Dioxolane Guanosine 5’-Triphosphate, an Alternative Substrate Inhibitor of Wild-type and Mutant HIV-1 Reverse Transcriptase

STEADY STATE AND PRE-STEADY STATE KINETIC ANALYSES*

The frequency of human immunodeficiency virus, type 1 (HIV-1) mutations in response to antiviral therapy and resulting drug resistance is of major concern. Amdoxovir ((−)-β-p-2,6-diaminopurine dioxolane), the prodrug of dioxolane guanosine (DXG), is currently in phase II/II clinical development for the treatment of HIV-1 infection. In vitro, HIV-1 mutants resistant to 3’-azido-3’-deoxythymidine (M41L/D67N/K70R/T215Y/K219Q) and (−)β-1’-2’,3’-dideoxy-3’-thiacytidine (3TC) (M184V) remain sensitive to DXG. HIV-1 with the reverse transcriptase mutations K65R, L74V, and/or Q151M were less sensitive to DXG, whereas the mutation K103N re-sensitized the virus to the inhibitory effect of DXG. In order to understand these observations at the enzyme level, we investigated the inhibition of the HIV-1 reverse transcriptase-catalyzed viral DNA synthesis by dioxolane guanosine 5’-triphosphate (DXG-TP), 3’-azido-3’-deoxythymidine-TP, and 3TC-TP by using steady state kinetic analysis and the incorporation of DXG-5’-monophosphate by using pre-steady state kinetic analysis. This mechanistic study provided detailed information on the amdoxovir-related drug resistance at a molecular level. Overall, the enzymatic data correlated well with the antiviral data obtained from cell culture experiments and further supported the use of amdoxovir for the treatment of nucleoside reverse transcriptase inhibitor-experienced patients.

Human immunodeficiency virus encodes an RNA-dependent DNA polymerase (reverse transcriptase, RT)1 that is essential for viral replication. The HIV-1 RT has been successfully exploited as a target for anti-human immunodeficiency virus therapy. However, monotherapy with antiviral agents has led to the rapid emergence of drug-resistant HIV-1 variants. Combination regimens are currently used to delay the emergence of drug-resistant viruses. Unfortunately, not all patients tolerate combination regimens well, and this leads to a lack of compliance. These issues have fueled the continuing search for compounds that maintain potency against drug-resistant viruses and are better tolerated by patients.

Amdoxovir ((−)-β-p-2,6-diaminopurine dioxolane) (Fig. 1) is currently in phase II/II clinical development for the treatment of HIV-1. This compound was designed as a pro-drug to enhance the oral bioavailability of the active compound, (−)-β-d-dioxolane guanosine (Fig. 1, DXG). Amdoxovir is deaminated by adenosine deaminase to DXG, which is further metabolized to its 5’-triphosphate (DXG-TP) by host cell enzymes, including deoxyguanosine kinase and high K₉₅ 5’-nucleotidase.2 DXG-TP is a potent alternative substrate inhibitor of the HIV-1 RT (1). Incorporation of DXG 5’-monophosphate (DXG-MP) into nascent DNA results in chain termination. Virus harboring HIV-1 RT mutations associated with resistance to AZT (M41L/D67N/K70R/T215Y/K219Q), 3TC (M184V), abacavir (M41L/D67N, M184V/L210WT215Y), efavirenz (K103N), and certain multi-drug resistance mutations remained sensitive to inhibition by DXG (2–4). This lack of cross-resistance makes amdoxovir a potential therapeutic agent for patients who have failed the currently available therapies.

The primary mutations that confer resistance to DXG are K65R and L74V in the HIV-1 RT (2, 4, 5). The K65R and L74V variants of HIV-1, constructed by site-specific mutagenesis of HIV-1_LAD showed a 5.6- and 3.5-fold increase in EC₅₀ to DXG, respectively, as compared with wild-type (WT) HIV-1 (Table 1). Additionally, the K65R mutation conferred partial to complete cross-resistance to 3TC, ddC, ddi, and [9-[2-[4-phosphonomethoxy]ethyl], adefovir] (4, 6), whereas L74V is associated with resistance to ddi and ddC (7).

Two other HIV-1 RT mutations of interest are Q151M and K103N. The Q151M mutation is involved in multiple dideoxynucleoside resistance (8). In vitro studies showed that recombinant viruses with the mutations Q151M or K65R/Q151M were 9.6- and >20-fold less sensitive to DXG (Table I). The K103N mutation is associated with resistance to non-nucleo-
DXG-TP as Substrate Inhibitor of WT and Mutant HIV-1 RT

**FIG. 1. Structure of amodoxor (\(-\beta\)-d-2,6-diaminopurine dioloxane) (DAPD) and DXG.**

**TABLE I**

| HIV-1LAm mutant with an HIV-1LAm backbone | Fold changes in E\text{C}_{50} from wild type |
|-----------------------------------------|------------------------------------------|
| AZT\textsuperscript{a}                    | 2.1\textsuperscript{a}                     |
| 3TC\textsuperscript{b}                   | 1.0\textsuperscript{c}                     |
| K65R                                   | 5.6                                       |
| L74V                                   | 3.5                                       |
| K103N                                  | 0.9                                       |
| Q151M                                  | 9.6                                       |
| 65R/103N                                | 8.5                                       |
| 65R/151M                                | >20                                       |
| 74V/103N                                | 1.8                                       |
| 153N/151M                               | ND\textsuperscript{d}                     |
| 65R/103N/151M                          | ND\textsuperscript{d}                     |

\textsuperscript{a} Ref. 4.
\textsuperscript{b} ND, not determined.

A clinical isolate of HIV that contains the K103N mutation was shown to be hypersensitive to DXG (2). Interestingly, K103N appears to partially reverse the resistance to DXG conferred by certain mutations (2).

