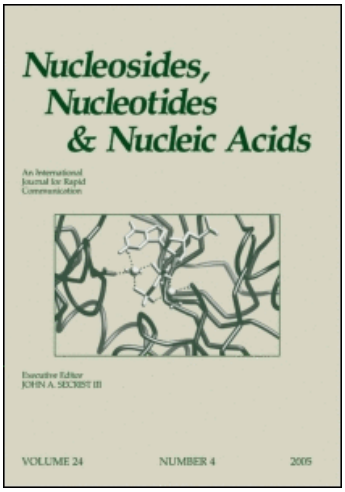

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VIROLOGIC AND ENZYMATIC STUDIES REVEALING THE MECHANISM OF K65R- AND Q151M-ASSOCIATED HIV-1 DRUG RESISTANCE TOWARDS EMTRICITABINE AND LAMIVUDINE

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□ *Emtricitabine (FTC) and lamivudine (3TC) are deoxycytidine analogues with potent and selective inhibition of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) replication. The K65R mutation in the HIV reverse transcriptase (RT) confers reduced susceptibility to 3TC, ddC, ddI, abacavir, and tenofovir* in vitro. *The Q151M mutation confers reduced susceptibility to many of the approved anti-HIV nucleoside analogues with the exception of 3TC and tenofovir. The double mutation K65R/Q151M has been shown to be more resistant to many NRTIs than either of the single mutations alone. In this study, we measured the antiviral activity of FTC and 3TC against HIV-1 containing K65R, Q151M, and K65R/Q151M mutations. We also studied the steady-state kinetic properties for the inhibition of dCTP incorporation by FTC 5′-triphosphate (TP) and 3TC-TP. In addition, we measured the incorporation of dCTP, FTC-TP, and 3TC-TP into a random sequence DNA/DNA primer/template by the HIV-1 RTs using pre-steady-state kinetic analysis. Finally, we studied the incorporation of these deoxycytidine analogues into a HIV-1 genomic DNA/DNA primer/template by K65R HIV-1 RT to address certain concerns associated with DNA sequence specificity. Overall, this study demonstrated that K65R and K65R/Q151M related drug resistance to FTC and 3TC was mainly due to a significant decrease in the rate of incorporation. There was little to no effect on the binding affinities of the mutant HIV-1 RTs for the deoxycytidine analogues. The Q151M mutation remained sensitive to both FTC and 3TC in both cell culture and enzymatic assays. At a molecular level, FTC-TP was incorporated at least as efficiently as 3TC-TP for all of the HIV-1 RT and primer/templates tested.*

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Keywords HIV-1 RT; K65R; Q151M; FTC; 3TC; Pre-steady state; Emtricitabine; Lamivudine

INTRODUCTION

Emtricitabine ((-)-β-2′,3′-dideoxy-5-fluoro-3′-thiacytidine, FTC) and lamivudine ((-)-β-2′,3′-didoexy-3′thiacytidine, 3TC) are L-cytidine nucleoside analogues that are potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) replication. These antiviral agents, once converted to their 5′-triphosphates (TP) by cellular enzymes, inhibit the DNA and RNA-dependent DNA synthesis catalyzed by HIV-1 reverse transcriptase (RT). The primary drug resistance mutation related to FTC and 3TC is M184V.[1–5] In addition, the K65R (AAA to AGA) mutant has also been shown to be 17- to 20-fold resistant to 3TC *in vitro*.[6,7] The K65R mutation is found infrequently (<4%) *in vivo*[8] and is selected for following treatment with ddI,[9] abacavir,[10] and tenofovir.[11] In cell culture, HIV-1 with the K65R mutation confers 3- to 10-fold resistance to zalcitabine (ddC),[7,12] 3-fold resistance to didanosine (ddI),[7] 3.7-fold to abacavir,[7] 3.2-fold to tenofovir,[13] and 5.6-fold to (-)-β-D-dioxolane guanosine (DXG).[6,13] No resistance to zidovudine (AZT) or stavudine (d4T) is observed with the K65R mutation.[12] The 2-bp mutation Q151M (CAG to ATG) is most often observed with a cluster of mutations (A62V, V75I, F77L, and F116Y) and confers high levels of resistance to most of the nucleoside analogues used in the clinic.[14–16] However, the prevalence of the Q151M mutation is low, varying from 0.85 to 3.5% in patients receiving long-term multiple NRTIs.[17–20] HIV-1 with the Q151M mutation alone has been reported to be 6-,[21] 10-,[22] and 30-fold[23] resistant to AZT, 3-[23] to 5-fold[22] resistant to ddI, 4- to more than 20-fold[21,22] resistant to ddC, 3-fold resistant to d4T,[22] and 9.6-fold resistant to DXG.[6] In contrast, Q151M HIV did not show resistance to 3TC or tenofovir.[6,23,24] There is a positive correlation between the presence of K65R and Q151M mutations in clinical isolates.[25] A recent study showed that viruses containing the double mutation K65R/Q151M were more resistant to many NRTIs than the viruses containing either of the single mutations as shown by the 17.8-, >20-, >20-, and >10-fold changes in loss of sensitivities to AZT, DXG, 3TC, and abacavir compared to the wild-type (wt) virus.[6] The combination also showed >10-fold resistance to tenofovir.[24]

Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZT^R, AZT-resistant; ddC, 2′,3′-dideoxycytosine; ddI, 2′,3′-dideoxyinosine; d4T, 2′,3′-didehydro-3′-deoxythymidine; DXG, (-)-β-D-dioxolane guanine; D30/D45, DNA/DNA primer/template 30/45-mer; D30/R45, DNA/RNA primer/template 30/45-mer; HBV, hepatitis B virus; HIV, human immunodeficiency virus; MP, 5′-monophosphate; NRTI, nucleoside reverse transcriptase inhibitor; dNTP, 2′-deoxynucleoside RT, reverse transcriptase; SDR, site-directed mutagenesis; 3TC, (-)β-L-2′,3′-dideoxy-3′-thiacytidine; $3TC^R$, 3TC-resistant; TP, 5′-triphosphate; wt, wild-type; XTT, 2, 3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino)carbonyl]-2H-terazolium hydroxide.

