# Comparative Enzymatic Study of HIV-1 Reverse Transcriptase Resistant to 2′,3′-Dideoxynucleotide Analogs Using the Single-Nucleotide Incorporation Assay

Takamasa Ueno[‡] and Hiroaki Mitsuya*

*Experimental Retrovirology Section, Medicine Branch, Division of Clinical Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892*

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ABSTRACT: Employing the single-nucleotide incorporation assay using a heteropolymeric RNA template and DNA primers, we defined enzymatic profiles of recombinant human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) containing a set of five mutations [A62V, V75I, F77L, F116Y, and Q151M] which confers resistance to multiple 2′,3′-dideoxynucleosides (ddNs) on HIV-1. RTs containing other drug-resistance-associated mutations were also examined. The $K_m$ for dNTPs, the $k_{cat}$, and the $k_{cat}/K_m$ ratios of mutant RTs were all comparable to those of wild-type RT ($RT_{wt}$). The processive primer extension activity of mutant RTs was also comparable to that of $RT_{wt}$ as examined in the presence of saturating concentrations of dNTPs and heparin. Determination of the $K_i$ values toward 5′-triphosphates (TP) of various ddNs [3′-azido-2′,3′-dideoxythymidine (AZT), 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T), 2′,3′-dideoxycytidine (ddC), (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC), (−)-β-L-2′,3′-dideoxy-5-fluorocytidine (FddC), 2′,3′-dideoxyadenosine (ddA), and 2′-β-fluoro-2′,3′-dideoxyadenosine (FddA)] and 9-(2-phosphonylmethoxyethyl)adenine diphosphate (PMEApp) revealed that $RT_{A62V/V75I/F77L/F116Y/Q151M}$ was insensitive to ddATP, AZTTP, D4TTP, FddATP, and ddCTP, but was sensitive to PMEApp, 3TCTP, and FddCTP. $RT_{K65R}$ was less sensitive to ddATP, FddATP, PMEApp, ddCTP, and 3TCTP, while $RT_{M184V}$ was less sensitive only to 3TCTP and ddCTP. The determination of $K_{i(ddNTP)}/K_{m(dNTP)}$ ratios showed that AZTTP, D4TTP, and ddCTP are, as substrates, as efficient for $RT_{wt}$ as their corresponding dNTPs, that ddATP, PMEApp, and 3TCTP are moderately efficient substrates for $RT_{wt}$, and that FddATP is the least efficient substrate among ddNTPs examined. The observed cross-resistance of HIV-1 RT to various ddNTPs should reflect the alteration of RT's substrate recognition and should provide insights into the molecular mechanism of RT discrimination of ddNTPs from natural substrates.

The error-prone polymerization (approximately one to ten misincorporations per genome per round of replication) mediated by the virally-encoded reverse transcriptase (RT)[1] and the high rates of replication of human immunodeficiency virus type 1 (HIV-1) (Preston et al., 1988; Roberts et al., 1988; Coffin, 1995) is thought to produce a myriad of HIV-1 variants (quasispecies), allowing the emergence of drug-resistant HIV-1 variants in the presence of drug pressure (Mitsuya et al., 1990; Nájera et al., 1995). Presently, five 2′,3′-dideoxynucleoside analogs (ddNs), 3′-azido-2′,3′-dideoxythymidine (AZT or zidovudine), 2′,3′-dideoxycytidine (ddC or zalcitabine), 2′,3′-dideoxyinosine (ddI or didanosine), 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T or stavudine), and (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC or lamivudine), are being used for therapy of HIV-1 infection, and several other ddNs are under clinical evaluation. HIV-1, however, has proved to be capable of developing resistance against virtually any of these therapeutic ddNs, and various amino acid substitutions have been found to be associated with reduced sensitivity to ddNs (Boucher et al., 1993; Gao et al., 1993a; Larder & Kemp, 1989; Schinazi et al., 1993; St. Clair et al., 1991; Tisdale et al., 1993; Kavlick et al., 1995).

The isolation of drug-resistant HIV variants have increased an interest in using multiple agents in combination for HIV-1 therapy; however, various mutations in the polymerase-encoding gene conferring resistance to multiple ddNs on HIV-1 have been identified in HIV-1 isolated from patients receiving combination chemotherapy with multiple ddNs. They include Q151M and a set of five mutations, A62V/V75I/F77L/F116Y/Q151M (Shirasaka et al., 1993, 1995; Shafer et al., 1994; Schmit et al., 1996), and K65R (Gu et al., 1994b; Zhang et al., 1994). We have previously reported enzymatic properties of RT carrying all or a subset of the five mutations using mostly synthetic homopolymeric template-primers, p(rA)·(dT)$_{12-18}$, p(rC)·(dG)$_{12-18}$, and p(rI)·(dC)$_{12-18}$, (Ueno et al., 1995). However, steady-state kinetic constants are greatly influenced by the dissociation of the enzyme−template-primer complex, the template-primers, and buffer conditions (Beard & Wilson, 1993; Klarmann et al., 1993; Reardon, 1992; Reardon & Miller, 1990). Moreover, steady-state rates determined with homopolymeric template-primers reflect only an average of the rates of polymerization and

* Corresponding author. Tel: (301) 496-9238. FAX: (301) 402-0709.

‡ Present address: Pharmaceuticals and Biotechnology Laboratory, Japan Energy Corporation, Toda, Saitama 335, Japan.

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[1] Abbreviations: 3TC, (−)-β-L-2′,3′-dideoxy-3′-thiacytidine; AZT, 3′-azido-2′,3′-dideoxythymidine or 3′-azidothymidine; D4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; ddA, 2′,3′-dideoxyadenosine; ddN, 2′,3′-dideoxynucleoside; dN, 2′-deoxynucleoside; FddA, 2′-β-fluoro-2′,3′-dideoxyadenosine; FddC, (−)-β-L-2′,3′-dideoxy-5-fluorocytidine; HIV-1, human immunodeficiency virus type 1; PBM, peripheral blood mononuclear cells; PHA, phytohemagglutinin; pp, diphosphate; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; RT, reverse transcriptase; $RT_{wt}$, wild type reverse transcriptase; TP, 5′-triphosphate.