In order to further understand the molecular mechanism of DXG drug resistance and its lack of cross-resistance toward HIV-1 variants that were resistant to standard nucleoside therapy, we investigated the inhibition of DNA synthesis by DXG-TP and incorporation of DXG-MP by wild-type and mutant HIV-1 RTs. Two aspects were evaluated. First, under the steady state conditions, we studied the inhibition of HIV-1 RT-catalyzed DNA synthesis by DXG-TP, AZT-TP, and 3TC-TP to assess the overall enzymatic reaction. Second, we used a transient pre-steady state kinetic approach to examine the incorporation of DXG-MP by HIV-1 RTs, which revealed the individual steps involving the binding of DXG-TP and its chemical catalysis. Wild-type HIV-1 RT and a series of mutant enzymes were used in these studies. The mutant RTs employed in our study possessed either the single mutations (K65R, L74V, K103N, and Q151M) or combinations of these mutations (K65R/K103N, L74V/K103N, K65R/Q151M, K103N/Q151M, and K65R/K103N/Q151M). AZT-resistant RT (D67N/K70R/T215Y/K219Q) and 3TC-resistant RT (M184V) were also used in this study.

For the steady state kinetic analysis, \( K_m \) (the apparent substrate dissociation constant), \( K_i \) (the apparent inhibitor dissociation constant), and \( k_{cat} \) (the catalytic constant) values were measured. For pre-steady state kinetic analysis, two kinetic parameters \( K_d \) (the dissociation constant, which reflects the binding affinity of dNTP to the enzyme-DNA complex) and \( k_{cat} \) (the maximum rate of dNMP incorporation) were determined. These two complementary kinetic analyses provide us with the following: 1) a molecular basis for the lack of cross-resistance between the AZT\textsuperscript{p} and 3TC\textsuperscript{p} viruses with DXG; 2) detailed information on DXG-related drug resistance; and 3) a further understanding of the collective effects of multiple mutations.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The non-radioabeled deoxynucleoside 5'-triposphates (dNTPs), radiolabeled dNTPs ([\(\alpha^{32}\)]P)dCTP, [\(\alpha^{32}\)]P)dGTP, and [\(\alpha^{32}\)]P(dTTP) (3000 Ci/mmol); poly(rC)oligo(dG)\textsubscript{12-18} and poly(rA)-oligo(dT)\textsubscript{12-18}, template-primers were purchased from Amersham Biosciences. Polynucleosic acid (poly(rC)) and oligodeoxyctydylate acid (oligo(dC)\textsubscript{12-18}) were purchased from Sigma and resuspended in a solution of 20 mM Tris-Cl (pH 7.5) and 100 mM NaCl. The annealed poly(rC)-oligo(dC)\textsubscript{12-18} template-primers were prepared by mixing the homopolymers at a ratio of 1 mg of poly(rC), 10 nmol of oligo(dC)\textsubscript{12-18} and boiled for 2 min. The template-primer was allowed to cool to room temperature, aliquoted, and stored at \(-80^\circ\) C. PAGE-purified DNA primer (30-mer, 5'-GCC TCG CAG CCG TCC AAC CAA CTC AAC CTC-3') and DNA template (45-mer, 3'-CGG AGC GTC GCC AGG TTG GTT GAG TGG GAG CTA GGT TAC GGC AGG-5') were obtained from Integrated DNA Technologies, Inc. (Corailville, IA). The RNA template (45-mer, 3'-CGC AGG GUC GCC AGG UUG UUG GAG UUG GAG CUA GGU UAC GGC AGG-5') was synthesized and gel-purified by New England Biolabs (Beverly, MA). The concentration of the oligonucleotides were estimated by UV absorbance at 260 nm using the following calculated extinction coefficients: DNA 30-mer, \( \epsilon = 294,000 \text{ M}^{-1} \text{ cm}^{-1} \); DNA 45-mer, \( \epsilon = 492,000 \text{ M}^{-1} \text{ cm}^{-1} \); and RNA 45-mer, \( \epsilon = 508,000 \text{ M}^{-1} \text{ cm}^{-1} \).

**Nucleotide Analogs—**DXG was synthesized using published procedures (11). The 5'-triphosphate of DXG (DXG-TP) was synthesized by Inspire Pharmaceuticals (Durham, NC). 3'-Azido-3'-deoxythymidine 5'-triphosphate (AZT-TP) and \((\beta\)-d-2,3'-dideoxy-3'-thiacytidine 5'-triphosphate (3TC-TP) were purchased from Moravek Biochemicals (Brea, CA).

**Site-directed Mutagenesis—**The plasmid, p6HRT-PROT (12), 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 Institutes of Health. The plasmid was used as the template for site-directed mutagenesis using the QuikChangeTM Site-directed Mutagenesis kit following the manufacturer's instruction (Stratagene, La Jolla, CA). The entire RT coding region of each mutant plasmid was analyzed by dideoxy sequencing to confirm the accuracy of the sequenced nucleotide. Purified HIV-1 RT harboring either the M184V mutation (3TC\textsuperscript{p} RT) or a combination of D67N/K70R/T215Y/K219Q RT mutations (AZT\textsuperscript{p} RT) was purified at Dr. K. S. Anderson's laboratory at Yale University (New Haven, CT).

**Enzyme Expression and Purification—**The wild-type and mutant HIV-1 RT proteins were expressed in Escherichia coli and purified as described previously (13, 14). Protein concentration was measured spectrophotometrically at 280 nm using an extinction coefficient (\( \epsilon_{280} \)) of 260,450 M\(^{-1}\) cm\(^{-1}\). The concentration of active RT in each preparation was determined by pre-steady state burst kinetics that yielded the active site concentration (30–40% of enzyme concentration). The active site concentrations were used to calculate the \( k_{cat} \) values reported in this study.

**Steady State Kinetic Analysis—**The steady state concentrations were measured using initial rates. Reaction mixtures contained 50 mM Tris-HCl (pH 7.8), 5 mM MgCl\textsubscript{2}, 0.025% Triton X-100, 0.012 units of homopolymeric RNA/DNA template-primer, 1 \( \mu \) M of [\(\alpha^{32}\)]P-dNTP, and varying dNTP concentrations in a final volume of 50 \( \mu \)l. All reactions were carried out at 37 °C and initiated by the addition of enzyme. Aliquots of the reaction were collected at five time points ranging from 0.5 to 5 min in the linear phase of the reaction. Aliquots (5 \( \mu \)l) were spotted on DE81 ion-exchange paper and allowed to dry. The DE81 paper was washed with 50 mg/ml Na\textsubscript{2}HPO\textsubscript{4} (3 times, 5 min/ wash) and ethanol (reagent grade, 2 min) and dried. Product quantification was performed using a Amersham Biosciences Phosphor-Imager. The \( K_m \) and \( V_{max} \) values were determined by fitting the data to the Michaelis-Menten equation through non-linear regression using Grafit (version 3.09b, Erithacus software, UK). Values reported are means ± S.E.