The aim of this study is to fully understand the impact of K65R, Q151M, and K65R/Q151M mutations on the viral susceptibility to FTC and the mechanism of resistance towards FTC and 3TC. First, we measured the antiviral activity of FTC and 3TC against K65R, Q151M, and K65R/Q151M mutant HIV-1 in cell culture assays. Secondly, we analyzed the kinetics of FTC-TP and 3TC-TP inhibition of dCMP incorporation by wt and mutant recombinant HIV-1 RT containing K65R, Q151M, and K65R/Q151M using steady-state analysis. To do so, we determined the catalytic rate constant (k_{cat}) for dCTP, the apparent binding constant (K_m) for dCTP, and the apparent inhibition constant (K_i) for FTC-TP and 3TC-TP. Thirdly, we used pre-steady-state kinetic analysis to assess the DNA-dependent incorporation of dCTP, FTC-TP, and 3TC-TP into a DNA/DNA 23/45-mer by the wt and mutant RTs. Two pre-steady-state kinetic constants, the maximum rate of incorporation (k_{pol}) and the dissociation constant (K_d), were reported for each of the deoxycytidine analogue. A third pre-steady-state rate constant, k_{ss}, which reflects the rate of product release, was also reported for the dCTP incorporation. Finally, in order to address whether the observed pre-steady-state kinetic parameters are specifically associated with the random sequence DNA/DNA 23/45-mer, we conducted a pre-steady-state analysis using a HIV-1 genomic DNA/DNA 18/36-mer to study the incorporation of dCTP, FTC-TP, and 3TC-TP by wt and K65R HIV-1 RT.

EXPERIMENTAL PROCEDURES

Reagents

The non-radiolabeled dCTP and [α-^{32}P]-dCTP were purchased from Amersham Biosciences. FTC-TP and 3TC-TP were synthesized by Moravek Biochemicals (Brea, CA) and quantified using UV absorbance. Polyinosinic acid (poly(rI)) (Sigma P-4154) and oligo deoxycytidylic acid (oligo$(dC)_{12}$) (Sigma O-9128) were annealed as previously reported.[26] PAGE-purified DNA primers (18- and 23-mer) and DNA templates (36- and 45-mer) were obtained from Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). The concentrations of the oligonucleotides were estimated by UV absorbance

TABLE 1 Sequences of Hetero DNA/DNA Primer/Template Used in the Study

Primer/template	Origin	Sequences
D23/D45	Random sequence	5′-GCC TCG CAG CCG TCC AAC CAA CT-3′ 3′-CGG AGC GTC GGC AGG TTG GTT GAG TTG GAG CTA GGT TAC GGC AGG-5′
D18/D36	HIV-1 genome	5′-G TCC CTG TTC GGG CGC CA-3′ 3′-CGA AAG TCC AGG GAC AAG CCC GCG GTG ACG ATC TCT-5′

at 260 nm as previously reported.[5,27] 5′-^{32}P-Labeling of DNA 23-mer and annealing of DNA/DNA primer/template 23/45-mer (D23/D45) were also conducted as previously reported.[27] All other buffers and reagents were of analytical grade.

Phenotypic Characterization

Recombinant viruses containing the K65R mutation were generated by homologous recombination as previously described.[13] Viruses containing the Q151M mutation or the K65R/Q151 M mutations were created by site-directed mutagenesis (SDM) in the wild-type LAI proviral clone using the Stratagene QuikChange™ SDM kit. All of the mutant viruses were analyzed for their phenotypic sensitivity to FTC and 3TC in MT2 cells as described previously.[6,13] Cells were infected at an M.O.I. (multiplicity of infection) of 0.03 for 3 to 6 h at 37°C in culture media and then plated at 3×10^4 cells/well into a 96-well plate containing various concentrations of drug in triplicate. The cultures were incubated for 5 days at 37°C in a humidified 5% CO_2 atmosphere. Antiviral activity was determined using the XTT (2, 3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino)carbonyl]-2H-terazoliumhydroxide)-based cytotoxicity assay. The plates were read on an Elisa plate reader at a wavelength of 450 nm and a dose-response curve was generated using the untreated infected cells as 0% protection. Fifty percent effective concentrations (EC_{50}s) were calculated as an average from at least three experiments.

Enzyme Expression and Purification

The plasmid, p66HIS-PROT,[28] which contains the 66-kDa coding region for HIV-1 RT and the HIV-1 protease coding region was obtained from Dr. Stephen Hughes at the National Institute of Health. The C-terminal of the 66-kDa subunit of RT included six consecutive histidine residues. The 51-kDa subunit was formed from the processed 66-kDa subunit and formed stable heterodimer p66/p51 enzyme. The plasmid was used as the template for site-directed mutagenesis using the Stratagene QuikChange™ SDM kit following the manufacturer's instruction. The entire RT coding region of each mutated plasmid was analyzed by dideoxy sequencing to confirm the accuracy of the sequence.

The wild-type and mutant clones were transformed into competent BL21 cells (Stratagene, La Jolla, CA) following the manufacturer's instruction. The cells were grown in 2 L LB broth containing ampicillin (100 mg/L) at 37°C to an optical density of 0.6 unit at 595 nm. The cultures were then induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) and the cells were allowed to grow for additional 4–5 h until the cell growth reached a plateau. The wt and mutant RTs were purified as previously described with modifications.[29] All purification steps were done at 0–4°C and cells