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Table 1: Nucleotide Sequences of an RNA Template and DNA Primers

| | | |
|---|---|---|
| **Template:** | **MS2 RNA** | 3'----- GCA AUC GGU GAG GCU UCA CGC AUA UUG CGC GUG -----5' |
| **Primers:** | **19C** | 5'- CGT TAG CCA CTC CGA AGT G -3' |
| | **20G** | 5'- CGT TAG CCA CTC CGA AGT GC -3' |
| | **21T** | 5'- CGT TAG CCA CTC CGA AGT GCG -3' |
| | **22A** | 5'- CGT TAG CCA CTC CGA AGT GCG T -3' |

[a] Nucleotide sequence (3315−3347) of the template, phage MS2 genomic RNA, is given at the top (Fiers et al., 1976).

dissociation of the enzyme−template-primer complex (Jaju et al., 1995; Johnson, 1993; Reardon, 1992). Therefore, in this study, we evaluated the biochemical properties of recombinant RTs with various mutations employing a single nucleotide incorporation assay using a heteropolymeric RNA template and DNA primers under the same buffer conditions. We also determined the profile of inhibition of various RTs with respect to eight different ddNTPs: AZTTP, ddCTP, ddATP, D4TTP, 3TCTP, 2′-$\beta$-fluoro-2′,3′-dideoxyadenosine 5′-triphosphate (FddATP), (−)-$\beta$-L-2′,3′-dideoxy-5-fluorocytidine 5′-triphosphate (FddCTP), and 9-(2-phosphonylmethoxyethyl)adenine diphosphate (PMEApp).

## MATERIALS AND METHODS

*Reagents.* dNTP, ddNTP, and CNBr-activated Sepharose 4B were purchased from Pharmacia Biotech Inc. (Piscataway, NJ). AZTTP, 3TCTP, and $^3$H-radiolabeled ([$^3$H]) dNTPs were purchased from Moravek Biochemicals (Brea, CA). Phage MS2 genomic RNA (single-stranded) was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Purified anti-RT monoclonal antibody, M33 (Di Marzo Veronese et al., 1986), was purchased from Advanced BioScience Laboratories, Inc. (Kensington, MD). 2′-Arafluoro-2′,3′-dideoxyadenosine 5′-triphosphate (FddATP; 85% pure and containing ∼10% of FddA 5′-monophosphate and diphosphate) and 9-(2-phosphonylmethoxyethyl)adenine diphosphate (PMEApp; >90% pure) were kindly provided by J. S. Driscoll (National Cancer Institute, Bethesda, MD) and M. Hitchcock (Gilead Sciences, Foster City, CA), respectively. D4TTP and (−)-$\beta$-L-2′,3′-dideoxy-5-fluorocytidine 5′-triphosphate [FddC; >90% pure] were kindly provided by Y. C. Chen and W. H. Prusoff (Yale University, New Haven, CT). All other reagents used in this study were of the commercially available analytical grade. Oligonucleotide synthesis, purification using polyacrylamide gel electrophoresis, and $^{32}$P-end-labeling with [$\gamma$-$^{32}$P]ATP by T4 polynucleotide kinase were carried out by Lofstrand Labs Ltd. (Gaithersburg MD).

*Plasmid Constructions.* Wild-type RT ($RT_{wt}$) and various mutant RT-expression vectors were constructed as previously described (Ueno et al., 1995). Briefly, desired mutations were introduced into the *Xma*I-*Nhe*I region (759 base pairs) of pTZNX1, which encoded Gly-15 to Ala-267 of HIV-1 RT (strain BH10), by oligonucleotide-directed mutagenesis using T7 DNA polymerase (US Biochemical Co., Cleveland, OH). The presence of these intended mutations and the absence of unintended mutations were confirmed by determination of nucleotide sequence of the entire *Xma*I-*Nhe*I region. To construct each RT expression vector, the *Xma*I-*Nhe*I fragment carrying the intended mutation(s) was replaced with the *XmNh* linker region of pKRT07 (Ueno et al., 1995).

*Enzyme Preparations. Escherichia coli* JM109 (Promega, Madison, WI) transformed with an $RT_{wt}$-expression vector or each of mutant RT-expression vectors was cultured in ampicillin (100 $\mu$g/mL)-containing 2× YT medium (1.6% tryptone, 1% yeast extract, and 0.5% NaCl) at 37 °C, exposed to 1 mM isopropyl 1-thio-$\beta$-D-galactoside (IPTG) at the late-log phase, and cultivated for an additional 7 h. Thus overproduced RT was recovered from the cytoplasm fraction and was purified by an immunoaffinity chromatography using the RT-specific M33 monoclonal antibody as described (Ueno et al., 1995). This RT preparation was dialyzed against 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 3 mM dithiothreitol (DTT) overnight at 4 °C and stored at −70 °C until use. Purified RTs were 66 kDa (p66)-dominant polypeptides with >90% purity as assessed by an SDS−polyacrylamide gel analysis (data not shown). Active enzyme concentrations of $RT_{wt}$ and mutant RTs were determined as described before (Reardon & Miller, 1990; Ueno et al., 1995).

*Template-Primer Annealing.* In the single-nucleotide incorporation assay, one heteropolymeric RNA template and one of four different DNA primers were employed depending on nucleotide substrates examined. MS2/19C, MS2/20G, MS2/21T, and MS2/22A were used with dCTP, dGTP, dTTP, and dATP, respectively. Nucleic acid sequences of the template and primers are illustrated in Table 1. DNA primer 22A is complementary to nucleotides 3326−3347 of phage MS2 genomic RNA (Fiers et al., 1976). All DNA and RNA were dissolved in a buffer containing 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA. The template and primer were combined at a 1:1 molar ratio, heated to 75 °C for 5 min, and cooled slowly to room temperature over an hour. These annealed template-primers were stored at −20 °C until use.

