HIV Reverse Transcriptase Pre-Steady-State Kinetic Analysis of Chain Terminators and Translocation Inhibitors Reveals Interactions between Magnesium and Nucleotide 3′-OH

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ABSTRACT: Deoxynucleoside triphosphate analogues with various 3′ substituents in the sugar ring (−OH (dTTP)), −H, −NH₂, −F, −O−CH₃, no group (2′,3′-didehydro-2′,3′-dideoxynucleoside triphosphate (dTTP)), and those retaining the 3′-OH but with 4′ additions (4′-C-methyl, 4′-C-ethy1) or sugar ring modifications (δ-carba dTTP) were evaluated using pre-steady-state kinetics in low (0.5 mM) and high (6 mM) Mg²⁺ with HIV reverse transcriptase (RT). Analogues showed diminished observed incorporation rate constants (k_{obs}) compared to dTTP ranging from about 2-fold (3′-H, −N₂, and d4TTP with high Mg²⁺) to >10-fold (3′-NH₂ and 3′-F with low Mg²⁺), while 3′-O-CH₃ dTTP incorporated much slower than other analogues. Illustrating the importance of interactions between Mg²⁺ and the 3′-OH, k_{obs} using 5 μM dTTP and 0.5 mM Mg²⁺ was only modestly slower (1.6-fold) than with 6 mM Mg²⁺, while analogues with 3′ alterations incorporated 2.8−5.1-fold slower in 0.5 mM Mg²⁺. In contrast, 4′-C-methyl and δ-carba dTTP, which retain the 3′-OH, were not significantly affected by Mg²⁺. Consistent with these results, analogues with 3′ modifications were better inhibitors in 6 versus 0.5 mM Mg²⁺. Equilibrium dissociation constant (K_{D}) and maximum incorporation rate (k_{pol}) determinations for dTTP and analogues lacking a 3′-OH indicated that low Mg²⁺ caused a several-fold greater reduction in k_{pol} with the analogues but did not significantly affect K_{D}, results consistent with a role for 3′-OH/ Mg²⁺ interactions in catalysis rather than nucleotide binding. Overall, results emphasize the importance of previously unreported Mg²⁺ interactions between Mg²⁺ and the 3′-OH of the incoming nucleotide and suggest that inhibitors with 3′-OH groups may have advantages in low free Mg²⁺ in physiological settings.

INTRODUCTION

Nucleoside reverse transcriptase inhibitors (NRTIs) are a hallmark of antiretroviral therapy (ART). All NRTIs currently approved for ART are “chain terminators” and either lack a hydroxyl group at the 3′ ribose position or have a substituted sugar or other group replacing ribose. In all cases, there is no group present for addition of the next base by reverse transcriptase (RT) (reviewed in refs 1−3). A second class of emerging NRTIs referred to as “translocation inhibitors” retain the 3′-OH but contain modifications at other positions that dramatically slow the addition of the next base.4−7 The first approved HIV drug, azidothymidine (also referred to as zidovudine, AZT, or ZDV), is essentially dTTP with the 3′-OH replaced by an azido group. Dideoxynucleosides such as d4T and dideoxycytidine (formerly approved HIV drug also referred to as zalcitabine or ddC). Other drugs remove both the 3′-OH and a hydrogen atom from the 3′ position by addition of a carbon double bond to the ribose ring (2′,3′-didehydro-2′,3′-dideoxythymidine) (also referred to as stavudine or d4T) and the guanosine analogue abacavir (ABC). Finally, some approved drugs substantially modify or remove the ribose sugar including lamivudine (3TC), emtricitabine (FTC), and tenofovir disoproxil fumarate (TDF). Despite the success of drugs lacking a 3′-OH, for some translocation inhibitors, this group is pivotal in promoting the phosphorylation of the prodrug in cells.8

Others have examined the effect of different groups at the 3′ positions of thymidine and adenosine analogues for inhibition of HIV RT.9,10 For thymidine, inhibition assays using steady-state kinetics and measuring incorporation on poly(rA)-oligo(dT₁₀) indicated that substitutions of fluorine (F), amino (NH₂), azido (N₃), and hydrogen (H) at the 3′ position were all effective inhibitors of dTTP incorporation with modest overall differences, with 3′-F and 3′-N₃ being the most and least effective, respectively.9 Despite this, only the 3′-N₃ derivative (marketed as AZT) has been an effective HIV drug. Drug effectiveness is complicated by several factors including stability, uptake, cellular phosphorylation, target and
Both analogues had lower potencies. Still, the selectivity index for 3′-H (ddATP) was only about 4.5-fold lower than that for AZT suggesting that it could be an effective drug.