were harvested by centrifugation for 5 min at 8000 rpm. After resuspension in 40 mL (20 mL/liter growth) lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM β-mercaptoethanol, 0.1 mg/mL lysozyme), cells were stirred at room temperature for 30 min and sonicated on ice for 3 min. The lysed cells were then centrifuged for 1 h at 10,000 rpm. The supernatant was treated with 6.0% polyethylene imine (PEI) to a final concentration of 0.4% to precipitate nucleic acids and followed by centrifugation at 8000 rpm for 45 min. The supernatant was then precipitated with 70% $(NH_4)_2SO_4$ and centrifuged at 8000 rpm for 1 h. The resulting protein pellet was resuspended in and dialyzed against Buffer A (50 mM Tris-HCl, 10% (v/v) glycerol, 0.5 M NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol). The crude RT was purified by Ni-affinity column using Ni-NTA Superflow resin (Qiagen, Valencia, CA). The dialyzed protein solution was mixed with 1 mL (bed volume, 0.5 mL/liter growth) pre-equilibrated resin, gently rotated on a rotor at 4°C for 1 h, and loaded onto a 3-cm × 13-cm column. The resin was then washed with Buffer A until no protein ($UV_{280} < 0.07$) was detected in the flow-through. The column was eluted with 100-mL step gradient from 5 mM to 0.1 M imidazole in Buffer A (5 mM imidazole increment per step) and 5-mL fractions were collected. Fractions containing RT (as determined by SDS-PAGE analysis) were pooled, concentrated down to 2 mL using a 250-mL Amicon Stirred Cells Concentrator with a YM30 membrane (Millipore, Bedford, MA), and dialyzed against Buffer B (50 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 2 mM dithiothreitol). The resulting protein was then further purified on a pre-equilibrated Q-Sepharose™ Fast Flow column (1.5 mL bed volume, Amersham Biotech, Piscataway, NJ). The column was washed with 10 mL Buffer B and eluted with a 50-mL step gradient from 0 to 50 mM NaCl in Buffer B (5 mM NaCl increment per step) and 5-mL fractions were collected. Fractions containing RT as determined by SDS-PAGE analysis were pooled, concentrated, and exchanged into RT storage buffer (50 mM Tris-HCl, pH 8.0, 50% glycerol (v/v), 50 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol). Protein concentration was measured spectrophotometrically at 280 nm using an extinction coefficient of 260,450 $M^{-1}cm^{-1}$. The concentration of active RT in each preparation was determined by pre-steady-state burst experiments that yielded the active site concentration (~30% of enzyme concentration).

Steady-state Kinetic Analysis for Substrate Incorporation and Analogue Inhibition

The steady-state kinetic constants for dCTP incorporation by HIV-1 RT were determined by measuring the initial rates of product formation. Reaction mixtures contained 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 0.025% Triton X-100, 18 nM enzyme (protein concentration), 0.012 units

of poly(rI):dC_{12-18}, 1 μCi of [α-^{32}P]-dCTP, and varying concentrations of dCTP in a final volume of 50 μL. All reactions were carried out at 37°C and initiated by the addition of enzyme. Five microliter aliquots of the reaction mixture were quenched by being spotted on DE81 ion exchange paper following a time course of 0.5, 1, 1.5, 2, 2.5, and 3 min, which was in the linear phase of the reaction. The DE81 ion exchange paper was washed with 50 mg/mL Na_2HPO_4 (3 times, 5 min/wash), rinsed with water and ethanol, and air dried. Product quantification was performed using a Bio-Rad Personal Imager FX. The observed rates were obtained by plotting the product formation over time of the reaction and fitting the data using linear regression. The K_m and V_{max} values were determined by plotting the observed rate over the substrate (dCTP) concentration through 3D nonlinear regression using Grafit (Version 3.09b, Erithacus software, UK) and fitting the data to the Michaelis-Menten equation:

$$v = [E]_0[S]k_{cat}/([S] + K_m)$$

Values were reported as means ± standard error from at least three experiments.

Inhibition analysis of FTC-TP and 3TC-TP were carried out under conditions similar to the ones mentioned above, except that at each concentration of dCTP, varying concentrations of FTC-TP or 3TC-TP were also included in the reaction mixture. The K_i values were determined by plotting the observed rate over the substrate (dCTP) concentration under each inhibitor (FTC-TP or 3TC-TP) concentration and by fitting the observed rate to the modified Michaelis-Menten equation for competitive inhibition through 3D non-linear regression using Grafit (Version 3.09b, Erithacus software, UK):

$$v = [E]_0[S]k_{cat}/[[S] + K_m(1 + [I]/K_i)]$$

Values were reported as means ± standard error from at least three experiments.

Pre-steady-state Kinetic Analysis

Transient kinetic experiments were performed by the rapid quench method as described previously using a KinTek Instrument Model RQF-3 rapid–quench-flow apparatus.[30] All reactions were carried out at 37°C. Unless noted otherwise, all concentrations referred to the final concentration after mixing. Under pre-steady state burst conditions, the reactions were carried out by mixing a solution containing the pre-incubated complex of HIV-1 RT (100 nM) and 5′-^{32}P-labeled D23/D45 (or D18/D36) primer/template duplex (300 nM) with a solution containing 10 mM $MgCl_2$ and various concentrations of the dNTP. When single turnover conditions were used, the

reactions were carried out either on the quench apparatus or performed manually by mixing a solution containing the pre-incubated complex of HIV-1 RT (200–250 nM) and 5′-^{32}P-labeled D23/D45 (or D18/D36) duplex (50 nM) with a solution of 10 mM $MgCl_2$ and various concentration of the dNTP. The reactions were quenched with 0.3 M EDTA at time intervals ranging from 3 ms to 60 min.