*Steady-State Enzymatic Assays.* The reaction mixture for conducting the single nucleotide incorporation assay contained 50 mM Tris-HCl, pH 7.8, 6 mM $MgCl_2$, 150 mM KCl, 0.01% Triton X-100, 0.5 $\mu$M template-primer (expressed as 3′-hydroxyl primer termini), [$^3$H]dNTP, and approximately 1 pmol of RT in the presence or absence of ddNTP in a total volume of 100 $\mu$L. After the reaction mixture was equilibrated at 22 ± 1 °C, the reaction was initiated by the addition of enzyme at 22 ± 1 °C. We confirmed that the steady-state conditions continued following the burst formation for at least 5 min after the initiation of the reaction. A portion of the reaction mixture (15 $\mu$L) was removed five times (0.5, 1.0, 1.5, 2.0, and 2.5 min after the initiation of the reaction) during the course of the assay and was spotted on a DE81 filter (Whatman), and the radioactivity in the product formed was counted as described before (Ueno et al., 1995). This method provided recovery of the extended primers by 95 ± 2% after thoroughly

Table 2: Substrate Analysis of $RT_{wt}$ and Mutant RTs Using the Single-Nucleotide Incorporation Assay[a]

| | dATP | | | dCTP | | | dGTP | | | dTTP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mutation | $K_m$[b] | $k_{cat}$[c] | $k_{cat}/K_m$[d] | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
| wild type | 0.035 ± 0.006 | 0.016 | 0.46 | 0.14 ± 0.02 | 0.0063 | 0.045 | 0.022 ± 0.005 | 0.029 | 1.3 | 0.11 ± 0.01 | 0.019 | 0.17 |
| Q151M | 0.041 ± 0.004 | 0.026 | 0.63 | 0.14 ± 0.03 | 0.0068 | 0.049 | 0.030 ± 0.004 | 0.026 | 0.87 | 0.068 ± 0.009 | 0.021 | 0.31 |
| A62V/V751/F77L/ F116Y/Q151M | 0.064 ± 0.011 | 0.018 | 0.28 | 0.15 ± 0.03 | 0.0074 | 0.049 | 0.029 ± 0.003 | 0.021 | 0.72 | 0.16 ± 0.03 | 0.019 | 0.12 |
| K65R | 0.078 ± 0.011 | 0.014 | 0.18 | 0.17 ± 0.03 | 0.0058 | 0.034 | 0.012 ± 0.001 | 0.029 | 2.4 | 0.085 ± 0.017 | 0.023 | 0.27 |
| L74V | 0.11 ± 0.01 | 0.016 | 0.15 | 0.40 ± 0.06 | 0.0076 | 0.019 | 0.038 ± 0.005 | 0.028 | 0.74 | 0.12 ± 0.02 | 0.022 | 0.18 |
| M184V | 0.036 ± 0.006 | 0.0096 | 0.27 | 0.28 ± 0.05 | 0.012 | 0.043 | 0.042 ± 0.008 | 0.023 | 0.55 | 0.085 ± 0.014 | 0.016 | 0.19 |
| T215Y | 0.084 ± 0.024 | 0.018 | 0.21 | 0.55 ± 0.14 | 0.0065 | 0.012 | 0.054 ± 0.008 | 0.042 | 0.78 | 0.093 ± 0.03 | 0.028 | 0.30 |

[a] Assays were conducted as described in Materials and Methods. For the determination of steady-state constants for dATP, dCTP, dGTP, and dTTP, MS2/22A, MS2/19C, MS2/20G, and MS2/21T were employed as template-primers, respectively. $K_m$ and $V_{max}$ values were determined by linear steady-state velocities using Lineweaver−Burk plot analyses. $k_{cat}$ values were calculated using the equation $k_{cat} = V_{max}/[E]$. One standard for each $k_{cat}$ value shown was all within <15% of the value. [b] In $\mu$M. [c] In $s^{-1}$. [d] In $\mu M^{-1}$ $s^{-1}$.

washing away unincorporated [$^3$H]dNTPs. The $K_m$ and $V_{max}$ values were determined from linear steady-state velocities with Lineweaver−Burk plot analyses, and the $K_i$ values were determined using Dixon plot analyses. The $k_{cat}$ values were calculated by dividing $V_{max}$ values by active enzyme concentrations.

*Processivity Assays.* For a single processive cycle of RT-mediated primer extension, various RTs were incubated in a solution (28 $\mu$L) containing 8 pmol of MS2/[5′-$^{32}$P]22A, 50 mM Tris-HCl, pH 7.8, 150 mM KCl, and 0.01% Triton X-100 for 10 min at 22 ± 1 °C. The reaction was then initiated by the addition of a solution (12 $\mu$L) containing 6 mM $MgCl_2$, 100 $\mu$M each of four dNTPs, and heparin (1 mg/mL; as an RT trapping agent; US Biochemical Co.) at 22±1 °C. A portion (12 $\mu$L) of the reaction mixture was removed three times (5, 10, and 30 min after initiation of the reaction), and the reaction was quenched by the addition of an equal volume of gel loading buffer (90% formamide, 89 mM Tris base, 89 mM boric acid, and 2 mM $Na_2EDTA$). The [5′-$^{32}$P]-labeled primers elongated by RT were subjected to electrophoresis in 8% Long Ranger sequencing gel (AT Biochem, Malvern, PA) containing 7 M urea, and the dried gel was exposed to Kodak XAR-2 film. The density of all bands above the primer on the X-ray film was measured by densitometry and integrated by using a software package for analysis of one-dimensional gels and films (Quantity One; PDI, Huntington Station, NY). To confirm the trap efficiency, $RT_{wt}$ was first incubated with MS2/[5′-$^{32}$P]22A and heparin (1 mg/mL) for 10 min at 22 ± 1 °C and the reaction was initiated by the addition of dNTPs and $MgCl_2$. For comparison of the integrated density products/enzyme values were determined by dividing the integrated density (OD) of the products formed in 30 min by the amount of RT (pmol) used in the reaction.

