

Mechanism of Inhibition of the Human Immunodeficiency Virus Type 1 Reverse Transcriptase by d4TTP: an Equivalent Incorporation Efficiency Relative to the Natural Substrate dTTP†

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Received 12 April 1999/Returned for modification 31 July 1999/Accepted 2 October 1999

Among the clinically used nucleoside analogue inhibitors that target human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), there is little detailed mechanistic information on the interactions of 2',3'-dideohydro-2',3'-dideoxythymidine-5'-triphosphate (d4TTP) with the enzyme · primer-template complex and how these interactions compare with those of the natural substrate, dTTP. Using a pre-steady-state kinetic analysis, we found that d4TTP was incorporated by HIV-1 RT just as efficiently as dTTP during both DNA- and RNA-dependent DNA synthesis. To our knowledge, these results represent the first observation of a 3'-modified nucleoside triphosphate analogue that has an incorporation efficiency comparable to that observed for the natural substrate during DNA synthesis by HIV-1 RT. This information provides a mechanistic basis for understanding the inhibition of HIV-1 RT by d4TTP as well as insight into the clinically observed lack of d4T resistance mutations in HIV-1 RT isolated from AIDS patients.

The replication of human immunodeficiency virus (HIV), which causes AIDS, requires the virally encoded enzyme reverse transcriptase (RT). RT converts the single-stranded HIV RNA genome to a double-stranded DNA copy by catalyzing both DNA-dependent and RNA-dependent DNA polymerization as well as RNase H cleavage activity to remove the RNA template once the DNA has been synthesized. Because of its unique catalytic properties, RT has been the target enzyme for many antiviral therapeutic agents used in the treatment of AIDS, including nucleoside and nonnucleoside analogues (2–4, 8, 26). The nucleoside analogues that are used clinically lack a 3' hydroxyl group and are metabolically activated by host cellular kinases to their corresponding 5'-triphosphate forms, which are subsequently incorporated into DNA by HIV type 1 (HIV-1) RT and which act as chain terminators of DNA synthesis. Among the nucleoside inhibitors currently used in the clinic, two compounds are deoxythymidine (compound 1) analogues: 3'-azido-3'-deoxythymidine (AZT; compound 2) and 2',3'-dideohydro-2',3'-dideoxythymidine (d4T; compound 3) (Fig. 1). The structures of d4T and abacavir (36) are unique among the U.S. Food and Drug Administration (FDA)-approved nucleoside analogues currently used, in that they contain a 2',3'-unsaturated bond. An X-ray crystallographic analysis of d4T has shown that the unusual unsaturation in the ribose ring provides a novel ring conformation (11). However, the structure of d4T triphosphate (d4TTP) bound to the active site of HIV-1 RT in the presence of a primer-template substrate is not available.

While AZT was the first compound approved by the FDA in 1987 for the treatment of AIDS, d4T was also shown to have antiretroviral activity (24) and was approved more recently in 1994. From a therapeutic standpoint, d4T is less toxic, particularly to bone marrow cells, than AZT (35) and has a more predictable pharmacokinetic profile in forming the biologically

active triphosphate (27). Because of its high degree of oral bioavailability and relatively low level of toxicity (27), d4T has become an attractive therapeutic alternative. Clinical studies have also shown a low frequency of appearance of drug-resistant virus in patients receiving long-term d4T therapy (5, 23, 25). Furthermore, most isolates from patients that do acquire resistance exhibit only moderate decreases in sensitivity to the drug. In a recent clinical study, however, a multidrug-resistant virus with a mutant HIV-1 RT containing T215Y and N67E/S substitutions and a two-amino-acid insertion between residues 68 and 69 was found in 3% of patients extensively pretreated with anti-HIV drugs (5). This mutant was observed to confer a high level of resistance to a number of drugs including d4T.