**In vitro Incorporation Experiments—**In vitro incorporation experiments used to measure the inhibition of dNMP incorporation into the homopolymeric RNA/DNA template-primer by a dideoxynucleoside analog 5'-triphosphate (ddNTP) were similar to the steady state reaction mixtures described above, except that the dNTP substrate concentration was fixed, and the concentration of ddNTP was varied. \( I_{50} \) values were obtained by fitting the inhibition data to the following Equation 1 by using nonlinear least squares regression,

\[
\% \text{ inhibition} = a(1 + \left(\text{ddNTP} / IC_{50\text{ additive}}\right))^b
\]

where \( a \) is the maximum percent inhibition at a saturating concentration of ddNTP, \( \text{[ddNTP]} \) is the concentration of the nucleoside analog

% inhibition = a/(1 + ([ddNTP]/IC\textsubscript{50 additive})) ^ b (Eq. 1)
Table II

Steady-state kinetic parameters for dNMP incorporation into homopolymeric template-primers by mutant HIV-1 RTs

| HIV RT          | dC12 | dG12 | dT12 | Kd, Kcat/Km | dC12 | dG12 | dT12 |
|-----------------|------|------|------|-------------|------|------|------|
| WT              | 0.50 ± 0.09 | 1.14 ± 0.25 | 4.72 ± 0.83 | 19.6 ± 2.8 | 12.7 ± 1.1 | 156 ± 25 | 39.2 ± 11.2 | 33.1 |
| K65R            | 1.10 ± 0.25 | 1.49 ± 0.27 | 3.06 ± 0.87 | 15.5 ± 5.4 | 14.1 ± 2.0 | 57 ± 13 | 14.1 ± 9.5 | 18.7 |
| L74V            | 0.45 ± 0.06 | 1.34 ± 0.25 | 2.73 ± 0.40 | 20.2 ± 4.6 | 18.7 ± 1.6 | 72 ± 17 | 45.3 ± 14.0 | 28.5 |
| K103N           | 0.50 ± 0.09 | 0.82 ± 0.10 | 2.18 ± 0.52 | 31.9 ± 5.9 | 17.8 ± 5.0 | 114 ± 28 | 63.4 ± 21.7 | 52.5 |
| Q151M           | 0.73 ± 0.26 | 0.40 ± 0.18 | 5.78 ± 0.94 | 13.6 ± 1.7 | 14.7 ± 4.5 | 166 ± 45 | 18.6 ± 37.0 | 28.7 |
| K65R/K103N      | 1.28 ± 0.23 | 0.96 ± 0.14 | 3.33 ± 0.90 | 30.4 ± 3.6 | 38.7 ± 4.5 | 203 ± 39 | 37.3 ± 40.5 | 61.1 |
| L74V/K103N      | 0.21 ± 0.01 | 0.30 ± 0.01 | 2.40 ± 0.33 | 12.6 ± 2.2 | 11.6 ± 1.8 | 121 ± 12 | 58.7 ± 38.9 | 50.5 |
| Q151M/K103N     | 0.75 ± 0.16 | 0.55 ± 0.08 | 3.88 ± 0.92 | 8.7 ± 0.9 | 13.1 ± 0.8 | 162 ± 26 | 11.6 ± 23.9 | 41.8 |
| K65R/Q151M      | 0.95 ± 0.07 | 1.18 ± 0.34 | 4.40 ± 1.22 | 12.9 ± 1.4 | 23.1 ± 6.5 | 147 ± 47 | 13.6 ± 19.5 | 33.3 |
| K65R/K103N/Q151M| 1.49 ± 0.27 | 0.65 ± 0.15 | 4.39 ± 0.99 | 18.6 ± 3.5 | 22.9 ± 6.5 | 197 ± 43 | 12.4 ± 35.3 | 44.9 |
| AZT<sub>⌧</sub>  | 0.86 ± 0.16 | 1.68 ± 0.61 | 6.00 ± 1.18 | 19.9 ± 3.5 | 16.5 ± 4.3 | 117 ± 25 | 23.1 ± 9.8 | 19.5 |
| 3TC<sub>⌧</sub> | 0.73 ± 0.20 | 1.14 ± 0.39 | 4.70 ± 0.71 | 15.0 ± 0.5 | 23.5 ± 4.8 | 123 ± 21 | 20.6 ± 20.6 | 26.2 |

a Values are mean ± S.E.

b AZT<sub>⌧</sub>, D67N/K70R/T215Y/K219Q.

c 3TC<sub>⌧</sub>, M184V.

Inhibition of HIV-1 RT by DXG-TP. AZT-TP, and 3TC-TP


dGMP Incorporation by DXG-TP—Inhibition of dGMP incorporation into homopolymeric RNA/DNA template-primer by DXG-TP was not affected by the following mutations: K65R, L74V, K103N, K65R/K103N, L74V/K103N, and K103N/Q151M, nor were AZT-resistant (AZT<sup>⌧</sup>) or 3TC-resistant (3TC<sup>⌧</sup>) RTs. However, the K<sub>Km</sub>/K<sub>cat</sub> ratios for the Q151M, K65R/Q151M, and K65R/K103N/Q151M mutant RTs were increased by 5.3-, 15-, and 7.8-fold, respectively, over the wild-type enzyme, indicating that these mutant enzymes were less sensitive to the inhibition of dGMP incorporation by DXG-TP than the WT enzyme. Recently, Mewshaw et al. (2) reported a possible re-sensitization effect of K103N mutation on the antiviral activity of DXG against clinical isolates of HIV-1. In this study, the presence of the K103N mutation in a background of K65R, Q151M, or K65R/Q151M caused a re-sensitization effect over the corresponding non-K103N-containing mutants. Compared with the K65R, Q151M, or K65R/Q151M RTs, the K<sub>Km</sub>/K<sub>cat</sub> ratios were reduced by 2.2-, 3.7-, and 1.9-fold (p values < 0.1) when the K103N mutation was introduced.