The products from each quench reaction were resolved by electrophoresis (14% acrylamide, 8 M urea) followed by phosphor imaging on a Molecular Imager (Bio-Rad Personal FX). Data were fitted by nonlinear regression (KaleidaGraph 3.51, Synergy Software, Reading, PA). Under burst conditions, the product formation occurred in a fast exponential phase followed by a slower linear phase. Data from burst experiments were fit to a burst equation:

$$[\text{Product}] = A[1 - \exp(-k_{\text{obsd}}t) + k_{\text{ss}}t]$$

where A represents the amplitude of the burst that correlates with the concentration of enzyme in the active form, k_{obsd} is the observed first-order rate constant for dNTP incorporation, k_{ss} is the observed steady state rate constant, and t is the reaction time. A typical burst curve fitting is plotted in Figure 1. Data from single-turnover experiments were fit to a single

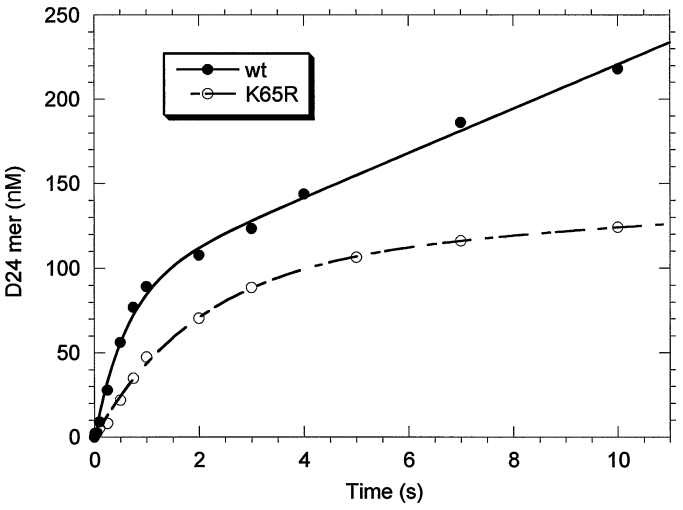

FIGURE 1 Pre-steady-state kinetics for the incorporation of 50 μM dCTP into a DNA/DNA 23/45 primer/template by wt (●) and K65R (○) HIV-1 RT. The solid line represents the best fit of the wt data (●) to a burst equation with an amplitude $A = 100 \pm 5$ nM, an observed first-order rate constant for the burst phase $k_{\text{obsd}} = 1.4 \pm 0.2\ \text{s}^{-1}$, and an observed rate constant for the linear phase $k_{\text{ss}} = 0.11 \pm 0.01\ \text{s}^{-1}$. The dashed line represents the best fit of the K65R data (○) to a burst equation with $A = 107 \pm 9$ nM, $k_{\text{obsd}} = 0.50 \pm 0.05\ \text{s}^{-1}$, and an observed rate constant for the linear phase $k_{\text{ss}} = 0.02 \pm 0.01\ \text{s}^{-1}$.

exponential equation:

$$[\text{Product}] = A[1 - \exp(-k_{\text{obsd}}t)]$$

An example of a single turnover curve fitting is shown in Figure 2. The dissociation constant, K_d, for dNTP binding to the enzyme-DNA complex was calculated by fitting the data to the hyperbolic equation:

$$k_{\text{obsd}} = (k_{\text{pol}} \times [\text{dNTP}])/(K_{\text{d}} + [\text{dNTP}])$$

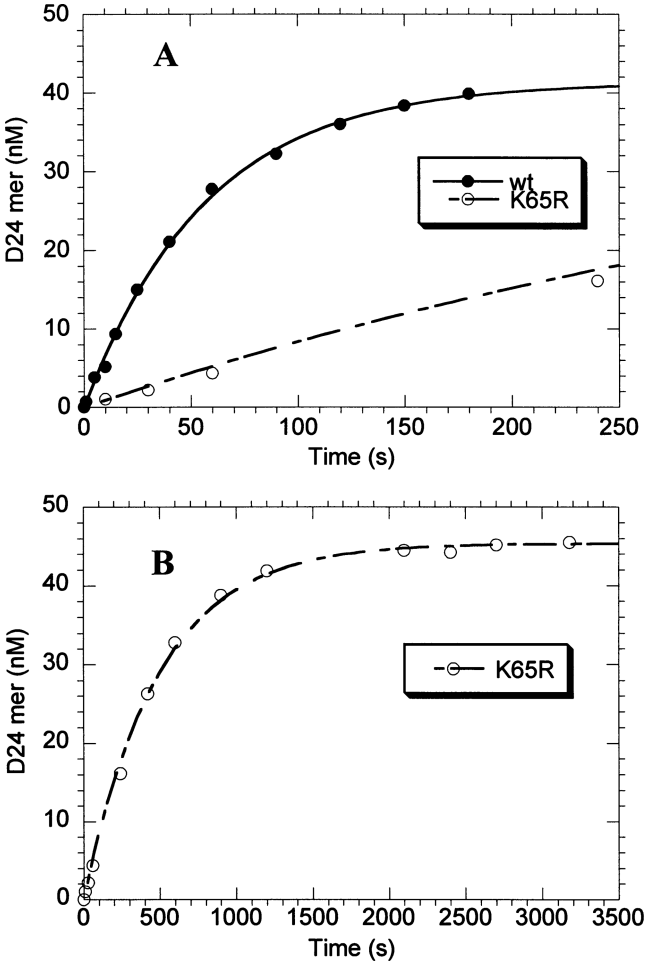

FIGURE 2 Pre-steady-state kinetics for the incorporation of 50 μM FTC-TP into a DNA/DNA 23/45 primer/template by wt (●) and K65R (○) HIV-1 RT. (A) The solid line represents the best fit of the wt data (●) to a single turnover equation with an amplitude $A = 41 \pm 1$ nM and an observed first-order rate constant for the burst phase $k_{\text{obsd}} = 0.0175 \pm 0.0006\ \text{s}^{-1}$. The dashed line represents the best fit of the K65R data (○) to a single turnover equation with $A = 45 \pm 1$ nM and $k_{\text{obsd}} = 0.0020 \pm 0.0001\ \text{s}^{-1}$. (B) The curve fitting for K65R HIV-1 RT was re-plotted to reveal its full scale.

