184I RT by restoring dNTP binding (Tables 1 and 2) (51, 53). The structural basis of this reversal appears to involve restoration of Tyr-183 in a WT-like conformation, as shown in the superposition of the M184I/E138K RT-RPV and WT-RPV models in Fig. 8B. Restoration of the Tyr-183 interactions is also likely to help overcome the processivity defect of M184I RT (51). The effect is likely to be the result of improved dNTP binding, which is also known to affect the processivity of polymerases (54, 96, 97).

Until now, there have been no transient kinetics biochemical studies with any of the second generation NNRTIs. Xu et al. (51) and Hu and Kuritzkes (52) determined IC₅₀ values for the inhibition of RTs by ETV, rather than with RPV, which was not widely available at the time. Here we determined that the E138K mutation in p51 confers low level reduced susceptibility to RPV in the presence and absence of the M184I mutation (~3–4-fold). Moreover, the molecular mechanism by which E138K causes resistance to RPV has not been known. Our work demonstrates, for the first time, that RPV resistance by the E138K mutation by itself or in combination with M184I is achieved through a large increase in the dissociation rate of the inhibitor ($k_{\text{off,RPV}}$), which overcomes a smaller enhancement in its rate of association ($k_{\text{on,RPV}}$) (Table 4). These data are in line with the molecular modeling observations, which highlight differences in accessibility to the NNRTI binding pocket. In conclusion, our study provides biochemical insights into the mechanism by which the E138K/M184I RT mutations confer resistance to RPV and into the mechanism by which these mutations determine changes in the catalytic efficiency of RT.

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