Inhibition of dTMP Incorporation by AZT-TP—Inhibition of dTMP incorporation into poly(rA):oligo(dT)<sub>12-18</sub> by AZT-TP was studied. The K<sub>cat</sub> values for the inhibition of dTMP incorporation...
by AZT-TP for AZT<sup>R</sup> (D67N/K70R/T215Y/K219Q) RT was similar to WT enzyme. For the mutant RTs with L74V, K103N, L74V/K103N, and M184V, there was no increase in the \( K/K_m \) ratio for the inhibition of dTMP incorporation by AZT-TP. Enzymes containing the K65R, Q151M, and K65R/Q151M mutations, however, did show significant resistance to AZT-TP inhibition, with fold changes of 6.4, 6.7, and 55, respectively. The K103N containing mutations K65R/K103N, K103N/Q151M, and K65R/K103N/Q151M caused 3.7-, 2.7-, and 18-fold increases for \( K/K_m \) ratio over WT RT. Again, the addition of the K103N mutation to K65R, Q151M, or K65R/Q151M RT mutants resulted in a moderate decrease in resistance to AZT-TP inhibition, with a 1.7- (K65R/K103N), 2.5- (K103N/Q151M), and 3.1-fold (K65R/K103N/Q151M) decrease in the \( K/K_m \) ratio for AZT-TP.

**Inhibition of dCMP Incorporation by 3TC-TP**—Inhibition of dCMP incorporation into poly(rI)-oligo(dC)12 by 3TC-TP was only marginally (0.5–2-fold change) affected by most of the mutations, with the exception of M184V. Similar to data reported previously (15), the M184V mutation caused an ~300-fold increase in the \( K/K_m \) ratio (Table III).

**Incorporation of DXG-MP by WT and Mutant HIV-1 RT**

**DNA-dependent Incorporation of dGMP by Wild-type and Mutant HIV-1 RTs**—Our first step in defining the kinetics for the incorporation of natural substrate dGTP was to conduct a pre-steady state burst experiment with WT and mutant HIV-1 RTs. In this experiment, the DNA substrate was in excess of DNA 30/45-mer primer-template (300 nM) and HIV-1 RT (active site concentration, ~100 nm) was mixed with 10 μM dGTP in 10 mM MgCl<sub>2</sub> containing buffer to start the reaction. The reactions were quenched at the indicated time interval with EDTA (0.3 mM) and were analyzed by sequencing gel electrophoresis. The solid line represents the fit to the burst equation as described in the text. The curves for WT RT represent fits with \( A = 84 \pm 4 \) nm, \( k_{obs} = 11 \pm 1 \) s<sup>−1</sup>, and \( k_{in} = 0.26 \pm 0.05 \) s<sup>−1</sup>, and the curves for the AZTR RT represents fits with \( A = 78 \pm 2 \) nm, \( k_{obs} = 12.3 \pm 0.7 \) s<sup>−1</sup>, and \( k_{in} = 0.19 \pm 0.04 \) s<sup>−1</sup>.

**dGMP incorporation (\( k_{pol} \)), and the efficiency of incorporation (\( k_{pol}/K_p \)).** Fig. 3 shows the dGTP concentration dependence of \( k_{obs} \) for incorporation into the D30/D45 primer-template using WT and AZT<sup>R</sup> HIV-1 RT. The \( k_{pol} \) value for dGMP incorporation by AZT<sup>R</sup> RT (18.3 ± 0.9 s<sup>−1</sup>) is very similar to that of WT RT (15.9 ± 0.5 s<sup>−1</sup>). The \( K_p \) value for the AZT<sup>R</sup> RT (4.8 ± 0.8 μM) also is similar to that of WT RT (6.1 ± 0.9 μM), indicating dGTP binds to the (AZT<sup>R</sup>)-DNA complex as tightly as it binds to the (WT RT)-DNA complex. As a result, dGTP is incorporated by AZT<sup>R</sup>-RT as efficiently as the WT enzyme, as indicated by the \( k_{pol}/K_p \) value (Table IV).

The \( k_{pol} \), \( K_p \), and \( k_{pol}/K_p \) values determined for dGMP incorporation during DNA-dependent DNA synthesis catalyzed by WT and mutant HIV-1 RTs are summarized in Table IV. Overall, the binding affinities of different (mutant RT)-DNA com-

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**TABLE III**

| HIV RT       | DXG-TP | AZT-TP | 3TC-TP |
|--------------|--------|--------|--------|
|              | \( k_i \) | \( K/K_m(\text{ATP}) \) | Fold change<sup>a</sup> | \( k_i \) | \( K/K_m(\text{ATP}) \) | Fold change<sup>b</sup> | \( k_i \) | \( K/K_m(\text{ATP}) \) | Fold change<sup>c</sup> |
| WT           | 50 ± 7<sup>b</sup> | 0.0439 | 1.0 | 37 ± 1 | 0.0078 | 1.0 | 233 ± 28 | 0.4664 | 1.0 |
| K65R         | 116 ± 33 | 0.0777 | 1.8 | 152 ± 34 | 0.0498 | 6.4 | 886 ± 171 | 0.8048 | 1.7 |
| L74V         | 67 ± 17 | 0.0500 | 1.1 | 38 ± 2 | 0.0140 | 1.8 | 354 ± 22 | 0.7935 | 1.7 |
| K103N        | 20 ± 2 | 0.0244 | 0.55 | 14 ± 2 | 0.0066 | 0.85 | 123 ± 24 | 0.2449 | 0.53 |
| Q151M        | 93 ± 7 | 0.2341 | 5.3 | 300 ± 22 | 0.0520 | 6.7 | 203 ± 25 | 0.2774 | 0.59 |
| K65R/K103N   | 34 ± 7 | 0.0356 | 0.81 | 97 ± 15 | 0.0290 | 3.7 | 1184 ± 272 | 0.9524 | 2.0 |
| L74V/K103N   | 22 ± 1 | 0.0738 | 1.1 | 15 ± 1 | 0.0064 | 0.83 | 150 ± 60 | 0.6970 | 1.5 |
| Q151M/K103N  | 35 ± 7 | 0.0639 | 1.5 | 81 ± 15 | 0.0209 | 2.7 | 159 ± 41 | 0.2109 | 0.45 |
| K65R/Q151M   | 766 ± 74 | 0.6472 | 15 | 1870 ± 896 | 0.4250 | 55 | 418 ± 57 | 0.4401 | 0.94 |
| K65R/K103N/Q151M | 221 ± 45 | 3.405 | 7.8 | 609 ± 225 | 0.1386 | 18 | 364 ± 95 | 0.2437 | 0.52 |
| AZTR<sup>e</sup>, 3TC<sup>d</sup> | 90 ± 1 | 0.0536 | 1.2 | 54 ± 1 | 0.0090 | 1.2 | 595 ± 130 | 0.6897 | 1.5 |
| AZTR<sup>e</sup>, 3TC<sup>d</sup> | 60 ± 2 | 0.0523 | 1.2 | 63 ± 15 | 0.0134 | 1.7 | 100790 | 138.0685 | 296 |

<sup>a</sup> Fold change represents the mutant RT \( K/K_m \) ratio divided by the WT \( K/K_m \) ratio.