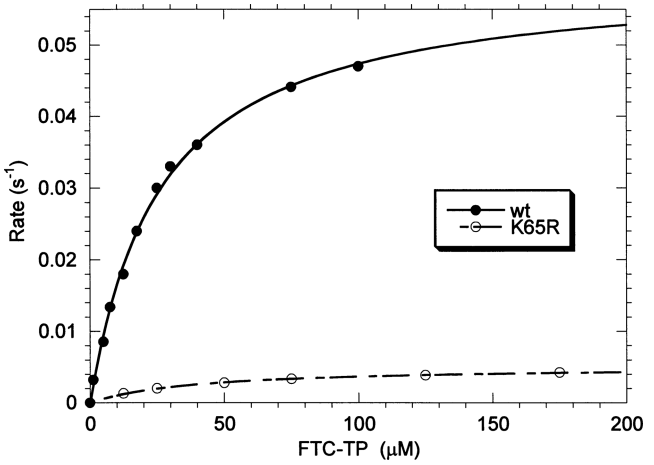

FIGURE 3 Concentration dependence of FTC-TP upon the observed first-order rate constant (k_{obsd}) for FTC-MP incorporation into a DNA/DNA 23/45 primer/template by wt (●) and K65R (○) HIV-1 RT. The data were fit to a hyperbola to yield a K_d value of 26 ± 1 μM and k_{pol} value of 0.060 ± 0.001 s^{-1} for the wt enzyme, and a K_d value of 40 ± 2 μM and k_{pol} value of 0.0051 ± 0.0001 s^{-1} for the K65R HIV-1 RT.

k_{pol} is the maximum rate of dNTP incorporation and [dNTP] is the corresponding concentration of dNTP. A typical K_d determination and curve fitting is shown in Figure 3.

RESULTS

In this study, we tested the antiviral activity of FTC and 3TC against viruses containing the K65R, Q151M, and K65R/Q151M mutations in HIV-1 RT. Fold changes in EC_{50} values for each of the mutant virus as compared to wild-type were evaluated. Because of the variability inherent in these assays, changes in EC_{50} less than 3-fold were considered to be insignificant. The results are summarized in Table 2. The K65R and K65R/Q151M mutations resulted in reduced susceptibility to both FTC and 3TC. For FTC, the K65R

TABLE 2 Antiviral Activity of FTC and 3TC against HIV-1 Containing K65R or Q151M Mutations

	Mean EC_{50} (μM) (fold change)	
Virus genotype	FTC	3TC
Wild-type (HXB2)	0.77 ± 0.09 (1.0)	3.9 ± 0.6 (1.0)
K65R (SDR[a], backbone HXB2)	6.4 ± 2.0 (8.4)	46 ± 16 (11.7)
Wild-type (LAI)[b]	0.6 ± 0.4 (1.0)	2.4 ± 1.4 (1.0)
Q151M (SDR[a], backbone LAI)	1.4 ± 0.9 (2.3)	3.7 ± 2.6 (1.5)
K65R/Q151M (SDR[a], backbone LAI)	>20 (>20)	>50 (>20)

[a]Site-directed mutant containing only the mutation indicated.
[b]EC_{50} values are averages ± standard errors for at least three experiments.

TABLE 3 Steady-state Kinetic Parameters for dCMP Incorporation into Homopolymeric Poly(rI):oligo(dC)$_{12}$ Template/Primers by Wild-type and Mutant HIV-1 RTs

HIV-1 RT	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min$^{-1}\mu$M^{-1})
wt	0.49 ± 0.04[a]	24 ± 1	49
K65R	1.1 ± 0.2	20 ± 1	18
Q151M	0.73 ± 0.3	22 ± 1	30
K65R/Q151M	0.95 ± 0.1	27 ± 2	28

[a]Values are mean $\pm$ standard error, as a result of at least three measurements.

mutation resulted in an 8.4-fold increase in EC_{50} as compared to wild-type while the double K65R/Q151M mutant had a >20-fold increase in EC_{50} value for FTC. Similarly, viruses containing the K65R and K65R/Q151M mutations demonstrated reduced susceptibility to 3TC with increases in EC_{50} values ranging from 11.7-fold (K65R) and >20-fold (K65R/Q151M) compared to wild-type. A virus containing only the Q151M mutation in HIV-RT remained sensitive to inhibition by both FTC and 3TC (fold changes <3-fold).

Using steady-state kinetic analysis, we first evaluated the incorporation of natural substrate dCTP into a homopolymeric template/primer poly(rI):oligo(dC)$_{12}$ by wt and mutant K65R, Q151M, and K65R/Q151M HIV-1 RT. The values for the catalytic constant (k_{cat}) and the apparent dissociation constant (K_m) are listed in Table 3. While the k_{cat} values for all of the mutant enzymes were similar to that of the wt enzyme, the K_m values were slightly (1.5- to 2.2-fold) higher than the wt. As the result, the catalytic efficiency (k_{cat}/K_m) for dCTP was 2.7-, 1.6-, and 1.8-fold lower than the wt enzyme for the mutant enzymes K65R, Q151M, and K65R/Q151M, respectively.

We also used the same system to examine the ability of FTC-TP to inhibit dCTP incorporation by the wt and mutant RTs. The apparent inhibition constant K_i and the specificity of inhibition (expressed as the ratio of K_i/K_m) are summarized in Table 4. In order to assess the effect of the

TABLE 4 Steady-state Kinetic Constants for FTC-TP and 3TC-TP Inhibition of dCMP Incorporation into Homopolymeric Poly(rI):oligo(dC)$_{12}$ Template/Primer by Wild-type and Mutant HIV-1 RTs

	FTC-TP			3TC-TP[c]		
HIV RT	K_i (μM)	K_i/K_{m-dCTP}	Fold change[b]	K_i (μM)	K_i/K_{m-dCTP}	Fold change[b]
wt	0.20 ± 0.06[a]	0.41	1.0	0.23 ± 0.03	0.47	1.0
K65R	0.42 ± 0.05	0.38	0.93	0.89 ± 0.17	0.80	1.7
Q151M	0.20 ± 0.02	0.27	0.66	0.20 ± 0.03	0.28	0.59
K65R/Q151M	0.75 ± 0.25	0.79	1.9	0.42 ± 0.06	0.44	0.94

[a]Values are mean $\pm$ standard error, derived from at least three measurements.
[b]"Fold change" represents the mutant RT K_i/K_m ratio divided by the wt K_i/K_m ratio.
[c]From reference (26).

mutations on nucleoside analogue inhibition, the K_i/K_m ratio of each of the RT mutants was compared with wt RT and the values were expressed as "fold change." As shown in Table 3, inhibition of dCTP incorporation into poly(rI):oligo$(dC)_{12}$ by FTC-TP was only marginally affected by the K65R, Q151M, and K65R/Q151M mutations, with respectively 0.93-, 0.66-, and 1.9-fold changes over the wt enzyme. Similarly, for 3TC-TP inhibition of dCTP incorporation, the mutations at K65R, Q151M, and K65R/Q151M only led to 1.7-, 0.59-, and 0.94-fold changes in the K_i/K_m ratio, respectively.