*Analysis of Steady-State Kinetic Data.* An employed kinetic model for RT, in which the concentration of template-primer is saturating and the template-primer dissociates from the enzyme after every nucleotide incorporation, is as follows:

$$E\cdot TP_n \overset{K_d}{\longleftrightarrow} E\cdot TP_n\cdot dNTP \overset{k_p}{\longrightarrow} E\cdot TP_{n+1} \overset{k_{off}}{\longrightarrow} E + TP_{n+1}$$

where $E\cdot TP_n$ is the enzyme−template-primer complex, dNTP is a nucleotide, $TP_{n+1}$ is the template-primer after the incorporation of a proper dNMP, $K_d$ is the equilibrium dissociation constant for dNTP, $k_p$ is the rate constant for nucleotide incorporation, and $k_{off}$ is the rate constant for dissociation of the enzyme−template-primer complex. The rate-limiting step for single nucleotide incorporation into a heteropolymeric template-primer represents the dissociation of the template-primer from the enzyme (*i.e.*, $k_{cat} = k_{off}$) (Kati et al., 1992; Reardon, 1992; Reardon & Miller, 1990). Under this condition, the Michaelis constants for dNTP and $k_{cat}/K_m$ are defined as follows:

$$K_m = k_{off}\cdot K_d/k_p$$

$$k_{cat}/K_m = k_p/K_d$$

## RESULTS

*Steady-State Kinetic Analysis.* The incorporation of a single [$^3$H]dNMP to heteropolymeric template-primers (MS2/19C, 20G, 21T, and 22A) mediated by either of $RT_{wt}$ or mutant RTs was biphasic with respect to time. Specifically, the burst formation was first observed, followed by the slow linear steady state for all RTs examined under the single-nucleotide incorporation assay, in agreement with the previous observation (Ueno et al., 1995; Kati et al., 1992; Reardon & Miller 1990). It was noted that the amplitudes of the burst formation mediated by $RT_{wt}$ and all mutant RTs were stoichiometric with regard to the amounts of the enzyme added in the reaction. Using thus determined amplitudes of the burst formation, we determined the concentrations of active enzyme in all RT preparations as previously described (Ueno et al., 1995; Reardon & Miller 1990). The steady-state kinetic constants determined under the single-nucleotide incorporation assay condition are summarized in Table 2. The $K_m$ and $k_{cat}$ values determined with $RT_{wt}$ at 22 ± 1 °C were comparable to those determined by others (Patel et al., 1995; Reardon, 1992), although the $k_{cat}$ value (0.016 $s^{-1}$) with dATP determined in this study was slightly less than our previously reported value (0.025 $s^{-1}$) which was determined at 37 °C (Ueno et al., 1995). It was noted that the $K_m$ values did not appreciably differ among the seven different RTs examined with regard to all dNTPs (<3.1-, <3.9-, <2.5-, and <1.6-fold for dATP, dCTP, dGTP, and dTTP, respectively; Table 2). The $k_{cat}$ value of $RT_{wt}$, which is equal to $k_{off}$ as described above (see Materials and Methods), was also virtually identical to those of mutant RTs examined. Thus, the $k_{cat}/K_m$ values of $RT_{wt}$ were comparable to those of mutant RTs examined (all differences being less than 4-fold). It is noteworthy that sequence (primer)-dependent differences wereobserved in $k_{cat}$ ($k_{off}$) values with RTs, consistent with the previous observation (Reardon,

1992). For example, the rate constant of dissociation of $RT_{wt}$ from the enzyme−MS2/20G complex (0.0063 $s^{-1}$, 20G = 19C + dCMP; see Table 1), which had been determined as the $k_{cat}$ value of dCMP incorporation to MS2/19C in this study, was 4.6-fold less than that from the enzyme-MS2/21T complex (0.029 $s^{-1}$, 21T = 20G + dGMP; see Table 1), which had been determined as the $k_{cat}$ value of dGMP incorporation into MS2/20G. A similar difference was observed with those values for other mutant RTs examined (Table 2).

*Processivity Analysis.* Since the steady-state kinetic constants determined in a single-nucleotide incorporation assay do not reflect the ability of RT to translocate itself along the template strand, we conducted a primer extension assay in the presence of saturating concentrations of dNTPs (100 $\mu$M of each dNTP) using a trapping agent, heparin, to allow only a single processive cycle of catalysis of RTs. Heparin has been shown to inhibit RT's polymerization activity through its binding to RT molecules not bound to the template-primer and those once dissociated from the template-primer (Beard & Wilson, 1993). In this study, when $RT_{wt}$ was incubated with the template-primer (MS2/22A) in the presence of heparin prior to the initiation of the reaction, no extended products were detected (Figure 1, lanes 22−24), confirming the findings by Beard and Wilson (1993). In order to compare the amount of extended products with each RT, products/enzyme values were determined by dividing the integrated density (OD) which represented the total products formed in 30 min with the amount (pmol) of each RT used. Those values were 46, 52, 43, 48, 44, 48, and 39 (OD/pmol) for $RT_{wt}$, $RT_{Q151M}$, $RT_{A62V/V75I/F77L/F116Y/Q151M}$, $RT_{K65R}$, $RT_{L74V}$, $RT_{M184V}$, and $RT_{T215Y}$, respectively (Figure 1), suggesting that the primer extension activity of mutant RTs examined was comparable to that of $RT_{wt}$. Also, the mobility and intensity of the pausing sites seen with mutant RTs including the predominant ones spanning the nucleotides 3315−3321 (GCGCGUG; see Table 1 for the sequence) of MS2 were virtually identical to those with $RT_{wt}$ (Figure 1).