Previous results indicated that the level of Mg$^{2+}$ in in vitro reactions can affect the fidelity of HIV RT, efficiency of reverse transcription, and the potency of both NRTIs and nonnucleoside reverse transcriptase inhibitors (NNRTIs). Fidelity increased significantly in reactions performed in low Mg$^{2+}$ (0.25–0.5 mM) that more closely mimics cellular free Mg$^{2+}$ ($\sim$6 mM) typically used in in vitro reactions with RT. The efficiency of reverse transcription, as judged by the length of products produced in primer extension reactions, was greater

Table 1. Comparison of Pre-Steady-State Incorporation of dTTP Analogues

| nucleotide ([Mg$^{2+}$])$^a$ | $k_{obs}$ (s$^{-1}$) ± standard deviation$^b$ | relative rate vs dTTP same [Mg$^{2+}$] | 6/0.5 mM Mg$^{2+}$ with the same nucleotide |
|-----------------------------|-------------------------------------------|----------------------------------------|---------------------------------------------|
| dTTP (0.5 mM)               | 8.6 ± 1.3                                  | 1                                      | 1.6                                         |
| dTTP (6 mM)                 | 14.1 ± 1.2                                 | 1                                      |                                             |
| ddTTP (0.5 mM)              | 1.4 ± 0.7                                  | 0.16                                   | 5.1                                         |
| ddTTP (6 mM)                | 7.2 ± 1.0                                  | 0.51                                   |                                             |
| AZT (0.5 mM)                | 2.0 ± 0.5                                  | 0.23                                   | 4.5                                         |
| AZT (6 mM)                  | 8.9 ± 1.9                                  | 0.63                                   |                                             |
| D4T (0.5 mM)                | 1.8 ± 0.7                                  | 0.21                                   | 4.6                                         |
| D4T (6 mM)                  | 8.3 ± 0.8                                  | 0.59                                   |                                             |
| 3′-amino dTTP (0.5 mM)      | 0.81 ± 0.13                                | 0.094                                  | 3.8                                         |
| 3′-amino dTTP (6 mM)        | 3.1 ± 0.1                                 | 0.22                                   |                                             |
| 3′-fluoro dTTP (0.5 mM)     | 0.54 ± 0.13                                | 0.063                                  | 2.8                                         |
| 3′-fluoro dTTP (6 mM)       | 1.5 ± 0.1                                 | 0.11                                   |                                             |
| 3′-O-methyl dTTP (0.5 mM)$^c$ | $3.3 \times 10^{-7} \pm 0.6 \times 10^{-7}$ | NA                                     | 5.4                                         |
| 3′-O-methyl dTTP (6 mM)$^c$ | $1.8 \times 10^{-7} \pm 0.2 \times 10^{-7}$ | NA                                     |                                             |
| 4′-C-methyl dTTP (0.5 mM)   | 4.5 ± 1.4                                  | 0.52                                   | 0.88                                        |
| 4′-C-methyl dTTP (6 mM)     | 3.9 ± 2.8                                  | 0.28                                   |                                             |
| 4′-C-ethyl dTTP (0.5 mM)    | 1.3 ± 0.1                                 | 0.15                                   | 3.1                                         |
| 4′-C-ethyl dTTP (6 mM)      | 3.9 ± 0.4                                 | 0.28                                   |                                             |
| N-carba dTTP (0.5 mM)       | 1.1 ± 0.5                                 | 0.13                                   | 1.2                                         |
| N-carba dTTP (6 mM)         | 1.3 ± 0.1                                 | 0.092                                  |                                             |

“All nucleotides were tested at 5 μM final concentration. The free Mg$^{2+}$ concentration in reactions is listed (see Materials and Methods). The nucleic acid primer—template used was: 20-nt primer, 5′-TTCCCCGTACGCTAGTAGAG-3′, and 36-nucleotide template, 5′-ATCGTCGTACTCAGCTAGGCTGACGGGAAATTGTAT-3′. $k_{obs}$ is the observed rate constant for nucleotide addition. Results are averages from three or more independent experiments ± standard deviations. See Figure 1 for an example of an experiment. $^a$Values for 3′-O-methyl dTTP are from steady-state experiments as the rate was too low to reliably measure by pre-steady-state methods. Units are nM/min. They cannot be directly compared to other values for $k_{obs}$. Conditions were 20 nM primer—template, 20 nM RT, and 100 μM 3′-O-methyl dTTP in the same buffer as pre-steady-state reactions, and reactions were measured over 30 min.