Using pre-steady-state kinetic analysis, we studied the incorporation of dCTP, FTC-TP, and 3TC-TP into the random sequence DNA/DNA 23/45-mer primer-template by wt and K65R, Q151M, and K65R/Q151M mutant HIV-1 RT. A summary of the kinetic constant k_{pol} and K_d is shown in Table 5. The k_{pol} value represents the maximum rate of incorporation at the saturating concentration of a dNTP analogue. The K_d value represents the nucleotide concentration that is required to reach half of k_{pol}. Therefore, a lower value of K_d indicates a tighter binding affinity of the enzyme-DNA complex to the dNTP analogue. The ratio of k_{pol}/K_d was defined as incorporation efficiency and a higher value of k_{pol}/K_d for a dNTP analogue indicates that it is a better substrate for HIV-1 RT. The ratio of the dCTP's k_{pol}/K_d value to the FTC-TP (or 3TC-TP)'s k_{pol}/K_d value was used to define a selectivity factor. A higher selectivity factor suggests a greater discrimination between the natural substrate and the nucleotide analogue by HIV-1 RT. The overall resistance factor of the RT was defined as the ratio between the selectivity factor of the mutant and the selectivity factor of the wt. A resistance factor >1 indicates that the mutant enzyme becomes more discriminating against the incorporation of nucleotide analogue versus the incorporation of the natural substrate. Subsequently, this altered substrate discrimination at the enzyme level could enable the corresponding mutant virus to be resistant to the nucleoside analogue *in vitro*.

As shown in Table 5, the binding affinities of K65R and K65R/Q151M RT for dCTP were 2.9- and 5.8-fold weaker than that of the wt, respectively. This led to a 3.5- and 8.8-fold decrease in the incorporation efficiency of this natural substrate for K65R and K65R/Q151M, respectively. Q151M mutation caused faster dCTP incorporation and a weaker binding affinity of the enzyme towards dCTP, resulting in unchanged incorporation efficiency for dCTP. The wt RT catalyzed incorporation of FTC-TP and 3TC-TP was 32-fold and 48-fold slower than the natural substrate dCTP. FTC-TP bound to the enzyme-DNA complex with an affinity similar to dCTP, while the affinity for 3TC-TP was 2.6-fold weaker than dCTP. The mutation of K65R resulted in 12- and 19-fold decreases in the maximum rate of incorporation for FTC-TP and 3TC-TP, accompanied by very little changes in the binding affinity. The overall resistance factor for K65R RT is 5.0 and 4.8 for FTC-TP and 3TC-TP, respectively. As a result of the combined effects of a 2.4 to 3.1-fold decrease in k_{pol} and a 3.2 to 4.3-fold increase in the binding affinity, incorporation of

TABLE 5 Kinetic Parameters and Differences between Wild-type, K65R, Q151M, and K65R/Q151M RTs in Incorporation of dCTP, FTC-TP, and 3TC-TP into DNA/DNA 23/45-mer Primer/Template

Nucleotide	RT	k_{pol} (s^{-1})	K_d (μM)	k_{ss} (s^{-1})	k_{pol}/K_d (μM^{-1} s^{-1})	Selectivity factor[a]	Resistance factor[b]
dCTP	wt	1.9 ± 0.1	24 ± 5	0.04 ± 0.01	0.08 ± 0.02	1	—
FTC-TP	wt	0.059 ± 0.001	26 ± 1	—	$(2.3 \pm 0.1) \times 10^{-3}$	35 ± 8	1
3TC-TP	wt	0.040 ± 0.003	63 ± 14	—	$(6 \pm 1) \times 10^{-4}$	125 ± 39	1
dCTP	K65R	1.6 ± 0.1	70 ± 15	0.07 ± 0.01	0.023 ± 0.005	1	—
FTC-TP	K65R	0.0051 ± 0.0001	40 ± 2	—	$(1.3 \pm 0.1) \times 10^{-4}$	176 ± 42	5.0 ± 1.6
3TC-TP	K65R	0.0021 ± 0.0001	55 ± 9	—	$(3.8 \pm 0.7) \times 10^{-5}$	596 ± 172	4.8 ± 2.0
dCTP	Q151M	4.30 ± 0.3	71 ± 17	0.11 ± 0.01	0.08 ± 0.02	1	—
FTC-TP	Q151M	0.025 ± 0.001	8.2 ± 1.1	—	$(3.0 \pm 0.4) \times 10^{-3}$	20 ± 6	0.6 ± 0.2
3TC-TP	Q151M	0.0128 ± 0.0003	14.8 ± 1.6	—	$(8.6 \pm 0.9) \times 10^{-4}$	17.1 ± 2.6	0.6 ± 0.2
dCTP	K65R/Q151M	1.27 ± 0.07	139 ± 19	0.06 ± 0.01	$(9.1 \pm 1.3) \times 10^{-3}$	1	—
FTC-TP	K65R/Q151M	$(9.2 \pm 0.2) \times 10^{-4}$	26 ± 3	—	$(3.5 \pm 0.4) \times 10^{-5}$	260 ± 50	7.4 ± 2.2
3TC-TP	K65R/Q151M	$(4.3 \pm 0.1) \times 10^{-4}$	15.6 ± 1.4	—	$(2.7 \pm 0.2) \times 10^{-5}$	332 ± 58	2.7 ± 0.9

[a]Selectivity factor is defined as $(k_{pol}/K_d)_{dCTP}/(k_{pol}/K_d)_{FTC-TP\ or\ 3TC-TP}$.
[b]Resistance factor = (Selectivity $factor_{mutant\ RT}$)/(Selectivity $factor_{WT\ RT}$).