Despite the success of d4T in the clinic, there is a paucity of detailed mechanistic information available on its mode of inhibition of HIV-1 RT. In the present study, we used a transient kinetic approach to provide a detailed understanding of the mechanism of inhibition of HIV-1 RT by d4T, in part, with the expectation that such knowledge may lead to the development of even more effective antiviral drugs. A traditional steady-state kinetic analysis is limited by the fact that it examines only the rate-limiting step in the overall reaction pathway of an enzyme. On the other hand, a pre-steady-state kinetic analysis allows one to examine each of the individual steps in the reaction pathway for an enzyme including the identification of enzyme intermediates and conformational changes which might be associated with chemical catalysis (1, 15, 16, 18). Our laboratory (19, 21, 31) and others (14, 28, 29) have used a transient kinetic analysis to examine the mechanism of DNA polymerization by RT. These studies have shown that the reaction pathway is ordered. The first step involves the binding of the primer-template (P_n/T) substrate to the enzyme (E) to form a tight $E \cdot P_n/T$ complex with an equilibrium dissociation constant (K_d) value in the nanomolar range. This step is followed by the binding of the correct deoxynucleoside triphosphate. The enzyme checks for proper base-pairing geometry and then undergoes a rate-determining conformational change which limits chemical catalysis. It is this conformational change that governs the maximum rate of DNA polymerization, k_{pol} . The slowest step in the pathway involves the disso-

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† We dedicate this paper to William H. Prusoff, one of the codiscoverers of d4T, on the occasion of his 79th birthday.

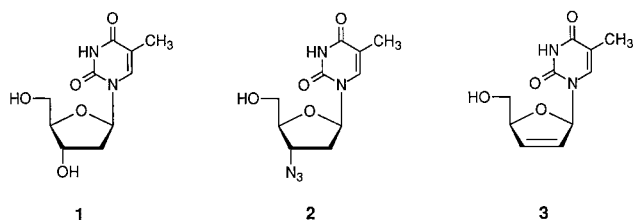


FIG. 1. Deoxythymidine (compound 1) and its nucleoside analogue HIV inhibitors AZT (compound 2) and d4T (compound 3).

ciation of the elongated primer-template substrate (P_{n+1}/T) from the enzyme and is the step which is being examined in a steady-state kinetic analysis (17).

A knowledge of the normal catalytic reaction pathway for HIV-1 RT facilitates a determination of the effect(s) that an inhibitor has on this pathway and, thus, an understanding of the mode of inhibition at a molecular level. Such an analysis has been extremely useful in providing an understanding of the mechanism of inhibition of AZT (20, 33), 2',3'-dideoxy-3'-thiacytidine (3TC) (6), and nonnucleoside inhibitors (31). In addition, such information enables an assessment of the functional consequences of drug-resistant mutant forms of RT, as demonstrated for AZT-resistant (20, 22, 33) and 3TC-resistant (7) RT. In the present study, we have extended this analysis to include an examination of the incorporation of d4TTP by HIV-1 RT compared to that of the natural substrate dTTP. The specific questions of this study include the following: (i) What are K_d and k_{pol} for d4TTP and dTTP? (ii) Are there differences in the efficiency of incorporation (k_{pol}/K_d) for d4TTP versus that for dTTP when either DNA/DNA or DNA/RNA primer-template substrates are used? (iii) Does the unusual ring conformation of d4TTP have any effect on the RNase H cleavage activity of RT?

In order to address these questions, we have compared the binding and incorporation of d4TTP and dTTP by HIV-1 RT during DNA- and RNA-dependent DNA polymerization. The random sequence, 5' end-labeled (asterisks), DNA/DNA and DNA/RNA primer-template substrates used in these studies are as follows: for the DNA/DNA 22- and 45-mers, respectively,