Figure 1. Example of pre-steady-state determination of the observed rate constant ($k_{obs}$) of nucleotide addition with 5 μM dTTP or AZTTP and 0.5 or 6 mM Mg$^{2+}$. (A) Assays were conducted as described in Materials and Methods with a 20-nt 5′-32P end-labeled DNA primer bound to a 36-nt DNA template using rapid quench analysis. Time points in these assays were 10, 20, 40, 80, 100, 200, and 500 ms and were conducted in duplicate. For nucleotide analogues with slower incorporation rates, longer time points were also used. Positions of the primer (P) and primer + 1 nucleotide (P+1) are shown for assays with dTTP and AZTTP at 0.5 or 6 mM Mg$^{2+}$. FE, full extension; –E, no enzyme added. (B) The data was fitted to an exponential equation as described in Materials and Methods to yield $k_{obs}$ (s$^{-1}$) used to produce the data in Table 1.
with physiological Mg\(^{2+}\) concentrations.\(^{18}\) The effect on NRTIs and NNRTIs was opposite, with the former being less effective in low Mg\(^{2+}\) and the latter being more effective.\(^{18,19}\)

In this report, we used pre-steady-state kinetic analysis at a fixed nucleotide concentration (5 \(\mu\)M) that approximated cellular dNTP concentrations\(^{20-22}\) and high (6 mM) or low (0.5 mM) Mg\(^{2+}\) concentrations to examine the incorporation rates of dTTP and several dTTP analogues with substitutions at the 3’ position. This included 3’-OH (dTTTP), –NH\(_2\) (AZT), –F, –O–CH\(_3\), –H (dddTTP), and d4T (no group at this position). Three translocation-type dTTP analogue inhibitors (4’-C-ethyl, 4’-C-ethyl, and d-carba dTTTP) that retained the 3’-OH were also tested. Determinations of \(k_{\text{pol}}\) and \(K_p\) were also performed for some analogues under pre-steady-state conditions. Overall, the results illustrate a potential benefit for NRTI drugs with 3’-OH groups, especially under physiological Mg\(^{2+}\) concentrations, while also uncovering a previously unreported direct or indirect interaction between the 3’-OH of incoming nucleotides and Mg\(^{2+}\).

## RESULTS

Substitution of the 3’-OH Differentially Affects the Incorporation Rates of dTTP Analogues. All the analogues tested, including chain terminators and translocation inhibitors, were incorporated more slowly than dTTP in both 0.5 and 6 mM Mg\(^{2+}\), in pre-steady-state assays with 5 \(\mu\)M nucleotide (Table 1, see Figure 1 for an example of an analysis). This amount was chosen as it approximately mimics the level of nucleotides in dividing T cells.\(^{20-22}\) The incorporation rate ordered from highest to lowest in 6 mM Mg\(^{2+}\) was as follows: 3’-OH > (–NH\(_2\) no group (d4TTTP), –H) > (4’-C-methyl, 4’-C-ethyl, –NH\(_2\)) > (–F, d-carba) \(\gg\) –O–CH\(_3\). In 0.5 mM Mg\(^{2+}\), the order was as follows: 3’-OH > (4’-C-methyl) > (–NH\(_2\) no group, –H, 4’-C-ethyl, d-carba, –NH\(_2\)) > –F \(\gg\) –O–CH\(_3\). The 3’-O–CH\(_3\) analogue was by far the slowest, and no data could be obtained using pre-steady-state conditions. However, steady-state analysis showed that the analogue could be incorporated by RT (Figure 2). Among the chain terminating analogues, at 6 mM Mg\(^{2+}\), a –NH\(_2\), –H, or no group at the 3’ position was most tolerated. The 3’-F was modestly slower than 3’-NH\(_2\), and the incorporation rate of 3’-O–CH\(_3\) was negligible in comparison (see above). Note that these results agree with a previous steady-state analysis of some of these analogues indicating that they were efficiently incorporated by HIV RT (see ref 9 and Introduction). However, the relative potency of the various inhibitors did not agree with our pre-steady-state analysis.

Incorporation rates for translocation inhibitors were less dependent on the Mg\(^{2+}\) conditions. Rates for the 4’-C-methyl and 4’-C-ethyl derivatives were similar in 6 mM Mg\(^{2+}\), and both were about 3-fold faster than d-carba dTTTP. In 0.5 mM Mg\(^{2+}\), only the incorporation rate for 4’-C-ethyl dTTTP declined significantly.