<sup>b</sup> Values are mean ± S.E.

<sup>c</sup> AZTR<sup>e</sup>, D67N/K70R/T215Y/K219Q.

<sup>d</sup> 3TC<sup>d</sup>, M184V.
and DXG-MP incorporation differed depending on the RT mutants. To analyze further the discrimination of DXG-TP over dGTP, we used the ratio of dGMP incorporation efficiency over DXG-MP incorporation efficiency to define the selectivity factor. In this study, a higher value for the selectivity factor indicates a higher degree of discrimination for DXG-TP over dGTP by the corresponding HIV-1 RT. The ratio of the selectivity factor for a mutant RT to that of WT RT reflects the overall fold change over the WT RT resulting from the mutation. For AZT<sup>Wt</sup> and 3TC<sup>R</sup> RTs, a marginal 2-fold change was observed. For the remaining RTs surveyed in this study, the fold change was within a range of 0.6–40, in the following order: K103N < WT < K103N/Q151M < K65R/K103N < K65R/K103N/Q151M = L74V < L74V/K103N < K65R/Q151M < K65R.

**RNA-dependent Incorporation of dGMP by Wild-type, AZT<sup>Wt</sup>, and 3TC<sup>R</sup> HIV-1 RTs**—By using a DNA/RNA primer-template 30/45-mer, the incorporation of dGMP by WT, AZT<sup>Wt</sup>, and 3TC<sup>R</sup> HIV-1 RT was investigated. In all cases, a biphasic burst of product formation was observed, indicating that compared with the WT HIV-1 RT, it is likely that the rate-limiting step for the incorporation of dGMP by AZT<sup>Wt</sup> and 3TC<sup>R</sup> HIV-1 RT was not changed during RNA-dependent DNA synthesis. The results summarized in Table V showed that AZT<sup>Wt</sup> and 3TC<sup>R</sup> RT incorporated dGMP at a rate that was 1.8- and 5.5-fold higher than WT RT. However, the binding affinity of dGTP to the enzyme-DNA complex is 2.8- and 11-fold weaker for AZT<sup>Wt</sup> and 3TC<sup>R</sup> RT compared with WT RT. Therefore, the net effects of the simultaneous changes in the <i>k<sub>pol</sub></i> and <i>K<sub>d</sub></i> values result in the following incorporation efficiencies, 2.8, 1.8, and 1.5 μm<sup>−1</sup>s<sup>−1</sup> for WT, AZT<sup>Wt</sup>, and 3TC<sup>R</sup> RT, respectively.

**RNA-dependent Incorporation of DXG-MP by Wild-type, AZT<sup>Wt</sup>, and 3TC<sup>R</sup> HIV-1 RTs**—The incorporation of DXG-MP into DNA/RNA primer-template 30/45-mer by WT, AZT<sup>Wt</sup>, and 3TC<sup>R</sup> HIV-1 RTs was studied in a manner similar to the one described above. The results summarized in Table V showed that the rate of incorporation of DXG-MP by AZT<sup>Wt</sup> RT (<i>k<sub>pol</sub></i>) and the binding affinity of DXG-TP for (AZT<sup>Wt</sup>-RT)-DNA (<i>K<sub>d</sub></i>) were identical to WT RT. The selectivity factors for WT, AZT<sup>Wt</sup>, and 3TC<sup>R</sup> RTs were 7.2, 11, and 3.8, respectively, which were not significantly different from each other, indicating that AZT<sup>Wt</sup> and 3TC<sup>R</sup> RT were not more discriminating against DXG-TP than the WT RT.

**DISCUSSION**

The high frequency of HIV-1 viral mutations in response to antiviral drug treatment makes the emergence of drug resistance one of the major concerns in drug discovery and development. The processes of nucleoside analog drug uptake, transport, metabolism, and incorporation are complex, and as a consequence these factors greatly contribute to the potencies of NRTI in naive and drug-experienced patients. For the most part, HIV-1 drug resistance can be accounted for by the structural changes in the targeted enzymes (16–18). One approach to better understand the molecular basis of drug resistance is the use of recombinant mutant enzymes as tools. Lys<sup>65</sup>, a residue located at the fingertip of the right hand configuration of HIV-1 RT, directly interacts with the incoming dNTP (or dNTP) by donating hydrogen bonds to the γ-phosphate of dNTP (19). After 13–14 passages of virus in the presence of increasing concentrations of DXG, a Lys<sup>65</sup> to Arg mutation (K65R) was identified that conferred partial resistance to amdoxovir (4). Site-directed mutagenesis studies of HIV-1 RT at Lys<sup>65</sup> showed that a positively charged residue at this position is essential for the correct catalytic functioning of the enzyme (20). Mutations at Lys<sup>65</sup> significantly changed the nucleotide binding selectivity of HIV-1 RT (20) and also increased the fidelity for DNA synthesis (21). Our steady state
DXG-TP as Substrate Inhibitor of WT and Mutant HIV-1 RT