*GCCTCGCAGCCGTCACCAAC
CGGAGCGTCGGCAGGTTGGTTGAGTTGAGCTAGGTTACGGCAGG*

and for the DNA/RNA 22- and 45-mers, respectively,

*GCCTCGCAGCCGTCACCAAC
CGGAGCGUCGGCAGGUUGGUUGAGUUGGAGCUAGGUUACGGCAGG*

We have previously used these substrates to examine the catalytic mechanism of RT as well as the kinetics of AZT triphosphate (AZTTP) incorporation with wild-type and AZT-resistant mutant RT. By using either the DNA/DNA or DNA/RNA primer-template substrate, rapid chemical quench experiments were performed as described previously (19, 20) with a KinTek Instruments Model RQF-3 rapid-quench-flow apparatus thermostated at 37°C. HIV-1 RT and the doubly 5'-³²P-labeled 22/45-mer duplex were preincubated on ice for 5 min in buffer (50 mM Tris chloride [pH 7.8], 50 mM NaCl). Polymerization was initiated at 37°C by the rapid addition of d4TTP or dTTP in buffer containing 10 mM MgCl₂, and the reactions were quenched at the indicated times with 0.3 M EDTA. The products were analyzed on a 20% polyacrylamide gel containing 8 M urea followed by phosphorimaging analysis on a Bio-Rad GS-525 Molecular Imager System. The data were fit by non-

linear regression with the program SigmaPlot, version 4.14 (Jandel Scientific), as described previously (33).

In the present study, a series of pre-steady-state burst and single-turnover experiments were conducted to determine the kinetic parameters for incorporation of either d4TTP or dTTP opposite a DNA template 2'-deoxyadenosine or an RNA template adenosine. The kinetic parameters determined for d4TTP and dTTP include k_{pol} , K_d , and k_{pol}/K_d . This information provides a quantitative basis for comparison of the incorporation of the nucleotide analogue d4TTP to that of the natural substrate dTTP by HIV-1 RT.

Pre-steady-state incorporation of d4TTP. The first step in defining the reaction kinetics of d4TTP incorporation was to conduct a pre-steady-state burst experiment to identify the rate-limiting step in the overall reaction pathway. In this type of experiment, the amount of DNA substrate is in slight excess over the amount of enzyme such that the first enzyme turnover as well as multiple turnovers can be examined. In earlier mechanistic studies, we have observed a burst of product formation under these conditions upon incorporation of dTMP by HIV-1 RT during DNA- or RNA-dependent DNA polymerization. This result is diagnostically important insofar as it indicates that the release of the elongated primer-template product limits the overall reaction pathway. In order to determine whether or not the reaction pathway had changed for d4TTP, we examined the time course for d4T monophosphate (d4TMP) incorporation by HIV-1 RT under pre-steady-state burst conditions by using either the DNA/DNA or DNA/RNA primer-