*Inhibitor Analysis of $RT_{wt}$.* Inhibition constants determined under different assay conditions and different template-primers cannot be directly compared for the inhibition strength of ddNTPs (Beard & Wilson, 1993; Jaju et al., 1995; Johnson, 1993; Klarmann et al., 1993; Reardon, 1992; Reardon & Miller, 1990). We therefore determined inhibition constants of $RT_{wt}$ and mutant RTs with respect to various ddNTPs using the same template and buffer conditions. Since chain-terminating ddNTPs examined can be alternate substrates for RT, the $K_i$ values for ddNTPs, determined as inhibitors of dNMP incorporation into the heteropolymeric template-primer, are equal to $K_m$ values of ddNTPs determined as substrates for RT under the single-nucleotide assay condition (Reardon, 1992; Ueno et al., 1995). Hence, $K_{i(ddNTP)}/K_{m(dNTP)}$ ratios determined in this study represent the Michaelis constant ratios of an inhibitor and a corresponding substrate (*i.e.*, $K_{i(ddNTP)}/K_{m(dNTP)} = K_{m(ddNTP)}/K_{m(dNTP)}$). As shown in Table 3, the $K_{i(ddNTP)}/K_{m(dNTP)}$ ratio of $RT_{wt}$ for AZTTP of 1.0 indicates that AZTTP is, as a substrate, as efficient for $RT_{wt}$ as is dTTP, consistent with the previous observation (Reardon, 1992). It is also noteworthy that ddC was virtually as efficient for $RT_{wt}$ as was dCTP (Table 3; $K_i/K_m$ being 1.9). PMEApp and ddATP were found to be relatively efficient substrates for $RT_{wt}$ (only ∼5-fold less efficient substrates compared to dATP), while FddATP was

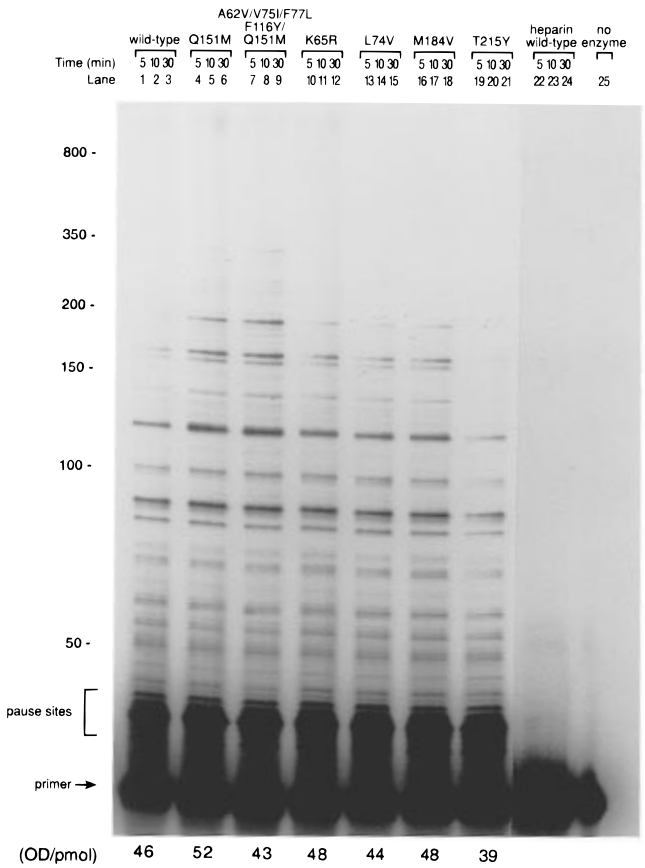


FIGURE 1: Processive primer extension activity of $RT_{wt}$ and mutant RTs. The reaction mixture contained MS2/[5′-$^{32}$P]22A, heparin, and $RT_{wt}$ (0.76 pmol; lanes 1−3), $RT_{Q151M}$ (0.92 pmol; lanes 4−6), $RT_{A62V/V75I/F77L/F116Y/Q151M}$ (1.1 pmol; lanes 7−9), $RT_{K65R}$ (0.92 pmol; lanes 10−12), $RT_{L74V}$ (1.0 pmol; lanes 13−15), $RT_{M184V}$ (0.88 pmol; lanes 16−18), or $RT_{T215Y}$ (0.80 pmol; lanes 19−21). A portion of the reaction mixture was removed three times (5, 10, and 30 min), and the [5′-$^{32}$P]-labeled primers elongated by RT were visualized by electrophoresis and autoradiography. To confirm the trap efficiency, $RT_{wt}$ (0.76 pmol; lanes 22−24) was incubated with MS2/[5′-$^{32}$P]22A and heparin for 10 min and the reaction was initiated by the addition of dNTPs and $MgCl_2$. Products/enzyme values (OD/pmol) determined for comparison of the density of the bands above the primer on the X-ray film were 46, 52, 43, 48, 44, 48, 39, and 1.4 for lanes, 3, 6, 9, 12, 15, 18, 21, and 24, respectively. No bands except the one representing the primer were detected when no enzyme was added (lane 25). Numbers on the left indicate product lengths that were determined using a $^{32}$P-end-labeled 50 bp DNA ladder (Life Technologies, Inc.). Note that the mobility and intensity of the pausing sites were virtually identical in all lanes.

a 12−14-fold less efficient substrate relative to PMEApp and ddATP (Table 3). This should explain, at least in part, the previous observation that FddA was approximately 10-fold less potent against HIV-1 *in vitro* than ddI when tested in phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBM), while FddA had a substantially higher intracellular FddATP/dATP ratio of 1.58 as compared to the ddATP/dATP ratio of 0.04 (Gao et al., 1994).