**TABLE IV**

| HIV-1 RT     | dGTP | DXG-TP | Selectivity factor | Fold changes |
|--------------|------|--------|--------------------|-------------|
|              | $k_{\text{pol}}$ | $K_d$ | $k_{\text{pol}}/K_d$ | $k_{\text{pol}}/K_d$ |
|              | s$^{-1}$ | $\mu M$ | $\mu M^{-1}$ s$^{-1}$ | $\mu M^{-1}$ s$^{-1}$ |
| WT           | 15.9 ± 0.6 | 6.1 ± 0.9 | 2.6 ± 0.4 | 1.46 ± 0.03 | 2.5 ± 0.2 | 0.58 ± 0.05 | 4.5 ± 0.8 | 1 |
| K65R         | 8.3 ± 0.6 | 2.7 ± 0.6 | 3.1 ± 0.7 | 0.19 ± 0.02 | 11 ± 4 | 0.017 ± 0.007 | 178 ± 79 | 40 |
| L74V         | 25.8 ± 0.5 | 7.5 ± 0.3 | 3.4 ± 0.1 | 0.50 ± 0.02 | 8.1 ± 0.9 | 0.06 ± 0.01 | 56 ± 7 | 13 |
| K103N        | 34 ± 2 | 16 ± 4 | 2.1 ± 0.6 | 2.89 ± 0.07 | 3.6 ± 0.4 | 0.80 ± 0.09 | 2.6 ± 0.7 | 0.6 |
| K151M        | 26 ± 2 | 6.8 ± 1.8 | 3.8 ± 1.0 | 0.34 ± 0.02 | 2.9 ± 0.4 | 0.12 ± 0.02 | 33 ± 10 | 7 |
| 65R/103N     | 9.4 ± 0.7 | 8.8 ± 2.1 | 1.1 ± 0.3 | 0.22 ± 0.03 | 5.1 ± 1.7 | 0.04 ± 0.02 | 25 ± 11 | 6 |
| 65R/151M     | 9.8 ± 0.4 | 10 ± 2 | 0.98 ± 0.11 | 0.045 ± 0.003 | 4.7 ± 1.2 | 0.010 ± 0.003 | 102 ± 29 | 23 |
| 74V/103N     | 242 ± 2.3 | 3.2 ± 0.2 | 7.6 ± 0.5 | 0.54 ± 0.03 | 4.3 ± 1.1 | 0.13 ± 0.03 | 60 ± 16 | 14 |
| 103N/151M    | 16 ± 2 | 3.7 ± 1.1 | 4.3 ± 1.4 | 0.38 ± 0.01 | 1.5 ± 0.1 | 0.25 ± 0.02 | 17 ± 6 | 4 |
| 65R/103N/151M| 6.3 ± 0.4 | 5.9 ± 1.3 | 1.1 ± 0.2 | 0.046 ± 0.002 | 1.3 ± 0.2 | 0.035 ± 0.006 | 30 ± 8 | 7 |
| AZT$^{a,b}$  | 18.3 ± 0.9 | 4.8 ± 0.8 | 3.8 ± 0.7 | 1.69 ± 0.08 | 4.8 ± 0.7 | 0.35 ± 0.06 | 11 ± 3 | 2 |
| 3TC$^{a,c}$  | 29 ± 1 | 8 ± 1 | 3.6 ± 0.5 | 2.3 ± 0.7 | 4.9 ± 0.8 | 0.47 ± 0.08 | 7.7 ± 1.7 | 2 |

$^a$ Defined as $(k_{\text{pol}}/K_d)_{\text{dGTP}}/(k_{\text{pol}}/K_d)_{\text{DXG-TP}}$.
$^b$ Defined as (selectivity factor)$_{\text{mutant RT}}$/selectivity factor$_{\text{WT RT}}$.
$^c$ Values are mean ± S.E. Errors in the values of $K_d$ were calculated using standard methods.

Kinetic differences between wild-type, AZT-resistant, and 3TC-resistant HIV-1 RTs in DNA-dependent incorporation of dGMP or DXG-MP

**TABLE V**

| HIV-1 RT     | dGTP | DXG-TP | Selectivity factor | Fold changes |
|--------------|------|--------|--------------------|-------------|
|              | $k_{\text{pol}}$ | $K_d$ | $k_{\text{pol}}/K_d$ | $k_{\text{pol}}/K_d$ |
|              | s$^{-1}$ | $\mu M$ | $\mu M^{-1}$ s$^{-1}$ | $\mu M^{-1}$ s$^{-1}$ |
| WT           | 34 ± 2 | 12 ± 2 | 2.8 ± 0.5 | 2.4 ± 0.1 | 9.6 ± 1.6 | 0.25 ± 0.04 | 11 ± 3 | 1 |
| AZT$^{a,b}$  | 61 ± 4 | 34 ± 6 | 1.8 ± 0.3 | 2.2 ± 0.03 | 8.9 ± 0.4 | 0.25 ± 0.01 | 7.2 ± 1.2 | 0.7 |
| 3TC$^{a,c}$  | 187 ± 7 | 127 ± 11 | 1.5 ± 0.1 | 11.24 ± 0.06 | 3.2 ± 0.5 | 0.39 ± 0.06 | 3.8 ± 0.6 | 0.4 |

$^a$ Defined as $(k_{\text{pol}}/K_d)_{\text{dGTP}}/(k_{\text{pol}}/K_d)_{\text{DXG-TP}}$.
$^b$ Defined as (selectivity factor)$_{\text{mutant RT}}$/selectivity factor$_{\text{WT RT}}$.
$^c$ Values are mean ± S.E. Errors in the values of $K_d$ were calculated using standard methods.

Recent studies (22–27) have shown that HIV-1 RT-catalyzed excision of the chain terminating nucleoside analogs contributes to NRTI drug resistance. White et al. (27) demonstrated that ATP-facilitated removal of chain terminators does not significantly contribute to DXG resistance. Our pre-steady state study demonstrated that weaker binding affinity of DXG-TP to the K65R RT-DNA complex (4.4-fold versus WT) and the slower rate of incorporation for DXG-MP (7.7-fold versus WT) is the main mechanism for DXG resistance. As a result, K65R RT was overall 40-fold more discriminating against DXG-TP than the WT RT. The 1.8-fold change in the $K_d$ values observed in this study is similar to the 2.8-fold change reported by White et al. (27) and in the same range as the 5.6-fold change in EC$_{50}$ value from antiviral studies (2). The apparent inconsistency between the data from the steady state and the pre-steady state studies is likely due to the different scope of analysis. The $K_m$ and $K_d$ values in the steady state analysis are apparent dissociation constants for the substrate and inhibitor that reflect the overall dissociation constants of all enzyme-bound species. In contrast, the $K_d$ and $k_{\text{cat}}$ values measured in our pre-steady state studies represent the true dissociation constant for the (RT-DNA)-dNTP complex and the rate of incorporation.