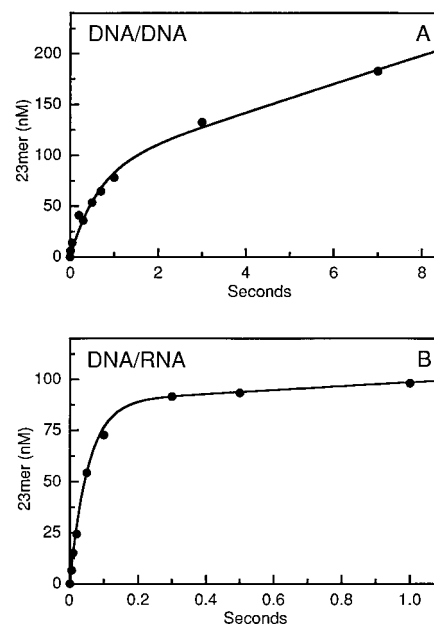


FIG. 2. Pre-steady-state kinetics of d4TTP incorporation by HIV-1 RT. (A) The incorporation of d4TTP (200 μ M d4TTP) into the 22/45-mer DNA duplex (300 nM) by HIV-1 RT (86 nM) was determined at 37°C in buffer containing 10 mM MgCl₂. The time course for d4TTP incorporation by HIV-1 RT was fit to a burst equation as described previously (34), and the curve is drawn for an amplitude (A) equal to 86 ± 13 nM, k_{obsd} equal to 1.6 ± 0.4 s⁻¹, and a steady-state rate (r) equal to 14 ± 5 nM s⁻¹. k_{ss} (which is equal to r/A) was calculated to be 0.16 ± 0.05 s⁻¹. (B) The incorporation of d4TTP (200 μ M d4TTP) into the 22/45-mer DNA/RNA heteroduplex (270 nM) by HIV-1 RT (89 nM) was determined at 37°C in buffer containing 10 mM MgCl₂. The time course for d4TTP incorporation by HIV-1 RT was fit to a burst equation, and the curve is drawn for an amplitude (A) equal to 89 ± 2 nM, k_{obsd} equal to 17 ± 2 s⁻¹, and a steady-state rate (r) equal to 9.5 ± 2.8 nM s⁻¹. k_{ss} (which is equal to r/A) was calculated to be 0.11 ± 0.03 s⁻¹.