TABLE 6 Kinetic Parameters for Incorporation of dCTP, FTC-TP, and 3TC-TP into DNA/DNA 18/36-mer Primer/Template by Wild-type and K65R HIV-1 RTs

Nucleotide	RT	k_{pol} (s^{-1})	K_d (μM)	k_{ss} (s^{-1})	k_{pol}/K_d (μM^{-1} s^{-1})	Selectivity factor[a]	Resistance factor[b]
dCTP	wt	24 ± 1	14 ± 3	1.1 ± 0.1	1.7 ± 0.3	1	—
FTC-TP	wt	0.071 ± 0.001	0.067 ± 0.005	—	1.05 ± 0.09	1.6	1
3TC-TP	wt	0.046 ± 0.003	0.18 ± 0.03	—	0.26 ± 0.05	6.5	1
dCTP	K65R	2.19 ± 0.09	1.5 ± 0.2	0.35 ± 0.06	1.5 ± 0.2	1	—
FTC-TP	K65R	0.012 ± 0.001	0.11 ± 0.03	—	0.11 ± 0.03	14	8.8
3TC-TP	K65R	0.0033 ± 0.0002	0.10 ± 0.01	—	0.033 ± 0.005	45	6.9

[a]Selectivity factor is defined as $(k_{pol}/K_d)_{dCTP}/(k_{pol}/K_d)_{FTC-TP\ or\ 3TC-TP}$.
[b]Resistance factor = (Selectivity factor $_{mutant\ RT}$)/(Selectivity factor $_{WT\ RT}$).

FTC-TP and 3TC-TP by Q151M RT were carried out at efficiencies similar to the wt enzyme. Kinetic data for the K65R/Q151M RT mutant indicated a 7.4- and 2.7-fold resistance for FTC-TP and 3TC-TP, respectively, resulting from significant 64 to 93-fold decreases in the rate of incorporation and 5.3- to 8.9-fold increases in binding affinity.

In an effort to address whether the observed kinetic parameters are specifically associated with the random sequence DNA/DNA 23/45-mer primer/template, we conducted another pre-steady state analysis using a HIV-1 genomic DNA/DNA 18/36-mer primer/template for the incorporation of dCTP, FTC-TP, and 3TC-TP by wt and K65R HIV-1 RT. The results are summarized in Table 6. In comparison to the wt enzyme, K65R RT bound dCTP 9.3-fold tighter and incorporated dCTP 11-fold slower, resulting in unchanged incorporation efficiency for dCTP. The binding affinities of K65R RT for FTC-TP and 3TC-TP were within 2-fold changes from that of the wt, however, the rates of incorporation for FTC-TP and 3TC-TP were 5.9 and 13.9-fold that of the wt enzyme. Consequently, FTC-TP and 3TC-TP were incorporated 8- to 10-fold less efficiently by the K65R RT than the wt enzyme.

DISCUSSION

AIDS (acquired immunodeficiency syndrome) has become a manageable infectious disease due to the development of anti-HIV therapies in the past two decades. Sustained effective treatment is achieved by the combination of therapies. However, the emergence of drug resistant HIV complicates treatment regimens and remains a major obstacle for long-term viral suppression. Extensive work has been done to reveal the mechanism of 3TC drug resistance associated with HIV-1 RT mutations K65R and Q151M. Using single-nucleotide incorporation steady state kinetic analysis, Ueno *et al.* showed that mutation K65R on HIV-1 RT led to a 9.5-fold change of the K_i/K_m ratio for 3TC-TP while there was only a 1.7-fold change caused by Q151M.[31] In the same study, it was also shown that neither K65R nor Q151M mutation

affected the k_{cat} and K_m values for dCTP incorporation. Using pre-steady state kinetic analysis, Selmi *et al.*[32] and Deval *et al.*[33] demonstrated that K65R had a general effect of decreasing the catalytic rate constant k_{pol} for ddCTP, 3TC-TP, ddATP, and tenofovir-DP, and was accompanied by a lesser or no effect on the binding affinity (K_d) of the incoming nucleotide. Similarly, another pre-steady-state study by Deval *et al.*[34] showed that the mechanism of multidrug resistance by Q151M for AZT-TP, ddATP, and ddCTP was mainly associated with a selective decrease of k_{pol} and little change on K_d values.

Emtricitabine is structurally closely related to lamivudine. However, it is distinctively different from lamivudine when FTC-TP and 3TC-TP served as alternative substrate inhibitors for wt HIV-1 RT and human DNA polymerase γ. In comparison to 3TC-TP, FTC-TP is incorporated 2.5- to 9.1-fold *more* efficiently by HIV-1 RT; however, it is incorporated 100-fold *less* efficiently by human DNA polymerase γ.[35] In the current study, we set out to evaluate the anti-HIV activity of FTC and 3TC against K65R and Q151M mutant viruses, to measure the inhibition of HIV-1 RT-catalyzed dCTP incorporation by FTC-TP and 3TC-TP, and to assess the ability and efficiency of mutant enzymes in utilizing FTC-TP and 3TC-TP as substrate during DNA synthesis. In this study, K65R and K65R/Q151M viruses showed moderate to high resistance to both FTC and 3TC. The K65R-associated resistance was 8.4-fold for FTC and 11.7-fold for 3TC. Viruses containing K65R/Q151M were more than 20-fold resistance to both FTC and 3TC. In agreement with earlier reports where Q151M remained sensitive to 3TC, our study showed that Q151M virus remained sensitive to both FTC and 3TC.