*Inhibitor Analysis of Mutant RTs.* Inhibition constants of various mutant RTs with respect to eight different ddNTPs were also determined in the single-nucleotide incorporation assay using the same heteropolymeric RNA template (MS2) and the same buffer condition (Table 3). RT with the Q151M mutation ($RT_{Q151M}$) and that with the five mutations, A62V/V75I/F77L/F116Y/Q151M, ($RT_{A62V/V75I/F77L/F116Y/Q151M}$), both of which have been shown to be associated with resistance to multiple ddNTPs (Ueno et al., 1995), showed

Table 3: Inhibition Analysis of $RT_{wt}$ and Mutant RTs Using the Single-Nucleotide Incorporation Assay[a,b]

| substrate | inhibitor | wild type | | Q151M | | A62V/V75I/F77L/F116Y/Q151M | | K65R | | L74V | | M184V | | T215Y | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $K_i$ | $K_i/K_m$ | $K_i$ | $K_i/K_m$ | $K_i$ | $K_i/K_m$ | $K_i$ | $K_i/K_m$ | $K_i$ | $K_i/K_m$ | $K_i$ | $K_i/K_m$ | $K_i$ | $K_i/K_m$ |
| dATP | ddATP | 0.19 ± 0.03 | 5.4 | 1.3 ± 0.1 | 32 | 3.3 ± 0.2 | 52 | 2.4 ± 0.2 | 31 | 0.61 ± 0.06 | 5.5 | 0.22 ± 0.02 | 6.1 | 0.52 ± 0.01 | 6.2 |
| | FddATP | 2.6 ± 0.2 | 74 | 6.4 ± 1.1 | 160 | 14 ± 1.4 | 220 | 29 ± 1 | 370 | 9.5 ± 1.7 | 86 | 2.5 ± 0.5 | 69 | 3.7 ± 0.4 | 44 |
| | PMEApp | 0.21 ± 0.01 | 6.0 | 0.22 ± 0.01 | 5.4 | 0.50 ± 0.01 | 7.8 | 1.7 ± 0.1 | 22 | 0.43 ± 0.11 | 3.9 | 0.12 ± 0.01 | 3.3 | 0.26 ± 0.05 | 3.1 |
| dCTP | ddCTP | 0.27 ± 0.02 | 1.9 | 0.66 ± 0.06 | 4.7 | 1.4 ± 0.1 | 9.3 | 1.4 ± 0.07 | 8.2 | 0.77 ± 0.03 | 1.9 | 1.8 ± 0.05 | 6.4 | 0.62 ± 0.10 | 1.1 |
| | 3TCTP | 1.3 ± 0.05 | 9.3 | 2.3 ± 0.1 | 16 | 2.4 ± 0.6 | 16 | 15 ± 2 | 88 | 2.1 ± 0.2 | 5.3 | 93 ± 4 | 330 | 3.4 ± 0.05 | 6.2 |
| | FddCTP | 2.8 ± 0.3 | 20 | 3.3 ± 0.1 | 24 | 4.6 ± 0.4 | 31 | ND[c] | | ND | | ND | | ND | |
| dTTP | AZTTP | 0.11 ± 0.02 | 1.0 | 0.34 ± 0.03 | 5.0 | 2.3 ± 0.4 | 14 | 0.33 ± 0.02 | 3.9 | 0.091 ± 0.002 | 0.76 | 0.076 ± 0.003 | 0.89 | 0.15 ± 0.01 | 1.6 |
| | D4TTP | 0.28 ± 0.03 | 2.5 | 0.85 ± 0.11 | 12.5 | 2.8 ± 0.7 | 17.5 | 0.78 ± 0.1 | 9.2 | 0.31 ± 0.02 | 2.6 | 0.19 ± 0.03 | 2.2 | 0.68 ± 0.12 | 7.3 |

[a] Assays were conducted as described in Materials and Methods. $K_i$ values were determined by linear steady-state velocities using Dixon plot analyses. $K_i/K_m$ ratios were calculated using the corresponding $K_m$ values given in Table 2. [b] $K_i$ values in $\mu M$. [c] $K_i$ values of mutant RTs (K65R, L74V, M184V, and T215Y) toward FddCTP were not determined (ND) because of the limited quantity of the compound.

substantially increased $K_i/K_m$ ratios to ddATP (6−10 fold), ddCTP (3−5-fold), AZTTP (5−14-fold), and D4TTP (5−7-fold) compared to $RT_{wt}$ (Table 3), suggesting that with these mutations, RT no longer recognizes these ddNTPs as adequate substrates. These two mutant RTs showed a slightly increased $K_i/K_m$ ratios to FddATP(2−3-fold) as compared to $RT_{wt}$. It was noted, however, that both $RT_{Q151M}$ and $RT_{A62V/V75I/F77L/F116Y/Q151M}$ showed comparable $K_i/K_m$ ratios to PMEApp (∼1-fold), 3TCTP (<2-fold), and FddCTP (<2-fold) as compared to $RT_{wt}$. In this regard, PMEA has been shown to be active against both wild-type HIV-1 and a recombinant infectious HIV-1 clone carrying all five mutations (HIV-$1_{A62V/V75I/F77L/F116Y/Q151M}$) in an *in vitro* drug sensitivity assay (M. Tanaka, and H. Mitsuya, submitted for publication).

Interestingly, all seven inhibitors tested proved to be less efficient substrates for RT containing the K65R mutation ($RT_{K65R}$) (Gu, 1994b), by 6-fold for ddATP, 5-fold for FddATP, 4-fold for PMEApp, ddCTP, AZTTP, and D4TTP, and 9-fold for 3TCTP (Table 3). In this respect, HIV-1 carrying the K65R mutation has been shown to be cross-resistant to ddC, 3TC, ddI, and PMEA *in vitro* (Gu et al., 1994a, 1995). It may also be worth noting that RT carrying the 3TC-associated M184V mutation ($RT_{M184V}$) (Boucher et al., 1993; Gao et al., 1993a; Schinazi et al., 1993; Tisdale et al., 1993) and RT carrying the AZT-related T215Y mutation ($RT_{T215Y}$) (Larder & Kemp, 1989) were both 2-fold more sensitive to PMEApp as compared to $RT_{wt}$ as assessed with the $K_i/K_m$ ratio (Table 3).