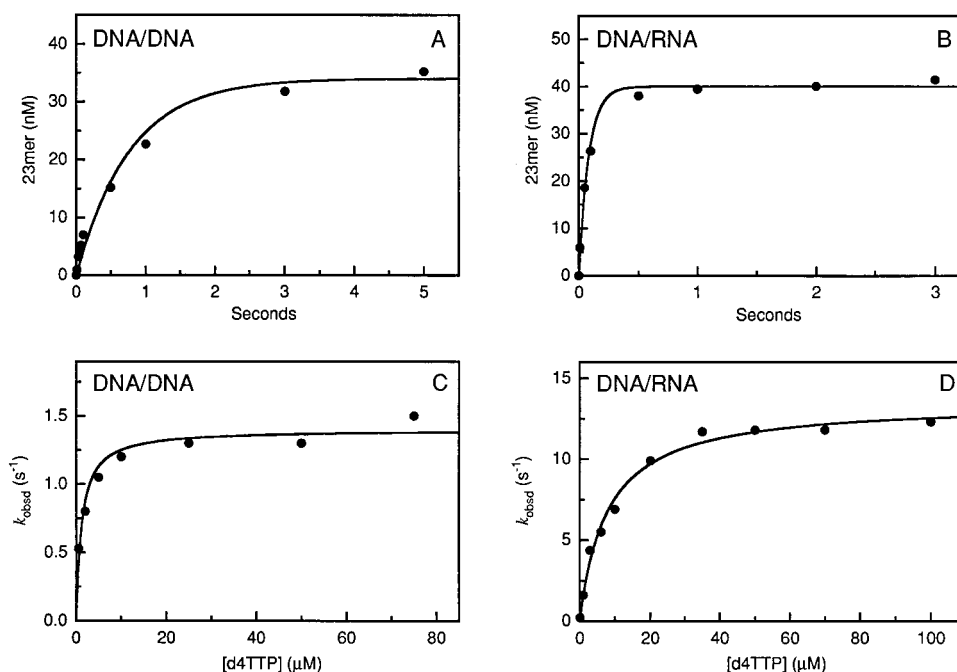


FIG. 3. Single-turnover d4TTP incorporation kinetics and d4TTP concentration dependency for HIV-1 RT with a DNA/DNA or DNA/RNA primer-template substrate. (A) The incorporation of d4TTP (25 μM d4TTP) into the 22/45-mer DNA duplex (50 nM) by HIV-1 RT (250 nM) was determined at 37°C in buffer containing 10 mM MgCl_2 . The time course for d4TTP incorporation by HIV-1 RT was fit to a single exponential equation as described (34), and the curve is drawn for an amplitude (A) equal to 34 ± 2 nM and k_{obsd} equal to 1.3 ± 0.2 s^{-1} . (B) The incorporation of d4TTP (50 μM d4TTP) into the 22/45-mer DNA/RNA heteroduplex (50 nM) by HIV-1 RT (390 nM) was determined at 37°C in buffer containing 10 mM MgCl_2 . The time course for d4TTP incorporation by HIV-1 RT was fit to a single exponential equation, and the curve is drawn for an amplitude (A) equal to 40 ± 1 nM and k_{obsd} equal to 12 ± 1 s^{-1} . (C) The dependence of k_{obsd} for incorporation of d4TTP into the 22/45-mer DNA duplex (50 nM) by HIV-1 RT (250 nM) on the concentration of d4TTP (0.5 to 75 μM) was determined at 37°C in buffer containing 10 mM MgCl_2 . The d4TTP concentration dependence of k_{obsd} for HIV-1 RT was fit to a hyperbolic equation as described previously (34), and the curve shown represents a fit for K_d equal to 1.2 ± 0.3 μM and k_{pol} equal to 1.4 ± 0.1 s^{-1} . Errors in the values of k_{obsd} were typically $\leq 17\%$. (D) The dependence of k_{obsd} for incorporation of d4TTP into the 22/45-mer DNA/RNA heteroduplex (50 nM) by HIV-1 RT (390 nM active sites) on the concentration of d4TTP (0.1 to 100 μM) was determined at 37°C in buffer containing 10 mM MgCl_2 . The d4TTP concentration dependence of k_{obsd} for HIV-1 RT was fit to a hyperbolic equation, and the curve shown represents a fit for K_d equal to 7.8 ± 0.8 μM and k_{pol} equal to 14 ± 1 s^{-1} . Errors in the values of k_{obsd} were typically $\leq 12\%$.

template substrate. Figure 2A shows that the incorporation of d4TTP (200 μM d4TTP) into the DNA/DNA 22/45-mer substrate occurs with an observed first-order rate constant (k_{obsd}) equal to 1.6 s^{-1} , followed by a slower linear steady-state rate constant (k_{ss}) equal to 0.16 s^{-1} . In Fig. 2B the incorporation of d4TTP (200 μM d4TTP) into the DNA/RNA 22/45-mer substrate is shown to occur with k_{obsd} equal to 17 s^{-1} and k_{ss} equal to 0.11 s^{-1} . In both cases the burst of product formation indicates that the reaction pathway for RT has not changed during d4TTP incorporation—the release of the extended primer-template product is still the rate-limiting step in the overall reaction pathway during DNA- and RNA-dependent DNA polymerization. Consistent with our previous studies (6, 19, 20, 34), the rate of d4TTP incorporation in the burst phase was faster for a DNA/RNA primer-template substrate than for a DNA/DNA substrate. However, the rates of extended primer-template product release (k_{ss}) were found to be similar (see Table 1) and are consistent with those from previous studies (6, 19, 20, 34).

Single-turnover incorporation of dTTP or d4TTP into a DNA/DNA 22/45-mer primer-template. A single-turnover experiment is performed under conditions in which the concentration of the enzyme is in excess of that of the substrate. These conditions ensure that a single enzymatic turnover at the active site can be measured directly without the concern that the substrate binding or product release step is rate limiting. In the current study, a series of single-turnover experiments were performed to examine the dependence of the observed rate of dTTP incorporation on the concentration of dTTP in order to

determine k_{pol} and K_d for dTTP during DNA-dependent DNA synthesis by HIV-1 RT. The values of k_{pol} (1.0 s^{-1}) and K_d (1.5 μM) for dTTP gave rise to a k_{pol}/K_d of 0.67 $\mu\text{M}^{-1} \text{s}^{-1}$ (see Table 1). In a similar manner, single-turnover experiments were used to determine the values of k_{pol} , K_d , and k_{pol}/K_d for d4TTP during DNA-dependent DNA synthesis by HIV-1 RT. A representative time course for d4TTP incorporation (25 μM d4TTP) into the DNA/DNA substrate under single-turnover conditions is shown in Fig. 3A. From this time course the value of k_{obsd} is determined at the given concentration of d4TTP. The hyperbolic dependence of k_{obsd} on the concentration of d4TTP illustrated in Fig. 3C is consistent with the formation of an $\text{RT} \cdot \text{DNA/DNA} \cdot \text{d4TTP}$ complex with K_d equal to 1.2 μM followed by the incorporation of d4TTP with k_{pol} equal to 1.4 s^{-1} , giving an incorporation efficiency (k_{pol}/K_d) of 1.2 $\mu\text{M}^{-1} \text{s}^{-1}$. Surprisingly, d4TTP is incorporated just as efficiently as dTTP (within experimental error; see Table 1).