Previous studies showed that HIV-1 K65R mutants were either not resistant (0.5–0.9-fold change for IC$_{50}$) (2, 27) or slightly resistant (3.3-fold change for IC$_{50}$) to AZT (4) in cell culture. Our steady state data showed that the mutant enzyme was 6.4-fold less sensitive to inhibition by AZT-TP, based on the $K/K_m$ ratio (Table III). This value was consistent with the level of resistance reported by Bazmi et al. (4) in their antiviral studies.

In cell culture, the HIV-1 K65R mutant was 20-fold less sensitive to 3TC (6). However, our enzymatic study showed no shift in the $K/K_m$ ratio for 3TC-TP/dCTP. This apparent contradiction could be partially due to the fact that HIV-1 RT catalyzes non-processive synthesis on poly(rI)·oligo(dC)$_{12}$ template-primer (Table II). The K65R mutation is not commonly observed in patients receiving nucleoside therapy. The low frequency of this mutation may be related to the impaired ability of the K65R-containing RT to catalyze dCMP incorporation.

Another mutation isolated when viruses were grown in the presence of increasing concentrations of DXG is the mutation Leu$^{74}$ to Val (L74V) (2, 4). Like Lys$^{65}$, Leu$^{74}$ is also located at the fingertip of the RT structure. However, instead of direct contact with the incoming nucleoside 5’-triphosphate it interacts with the template nucleotide paired with the incoming dNTP (19). As a result, the templating base is tightly locked in place, whereas the template is wedged between Leu$^{74}$ and Gly$^{152}$. Studies showed that the L74V mutation had no effect on fidelity but did cause a decrease in processivity (21, 29). Our steady state study showed that the L74V mutation did not affect the apparent binding of dCTP, dGTP, or dTTP, nor did
this mutation affect the incorporation of dNMPs into their respective homopolymeric template-primer (Table II). In addition, this mutant RT was not resistant to inhibition by DXG-TP, AZT-TP, or 3TC-TP, confirming the results of Boyer et al. (12), Martin et al. (30), and Bazmi et al. (4) for AZT-TP inhibition of an L74V RT mutant. Our pre-steady state analysis demonstrated that the binding affinity of L74V RT for DXG-TP was 3.2-fold weaker than the WT RT, and the rate of incorporation of DXG-TP by L74V RT was 2.9-fold slower than the WT RT. The resulting 13-fold change over WT RT for L74V is similar to the 12.3-fold change observed by Selmi et al. (31); however, it is higher than the 3.5-fold change in EC_{50} values observed from cell culture studies (Table I) and the 1.64-fold change in K_{i}/K_{m} values determined by steady state analysis. Because L74V differentiates incoming ddNTP through interaction with template, it is very likely that the different template-primer sequences used in different assays would result in different observations. When antiviral assays are performed, the compound is generally added 1 h post-infection. Therefore, in these assays viral inhibition by nucleoside analogs involves the heteropolymeric viral RNA genome and primarily focuses on the inhibition of RNA-dependent DNA synthesis. In the steady state study a homopolymeric RNA template was used, and in the pre-steady state study we used a heteropolymeric DNA as template.

With the exception of 3TC, the Gln to Met mutation at codon 151 of HIV-1 RT was shown to confer viral resistance to dideoxy analogs, including AZT, ddI, ddC, 2',3'-dideoxy-3'-deoxymethidine, and abacavir (32). In our steady state study, we showed the Q151M substitution caused marginal changes in the catalytic efficiency of RT (K_{cat}/K_{m}, Table II), similar to a report by Ueno et al. (33). Crystal structure studies showed that the side chain of Gln^{151} in the p66 RT subunit interacts not only with the 3'-OH of the incoming dNTP but also with the base of the incoming dNTP (19). In addition, Kaushik et al. (34, 35) proposed an interaction between Gln^{151} and Arg^{72} and a subsequent involvement in the conformational change in the ternary complex. The multiple interactions of the Gln^{151} with the dNTP-binding site might also be expected to differentially affect the sensitivity of the enzyme to NRTIs (36). The three NRTIs analyzed in our steady state study were different from their natural analogs by the structure of their sugar ring at the 3'-position, rather than the base itself. DXG-TP, which has a 1-β-D configuration, lacks a 3'-functional group, and the 3'-carbon was replaced by an oxygen atom and was a 5.3-fold less potent inhibitor of the Q151M RT than WT RT. 3TC-TP lacks a 3'-functional group but is in the 1-β-L configuration, has a sulfur atom in the place of the 3'-carbon, and remains a potent inhibitor of the Q151M RT. Finally, AZT-TP, with an azide group in the 3'-position of the β-D nucleoside analog, was impeded in the ability to inhibit dTMP incorporation by this mutant enzyme. These results suggest that the observed multidrug resistance due to the Q151M mutation may be the result of steric hindrance between the methionine residue and the modified sugar ring of D-configured nucleotides analogs (i.e. AZT-TP, ddCTP, ddATP, and DXG-TP). Compared with the WT RT, the Q151M enzyme was inhibited 5-fold less effectively by DXG-TP in our steady state studies (Table III) and was 7-fold more discriminating against DXG-TP in the pre-steady state studies (Table IV). These results are in the same range as the 9.6-fold resistance observed from in vitro studies using recombinant viruses.

Viruses containing the double mutation K65R/Q151M showed a significant increase in the level of resistance to inhibition by nucleoside analogs in cell culture assays, compared with the single mutants (2). The effect of this combination was evaluated by steady state and pre-steady state kinetic analysis. In general, the double mutation did not significantly affect the K_{m} and k_{cat} values for dCTP, dGTP, and dTTP incorporation. 3TC-TP remained an effective inhibitor of this enzyme; however, the enzyme was 55- and 15-fold less sensitive to inhibition by AZT-TP and DXG-TP, based on K_{i}/K_{m} values. Harris et al. (36) suggested that mutations around the dNTP-binding pocket, i.e. Q151M and K65R, may alter the size and shape of this pocket and result in altering the ability of the enzyme to select the correct dNTP during polymerization. The results of this study further indicated that this double mutation might allow the enzyme to discern structural modifications on the correctly base-paired nucleotide analogs, resulting in resistance to ddNTPs through a weaker apparent binding affinity (K_{i}). Based on the pre-steady state k_{pol}/K_{i} values, K65R/Q151M RT was 23-fold more discriminating against DXG-TP than the WT RT. This was mainly due to a significant decrease in the rate of incorporation of DXG-TP.