Since the 3TC-associated mutation, M184V, has been reported to suppress AZT resistance caused by various AZT-associated mutations including T215Y *in vitro* (Boucher et al., 1993; Larder et al., 1995; Tisdale et al., 1993), we determined the inhibition constants to 3TCTP with the RT carrying the five mutations, A62V/V75I/F77L/F116Y/Q151M, which renders RT less sensitive to AZTTP (Table 3). This mutant RT preserved the sensitivity to 3TCTP (<2-fold), consistent with the previous observation that recombinant HIV-1 containing the five mutations showed only a slightly decreased sensitivities to 3TC compared to wild type HIV-1 (Iversen et al., 1996). It is noted that $RT_{M184V}$ was slightly more sensitive to AZTTP and that $RT_{T215Y}$ was slightly more sensitive to 3TCTP (Table 3), in agreement with previous findings (Boucher et al., 1993; Tisdale et al., 1993). In this regard, it may be interesting to examine the sensitivity of HIV-1 and RT carrying both Q151M and M184V mutations. It should be noted, however, that the $K_{i(ddNTP)}/K_{m(dNTP)}$ ratios do not always predict the sensitivities of HIV-1 variants to all ddNTPs. Additional factors such as the cytoplasmic ddNTP/dNTP ratios and the cytoplasmic half-life of ddNTPs are also linked to the anti-HIV-1 potency of ddNs (Ahluwalia et al., 1993; Yarchoan et al., 1991). Indeed, $RT_{T215Y}$ which renders HIV-1 highly resistant to AZT was less sensitive to AZTTP only by 1.6-fold as assessed in the single-nucleotide incorporation assay (Table 3).

## DISCUSSION

In the present study, we defined the biochemical properties of RTs with amino acid substitutions which have been associated with reduced sensitivity to multiple ddNTPs by using the single-nucleotide incorporation assay. There is a large body of experimental data with regard to steady-state

kinetic constants of $RT_{wt}$ and various mutant RTs (Gu et al., 1994b; Martin et al., 1993; Lacey et al., 1992; Ueno et al., 1995). Such data, however, have been generated by using different conditions and, more importantly, using different template-primers. Steady-state kinetic constants are known to be greatly influenced by the dissociation of the enzyme−template-primer complex, and the $k_{off}$ values vary depending on the template-primers and salt concentrations used (Beard & Wilson, 1993; Klarmann et al., 1993; Reardon, 1992; Reardon & Miller, 1990). Furthermore, steady-state rates determined using homopolymeric template-primers represent only an average of the rates of multiple steps, *e.g.*, the polymerization and dissociation (Jaju et al., 1995, Johnson, 1993, Reardon, 1992), thus providing only general mechanistic information. These issues have rendered the comparison and interpretation of kinetic data complicated. Therefore, in the present study, we chose to use only a heteropolymeric RNA template and the same buffer conditions in determining all kinetic constants of $RT_{wt}$ and mutant RTs. It is worth noting that in this study, we did not separate the elongated products by the high-resolution polyacrylamide gel electrophoresis. However, when we tested whether [$^3$H]-dAMP was incorporated into MS2/21T, 20G, and 19G mediated by $RT_{wt}$, there was virtually no [$^3$H]dAMP incorporation to MS2/21T, 20G, or 19G, suggesting that misincorporation of incorrect nucleotide or contamination with other nucleotides did not occur in the assay conditions employed. Moreover, the incorporation of [$^3$H]dAMP and [$^3$H]ddAMP to MS2/22A mediated by $RT_{wt}$ occurred with virtually identical $k_{cat}$ values, as previously described (Ueno et al., 1995), indicating that no significant products longer than expected were generated under the assay conditions studied.

The present substrate analyses of $RT_{wt}$ and all mutant RTs examined using the single-nucleotide incorporation assay indicated, however, that the steady-state kinetic constants to natural substrates had not been significantly altered by such mutations, mostly consistent with previously published data generated by using different template-primers and different assay conditions (Gu et al., 1994b; Lacey et al., 1992; Martin et al., 1993; Ueno et al., 1995). It is not clear as yet, however, whether an insignificant difference in the kinetic constants produces a significant difference in the dynamics of HIV-1 through its continuous, long-standing replication *in vivo*.

By contrast, the $K_i$ values to various ddNTPs proved to substantially differ among mutant RTs, strongly suggesting that RT's altered recognition of the missing and/or additional groups in its substrates (*i.e.*, ddNTPs) caused by the mutations is associated with RT's reduced sensitivity to ddNTPs. Since the dissociation of RT from the enzyme−template-primer complex is not significantly affected by ddNMP *per se*, which is incorporated at the 3′ end of the elongating primer (Reardon, 1992; Ueno et al., 1995; Van Draanen et al., 1992), the increase in $K_i$ values is most likely to be due to an increase in $K_{d(ddNTP)}$ and/or a decrease in $k_{p(ddNTP)}$. Considering that the equation, $K_i = K_{m(ddNTP)} = k_{off} \cdot K_{d(ddNTP)}/k_{p(ddNTP)}$ stands in the single-nucleotide incorporation assay, this issue will have to be examined using pre-steady-state kinetic analysis. Nevertheless, the $K_{i(ddNTP)}/K_{m(dNTP)}$ ratio ($K_{m(ddNTP)}/K_{m(dNTP)}$) determined in the present work appear to serve as a useful indicator in examining the altered properties of mutant RTs. For instance, the $K_{i(ddNTP)}/K_{m(dNTP)}$ ratio of $RT_{wt}$ for AZTTP (1.0; Table 3) indicates that AZTTP is the most efficient ddNTP for $RT_{wt}$ among the ddNTPs examined in this study. This finding well explains why AZT is potent against HIV-1 despite its low to moderate AZTTP/dTTP ratio, 0.04 and 0.61 as assessed in resting peripheral blood mononuclear cells (R-PBM) and PHA-PBM, respectively (Gao et al., 1994). DdCTP was also found to be an efficient substrate for $RT_{wt}$ ($K_i/K_m$ being 1.9, Table 3), a finding consistent with the fact that ddC is among the most potent antiviral ddNs against HIV-1 on the basis of molarity although its high ddCTP/dCTP ratios [4.31 and 0.98 in R-PBM and PHA-PBM, respectively (Gao et al., 1994)], should also be accounted for its potency. It is of note that $RT_{A62V/V75I/F77L/F116Y/Q151M}$ was less sensitive against ddATP (10-fold), ddCTP (5-fold), AZTTP (14-fold), and D4TTP (7-fold) as previously published (Ueno et al., 1995), while this mutant enzyme was as sensitive to PMEApp as was $RT_{wt}$ (Table 3). RTs with L74V, M184V, or T215Y were found to be more sensitive to PMEApp, 3TCTP, and FddCTP than was $RT_{wt}$ (Table 3). An introduction of PMEA to the existing antiviral therapy, in particular to the combined use of AZT plus ddI, AZT plus ddC, and AZT plus 3TC, may be worth exploring.