Single-turnover incorporation of dTTP or d4TTP into a DNA/RNA 22/45-mer primer-template. Similar to the experiments described above, single-turnover experiments were used to determine the values of k_{pol} and K_d for dTTP during RNA-dependent DNA synthesis by HIV-1 RT. These values are shown in Table 1. A representative time course for d4TTP incorporation (50 μM d4TTP) into the DNA/RNA substrate under single-turnover conditions is shown in Fig. 3B. The hyperbolic dependence of k_{obsd} on the concentration of d4TTP illustrated in Fig. 3D is consistent with the formation of an $\text{RT} \cdot \text{DNA/RNA} \cdot \text{d4TTP}$ complex with a K_d of 7.8 μM , followed by the incorporation of d4TTP with k_{pol} equal to 14 s^{-1} ,

TABLE 1. Kinetic and equilibrium constants for binding and incorporation of dTTP or d4TTP by HIV-1 RT

Primer-template	Nucleotide	k_{pol} (s^{-1})	K_d (μM)	k_{ss} (s^{-1})	k_{pol}/K_d ($\mu\text{M}^{-1} \text{s}^{-1}$) ^a
DNA/DNA	dTTP	1.0 ± 0.1	1.5 ± 0.5	0.13 ± 0.03	0.67 ± 0.23
DNA/DNA	d4TTP	1.4 ± 0.1	1.2 ± 0.3	0.16 ± 0.05	1.2 ± 0.3
DNA/RNA	dTTP	60 ± 3	28 ± 5	0.07 ± 0.03	2.1 ± 0.4
DNA/RNA	d4TTP	14 ± 1	7.8 ± 0.8	0.11 ± 0.03	1.8 ± 0.2

^a Errors in the values of k_{pol}/K_d were calculated by standard methods (30).

giving an incorporation efficiency k_{pol}/K_d of $1.8 \mu\text{M}^{-1} \text{s}^{-1}$. While the value of k_{pol} was 4.3-fold slower for d4TTP incorporation than for dTTP incorporation, the affinity of d4TTP for the RT · DNA/RNA complex was 3.6-fold higher than that of dTTP. The overall result is that the incorporation efficiencies of d4TTP and dTTP are similar during RNA-dependent DNA polymerization by HIV-1 RT (Table 1). In addition, as we have previously observed in a comparison of RNA-dependent and DNA-dependent DNA synthesis by HIV-1 RT, k_{pol} is faster and the efficiency of incorporation is higher for a DNA/RNA primer-template substrate than for a DNA/DNA substrate.

Effect of d4TTP on RNase H cleavage. We have previously examined the rate of RNase H cleavage and the pattern of cleavage products during RNA-dependent DNA polymerization for several 2'-deoxynucleoside-5'-triphosphate analogues (6, 20). By radiolabeling the 5' end of the template strand, in addition to the primer, one can examine the pattern of template cleavage products as well as the rate at which these products are formed. The length of the RNA cleavage products provides an indication of how the template strand is positioned between the polymerase and RNase H active sites. In studies characterizing the RNase H cleavage activity of HIV-1 RT during the incorporation of dCTP or natural (+) or unnatural (-) isomers of 2',3'-dideoxy-3'-thiacytidine triphosphate (3TCTP), we found differential cleavage products with the unnatural 3TCTP isomer, indicating an altered position relative to that for the natural isomers (6). In the present study, we have extended this kind of analysis of RNase H cleavage to include an examination of the effect of the unsaturated ribose ring of d4TTP bound to the RT · DNA/RNA complex. In comparing the rate of RNase H cleavage of the DNA/RNA 22/44-mer substrate, we found that the rates of 45-mer template cleavage are similar during the incorporation of dTTP ($3.7 \pm 0.2 \text{ s}^{-1}$) or d4TTP ($3.9 \pm 0.5 \text{ s}^{-1}$) at saturating concentrations of these 2'-deoxynucleoside-5'-triphosphates. Moreover, the major cleavage products were the same (data not shown): a 41-mer and a 42-mer resulting from RNase H cleavage 18 and 19 nucleotides, respectively, from the polymerase active site (10, 19). These results are consistent with those previously observed in a study of the effect of AZTTP on the pattern and rate of RNase H cleavage (20).