The ability of FTC-TP and 3TC-TP to inhibit dCTP incorporation (through competition) by wt and mutant K65R, Q151M, and K65R/Q151M HIV-1 RTs was evaluated by measuring K_i values. The K_i value of FTC-TP for inhibition of wt RT was similar to a published study.[36] The inhibition of dCTP incorporation into poly(rI):oligo$(dC)_{12}$ by FTC-TP and 3TC-TP was only marginally affected by the K65R and K65R/Q151M mutations, in contrast to the resistance profile of HIV-1 mutants K65R and K65R/Q151M observed in cell culture. The lack of consistency between the steady-state study and the cell culture study was also observed earlier in a study on inhibition of dCTP incorporation by 3TC-TP using a similar testing system.[26] The apparent discrepancy may be due to the fact that HIV-1 RT catalyzes non-progressive synthesis on poly(rI):oligo$(dC)_{12}$. Therefore, the K_m values for dCTP and the K_i values for FTC-TP and 3TC-TP are only reflective of their respective K_d values (but not k_{pol} values).[37] Mutations that resulted in an altered binding affinity (K_d) of FTC-TP and 3TC-TP, such as the M184V mutation, led to a change in K_i value and a significant increase in resistance factor measured by the K_i/K_m ratio.[5,37] In contrast, mutations that do not have appreciable effect on the binding affinity, such as the K65R and K65R/Q151M mutation, showed little changes on the K_i/K_m ratio compared to that of

the wt. Under these circumstances, a defined DNA/DNA primer/template may be more suitable for the steady-state analysis, where K_m and K_i values for the single-nucleotide incorporation (forced termination mode) could reflect the combined effects of the changes in both k_{pol} and K_d values caused by mutations.[31,37]

Recently, Selmi *et al.* and Deval *et al.* reported the incorporation of a series of nucleotide analogues, including dCMP and 3TC-MP, by wt, K65R and Q151M HIV-1 RT using pre-steady-state analysis.[32,33] Similar to their results, our data with DNA/DNA 23/45-mer primer-template indicated that the overall mechanism for K65R-associated 3TC resistance was the significant decrease in the rate of incorporation. However, several of our results were different from the findings reported earlier.[32,33] First, for all the enzymes tested, the incorporation efficiency for dCTP measured by Deval *et al.* is one magnitude higher than our findings as a result of higher rates of incorporation and tighter binding affinities.[33] Secondly, the binding affinity of K65R for dCTP is reported to be 2.8-fold tighter by Deval *et al.* in contrast to the 2.4-fold increase in our data.[33] Thirdly, Selmi *et al.* reported that the Q151M HIV-1 RT has a similar binding affinity for dCTP to the wt enzyme, compared to the 3-fold increase reported here.[32] The sources of these discrepancies could be different HIV-1 RT protein preparations and/or different primer/template. We conducted the following studies to address these possibilities. First, we studied the incorporation of dCTP, FTC-TP and 3TP-TP into DNA/DNA 23/45-mer primer/template using K65R and Q151M HIV-1 RTs from the same source as described previously.[32,33] The resulting kinetic constants (unpublished data) were similar to the ones in Table 5, indicating that different RT proteins did not contribute to the observed discrepancies. Secondly, we studied the incorporation of dCTP, FTC-TP, and 3TC-TP into a HIV-1 genomic DNA/DNA 18/36-mer primer/template. As shown in Table 6, the kinetic parameters were significantly different from the ones where DNA/DNA 23/45-mer primer/template were used, but similar to the ones reported earlier.[32,33] This suggests that the primer/template sequences could affect the absolute values of k_{pol}, K_d and k_{pol}/K_d significantly. However, the "resistance factor" measured in both pre-steady-state studies were comparable to each other, as shown by the 8.8- and 6.9-fold differences for FTC-TP and 3TC-TP for incorporation into a HIV-1 genomic DNA *versus* the 5.0- and 4.8-fold differences for FTC-TP and 3TC-TP for incorporation into a random sequence DNA. Thus, comparisons of kinetic parameters, selectivity factors, and resistance fold change values obtained with the same primer/template are valid, whereas they may not be when different primer/template systems are used. This also indicates that kinetic parameters of single nucleotide incorporation into a hetero DNA duplex could be affected, even to a great extent, by the composition of the surrounding nucleotides on the primer/template. Despite the sequence differences between the primer/template used, both pre-steady-state analyses demonstrated that

the mechanism of action for K65R-associated FTC and 3TC resistance was mainly due to a significant decrease in the rate of incorporation of FTC-TP and 3TC-TP. The binding affinities of mutant RTs for these two nucleotide analogues were only slightly affected or not changed.

There was one discrepancy between the pre-steady-state data and the cell culture data. In cell culture studies, we observed enhanced resistance against both FTC and 3TC by the K65R/Q151M mutant compared to the K65R mutant. However, the pre-steady-state results showed very similar resistance factors for both of these mutants. Due to the fact that HIV-1 RT catalyzes both DNA-dependent and RNA-dependent DNA synthesis, further studies using a DNA/RNA primer/template would likely provide more information.

The two pre-steady-state kinetic analyses demonstrated that during DNA-dependent DNA synthesis, the incorporation efficiency of FTC-TP is consistently 3- to 4-fold higher than 3TC-TP for wt, K65R, and Q151M HIV-1 RTs. These findings are consistent with the reports that FTC-TP was incorporated 2.5-fold more efficiently than 3TC-TP during DNA-dependent DNA synthesis by wt RT[35] and 10-fold more efficiently than 3TC-TP during RNA-dependent DNA synthesis by wt RT.[38] Our mechanistic study suggests that the consistently higher incorporation efficiency of FTC-TP over 3TC-TP results from the combined effects of higher rate of incorporation and tighter binding affinity.

In conclusion, our studies provide the mechanism of resistance for K65R and K65R/Q151M mutant HIV-1 to FTC and 3TC. At the molecular level, this is mainly due to a significant decrease in the rate of analogue incorporation during viral DNA synthesis. Although the prevalence of the K65R mutation has increased recently with the increased usage of NRTIs that select for this mutation (abacavir and tenofovir), the prevalence in patients still remains low (<4%).[39] In contrast, the association of K65R with Q151M has decreased recently suggesting that current therapeutic approaches are minimizing the development of high-level multinucleoside resistance. A better understanding of the virology of mutant HIV and the biochemical properties of mutant HIV-1 RT offers valuable information for better treatment of HIV infection and discovery of new antiretroviral agents.

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