The mutation at Lys^{103} → Asn is associated with resistance to non-nucleoside RT inhibitors (NNRTI). The K103N mutation lies in the hydrophobic binding pocket of the NNRTIs (37). The structures of HIV-1 RT complexed with NNRTIs revealed that Lys^{103} contributes to the binding of NNRTI through electrostatic forces and van der Waals interactions (16). Comparison of unliganded WT and K103N mutant HIV-1 RT structures showed a network of hydrogen bonds in the mutant that is not...
present in the WT enzyme, suggesting that K103N stabilizes the closed pocket form of the enzyme and prevents the binding of NNRTIs to the enzyme (38). Mewshaw et al. (2) observed a hypersensitization of the response to DXG for HIV-1 harboring the K103N mutation, which is consistent with our steady state and pre-state steady kinetic analysis. As shown in Tables III and IV and illustrated in Fig. 4, K103N RT was less or equally discriminatory toward DXG-TP as was the WT RT. Addition of K103N to a background of K65R, Q151M, or K65R/Q151M led to a partial re-sensitization to DXG-TP compared with the parent mutants. Because Lys^{203} is >20 Å away from Lys^{65} and Gln^{151}, the structural mechanism for the seemingly long range effect of K103N on the other mutations remains a question. However, it has been suggested that changes in the non-nucleoside-binding pocket could affect the conformation of residues at the dNTP-binding site and result in partial reversal of drug resistance (39). A similar effect has been reported for HIV-1 RT mutations Y181C and L100I which were selected by a variety of NNRTIs and suppressed resistance to AZT (9). Current data suggest that resistance to AZT observed with the D67N/K70R/T215Y/K219Q RT is due to enhanced excision of AZT-MP from the end of the primer catalyzed by RT, through either pyrophosphate-dependent (22) or ATP-dependent (23, 24) pyrophosphorolysis. Consistent with these data, the K^{2} ratio for AZT inhibition of the AZT^{65} RT harboring the mutations D67N/K70R/T215Y/K219Q was similar to WT RT. The inhibition of 3TC^{65} RT by 3TC-TP has been well characterized (40–42). Our study showed that the M184V mutation increased the K_{obs} for 3TC-TP by ~300-fold. Both AZT^{65} and 3TC^{65} RT remained sensitive to the inhibition of DXG-TP. In our pre-steady state analysis of the AZT-resistant RT and the 3TC-resistant RT, a minimal change of 0.7–2-fold was observed for DXG-TP incorporation for DNA- and RNA-dependent DNA synthesis. Structurally, this lack of cross-resistance can be attributed to the following: 1) DXG-TP lacks a large 3'-substituent like the 3'-azido group of AZT-TP, so its interaction with the AZT resistance-specific site is likely to be minimal (19); 2) the natural configuration of DXG-TP makes it less likely to sterically interfere with the mutation M184V (43), as encountered for 3TC-TP.

By using pre-steady state kinetic methods and computer-aided structural studies, the molecular mechanism of K65R, L74V, and Q151M HIV-1 RT resistance to dideoxynucleotides has been presented by Selmi et al. (31, 44) and Deval et al. (45). In their studies, a decreased catalytic rate constant (k_{cat}) mostly accounted for the lower incorporation efficiency observed for ddATP and ddCTP. Our data suggest that the K65R, L74V, and Q151M enzymes discriminate DXG-TP through both a decrease in the rate of incorporation and a decrease in the binding affinity.

In conclusion, our steady state and pre-steady state kinetic studies on the DXG-TP inhibition of HIV-1 RT and incorporation of DXG-TP revealed a thorough and detailed molecular mechanism associated with DXG resistance. In general, the two kinetic analyses were in good agreement with each other, regardless of the different primer-template used and the different scope of kinetic parameters measured (Fig. 5). Overall, these kinetic data also reflect the drug potency and drug resistance observed in cell culture studies.

AZT and 3TC alone and in combinations account for more than 60% of all NNRTIs prescribed the United States (46). The wide use of these two drugs is also likely to result in an increasing population of AZT- and/or 3TC-resistant viruses. Our enzymatic studies showed that there is no cross-resistance between DXG-TP and the AZT^{65} and 3TC^{65} mutant HIV-1 RT; therefore, amdoxovir offers the possibility of therapy for those who have failed current nucleoside therapy.
P. A., Kleim, J. P., Rosner, M., Hughes, S. H., and Arnold, E. (2001) J. Mol. Biol. 309, 437–445

Laethem, K. V., Witvrouw, M., Pannecouque, C., Remoortel, B. V., Schmit, J.-C., Esnouf, R., Kleim, J.-P., Balzarini, J., Desmyter, J., De Clercq, E., and Vandamme, A. M. (2001) AIDS 15, 553–561

Schinazi, R. F., Lloyd, R. M., Jr., Nguyen, M. H., Cannon, D. L., McMillan, A., Ilksoy, N., Chu, C. K., Laotta, D. C., Barri, H. Z., and Mellors, J. W. (1993) Antimicrob. Agents Chemother. 37, 875–881

Krebs, R., Immendorfer, U., Thral, S. H., Wohrl, B. M., and Goody, R. S. (1997) Biochemistry 36, 10292–10300

Feng, J. Y., and Anderson, K. S. (1999) Biochemistry 38, 9440–9448

Sarafianos, S. G., Das, K., Clark, A. D., Jr., Ding, J., Boyer, P. L., Hughes, S. H., and Arnold, E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10027–10032

Selmi, B., Boretto, J., Sarfati, S. R., Guerreiro, C., and Canard, B. (2001) J. Biol. Chem. 276, 48466–48472

Deval, J., Selmi, B., Boretto, J., Egloff, M. F., Guerreiro, C., Sarfati, S., and Canard, B. (2002) J. Biol. Chem. 277, 42977–42104

National Data Corp. (2002) Health Retail Audit, National Data Corp., Atlanta
Dioxolane Guanosine 5'-Triphosphate, an Alternative Substrate Inhibitor of Wild-type and Mutant HIV-1 Reverse Transcriptase: STEADY STATE AND PRE-STEADY STATE KINETIC ANALYSES

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