Conclusions. While the incorporation of d4TTP by HIV-1 RT has been examined in a variety of steady-state kinetic assays (13, 32), detailed mechanistic information is not available since the value of k_{cat} reflects the slowest step in the overall kinetic pathway and K_m is a complex kinetic parameter whose value may or may not be equivalent to the K_d value. In the current study, we used a transient kinetic analysis to examine directly the events occurring at the enzyme active site and to determine the kinetic parameters k_{pol} and K_d for d4TTP. These parameters provide information on the rate of the conformational change involved in d4TTP incorporation as well as a direct measure of the affinity of d4TTP for the RT · primer-

template complex, resulting in a ternary complex poised for catalysis. In addition, the efficiency of incorporation (k_{pol}/K_d) takes into account compensatory changes in binding and catalysis and, thus, allows comparisons to be made between 3'-modified nucleoside triphosphate analogues and the corresponding natural substrates. It has been suggested that the potencies of these analogues as DNA chain terminators are due, in part, to their ability to compete effectively with the corresponding natural substrate (9). In previous mechanistic studies conducted in our laboratory we compared the incorporation efficiency of AZTTP or 3TCTP relative to those of the corresponding natural substrates and have shown that these nucleoside analogues are incorporated much less efficiently during either DNA- or RNA-directed DNA synthesis (6, 20, 33). Therefore, we were surprised to find that d4TTP was incorporated as efficiently as the natural substrate dTTP during both DNA- and RNA-dependent DNA polymerization. To our knowledge, this is the first time that a 3'-modified nucleoside triphosphate analogue has been shown to be incorporated just as efficiently as the corresponding natural substrate by HIV-1 RT.

The efficient incorporation for d4TTP may provide an explanation for the observed lack of d4T resistance mutations in HIV-1 RT isolated from AIDS patients receiving long-term therapy. Even though d4TTP and dTTP have different structures, they are kinetically equivalent with respect to their incorporation efficiencies by HIV-1 RT. Because of this similarity, it may be more difficult for resistance mutations in HIV-1 RT that would selectively reduce the incorporation efficiency of d4TTP relative to that of dTTP to develop in vivo. Interestingly, we have observed that AZTTP and 3TCTP, to which HIV-1 RT resistance develops more rapidly, have lower incorporation efficiencies than the natural deoxynucleotide triphosphates (6, 20, 33). However, in contrast to AZT and 3TC resistance, in which mutations in HIV-1 RT have been shown to arise after prolonged drug treatment, there is little documentation showing that a patient's failure to respond to d4T therapy is due to mutations that arise in HIV-1 RT (23). While we must exercise caution in drawing conclusions about clinical drug resistance based on data from in vitro experiments and while there is no doubt that other factors such as changes in metabolic activation and transport may come into play (12), further mechanistic studies are warranted.

This work was supported by NIH grant GM49551 to K.S.A. and ACS postdoctoral fellowship PF-4478 to J.A.V.

We thank Greg Yamanaka and Rich Colonno of Bristol-Myers Squibb Inc. for the generous gift of d4TTP, without which this study would not have been possible. We also thank Stephen Hughes, Paul Boyer, and Andrea Ferris for the generous gift of the RT clone.

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