In the present study, in order to examine the processivity (the ability to translocate the enzyme along the template strand without dissociation) of various mutant RTs, we conducted a primer extension assay using MS2/22A as a template-primer and heparin as a trapping agent. Despite the strong pausing site observed at the very beginning of the reaction, the results showed that at least in the presence of saturating concentrations of dNTPs (100 $\mu$M dNTP each), the processive primer extension activity of mutant RTs escaping from the pausing site was comparable to that of $RT_{wt}$, indicating that the interaction of RT with the template-primer was not appreciably altered by mutations examined with all enzymes. It should be noted, however, that the processivity of RT is affected by not only the template sequence (Abbotts et al., 1993; Klarmann et al., 1993) but also the concentrations of dNTPs in the reaction mixture (Arts et al., 1996; Gao et al., 1993b). It is possible that differences in the processivity are seen between $RT_{wt}$ and mutant RTs if the RT-mediated primer extension occurs in the presence of low concentrations of dNTPs, *e.g.*, those in R-PBM, and/or a proper HIV-1 RNA is used as a template.

It was noted in the present study that RT with the K65R mutation showed resistance to all ddNTPs tested, a finding consistent with those by Gu et al. (1994b, 1995), although the magnitude of its resistance to ddATP, ddCTP, and AZTTP was less than that of $RT_{A62V/V75I/F77L/F116Y/Q151M}$. Gu et al. have recently reported that, as compared to an infectious wild-type HIV-1 clone, an HIV-1 clone carrying the K65R mutation was less sensitive to PMEA, 3TC, ddC, and ddI but was comparably sensitive to AZT in culture (Gu et al., 1995). In this regard, the slight changes seen in the sensitivity to AZTTP of RT with the T215Y mutation (Table 3) and RT with the D67N/K70R/T215Y/K219Q mutations account only partly for the significantly reduced sensitivity of the virus carrying such mutations to AZT (Kellam et al., 1992; Lacey et al., 1992; Larder et al., 1989). Thus, it is possible that the cross-resistance profile of certain mutant RTs to various ddNTPs may not be directly linked to the cross-resistance profile of the virus carrying the same RT.

Although the development of HIV-1 variants with resistance to RT inhibitors has recently been demonstrated to be related to clinical deterioration in patients receiving RT inhibitors (D'Aquila et al., 1995; Kozal et al., 1993), clinical benefits of continuous use of RT inhibitors should still be considered possible since such mutations may impair RT's structure/function, resulting in the production of less replication-competent HIV-1 (Coffin, 1995). In this regard, Wainberg et al. recently reported that RT carrying the 3TC-associated M184V substitution had an increased fidelity of nucleotide insertion than $RT_{wt}$ (Wainberg et al., 1996). However, whether the enhanced fidelity observed in such misinsertion fidelity assays poses a significant impact on the pathogenesis of HIV-1 diseases remains to be examined. Indeed, HIV-1 carrying M184V yet appears to readily acquire other drug-related mutations during 3TC-containing therapy (Schmit et al., 1996). Nevertheless, it is interesting to examine the fidelity of RTs with various combined mutations (*i.e.*, $RT_{A62V/V75I/F77L/F116Y/Q151M}$) which may have substantial constraints due to multiple mutations. Other factors constituting of RT's catalytic activity such as its RNase H activity also remain to be analyzed.

Three of the five mutation sites (Val-75, Phe-77, and Gln-151) are located close to the first template base and form part of the "template grip" (Jacobo-Molina et al., 1993). It is worth noting that residue 151 (Gln) of HIV-1 RT is located in a highly conserved concatenated motif B, which has been identified in RTs of animal and human retroviruses (Poch et al., 1989). In fact, the *pol* genes from 16 of sixteen animal and human retroviruses examined by Poch and colleagues encode the Leu-Pro-Gln-Gly sequence in the corresponding region within motif B (Poch et al., 1989). As we demonstrated in this study, the Q151M mutation appears to be sufficient to account for significant resistance to multiple TPs (Table 3). In this regard, Yadav et al. have recently proposed that Gln-151 of HIV-1 RT is involved in its dNTP binding function on the basis of their molecular modeling study of the catalytic domains of HIV-1 RT and *E. coli* DNA polymerase I (Yadav et al., 1994). Subsequently, Sarafianos et al. introduced a Q151A mutation into HIV-1 RT and showed that the rate of the first nucleotide incorporation was severely affected by the mutation (Sarafianos et al., 1995), suggesting that Gln-151 indeed plays a critical role in the dNTP binding step. Sarafianos et al. also showed a failure of RT with the Q151A mutation to catalyze the nucleotidyl transferase reaction onto the primer terminus of the covalently immobilized template-primer and a significant alteration in the divalent cation preferences with the Q151A mutation (Sarafianos et al., 1995), strongly suggesting a critical role of Gln-151 in the dNTP binding site of RT. Considering that the Q151M mutation did not affect the steady state kinetic parameters for dNTP, it would be interesting to examine the replication competence and sensitivity to ddNs of HIV-1 carrying the Q151A mutation. It is also of interest to study the mechanisms of substrate recognition or discrimination of ddNTPs by three-dimensional structure analysis and X-ray crystallographic analysis involving various mutant RTs including $RT_{Q151M}$ and $RT_{Q151A}$. Such studies should provide insight to the understanding of molecular mechanisms of substrate recognition and catalytic function of HIV-1 RT.

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