Effect of Lower Mg\(^{2+}\) on Incorporation Is Largely Independent of the Type of 3’ Substitution. All analogues that did not have a 3’-OH were incorporated more slowly in 0.5 than 6 mM Mg\(^{2+}\). The magnitudes of the decreases were in a range between 2.8-fold and 5.1-fold (Table 1), with 3’-F and 3’-H (dddTTP) being the lowest and highest, respectively. However, the difference between the various 3’ analogues was small and, in most cases, not significant. This indicates that the low Mg\(^{2+}\) effect results more from the loss of the 3’-OH than a property of the substitution. The 4’-C-methyl and d-carba translocation inhibitor analogues were not strongly affected by lower Mg\(^{2+}\), while dTTTP showed just a 1.6-fold decrease in the incorporation rate with low Mg\(^{2+}\) (Table 1). Curiously, 4’-C-ethyl dTTTP behaved more like the chain terminators and was incorporated about 3 times more slowly in 0.5 mM Mg\(^{2+}\). It is notable, however, that despite possessing a 3’-OH, 4’-C-ethyl dTTTP actually behaves like a chain terminator as unlike 4’-C-methyl and d-carb dTTTP, a nucleotide cannot be added to this moiety (see ref 23 and see Discussion).

Analogues without a 3’-OH Demonstrate a More Profound Loss of Catalytic Efficiency in Lower Mg\(^{2+}\). The observed incorporation rate of a nucleotide or analogue at a particular concentration is dependent on its affinity for the enzyme and the overall rate of the catalytic steps. Several reports have shown that 3’-OH substitutions may alter both the binding affinity (i.e., equilibrium dissociation constant \(K_p\)) for HIV RT and the maximum incorporation rate (\(k_{\text{pol}}\))\(^{24-28}\) We choose three dNTPs/analogues, dTTP, AZTTP, and d4TTTP, to evaluate more thoroughly and determine both pre-steady-state \(K_p\) and \(k_{\text{pol}}\) values at 0.5 and 6 mM Mg\(^{2+}\). Although it would have been more complete to test all 10 analogues, a combination of limiting amounts of material and time made it more feasible to test a smaller representative set. The decision to focus on AZTTP and d4TTTP was made because there is extensive literature on these compounds, and they are approved HIV drugs. The \(K_p\) values for all three compounds were similar with d4TTTP showing a modestly higher \(K_p\) than the others at both Mg\(^{2+}\) concentrations (Figure 3 and Table 2). The level of Mg\(^{2+}\) did not strongly affect the \(K_p\) value for any of the nucleotides. Values for \(K_p\) at 6 mM obtained here were comparable to previous results with high Mg\(^{2+}\).\(^{24,28}\) As expected from the results in Table 1, changes in \(k_{\text{pol}}\) were more pronounced, especially for the nucleotides lacking a 3’-OH (AZTTP and d4TTTP). The decline in catalytic efficiency (\(k_{\text{pol}}/K_p\)), with 0.5 versus 6 mM Mg\(^{2+}\), was also more pronounced for these analogues. For example, AZTTP was incorporated only 1.5-fold less efficiently than dTTTP in 6 mM Mg\(^{2+}\) but 7.2-fold less efficiently in 0.5

Figure 2. Addition of 3’-O–CH\(_3\) dTTP in steady-state assays with HIV RT with 0.5 or 6 mM Mg\(^{2+}\). Reactions were carried out as described in Materials and Methods using a 20-nt 5’-32P end-labeled DNA primer bound to a 36-nt DNA template. All reactions contained 100 \(\mu\)M 3’-O–CH\(_3\) dTTP with either 0.5 or 6 mM Mg\(^{2+}\). Reactions were carried out for the indicated times and resolved on a 20% denaturing gel. The proportion of the extended primer was quantified on a phosphorimager and used to determine the observed rate constant (\(k_{\text{pol}}\)) of nucleotide addition. –E, no enzyme added; P, 5’-32P end-labeled DNA primer; P + 1, primer extended with 3’-O–CH\(_3\) dTTP.
mM Mg\(^{2+}\) (Table 2). The data again illustrates that the lack of a \(^3\)′-OH has a more pronounced negative effect on incorporation in low, more physiological Mg\(^{2+}\), and a decrease in the observed incorporation rate (\(k_{\text{obs}}\)) as opposed to changes in affinity (\(K_D\)) for the analogues is the predominant factor.

**Inhibition Assays on a Heteropolymeric DNA Template Are Generally Consistent with the Kinetic Analysis.** To evaluate the effectiveness of the various analogues at terminating DNA synthesis, a primer extension assay was performed on a 100-nt DNA template primed with a 20-nt 5′-32P-labeled DNA primer (Figure 4). Assays contained 5 μM dCTP, dGTP, dATP, and dTTP. Analogues were included at 3 μM when present. Consistent with the 6/0.5 mM Mg\(^{2+}\) ratios shown in Table 1, all chain terminators and 4′′-C-ethyl dTTP were less effective (as judged by a lower level of paused (terminated) products and an increase in fully extended products) in 0.5 mM Mg\(^{2+}\) than in 6 mM Mg\(^{2+}\). Among translocation inhibitors, 4′′-C-methyl dTTP was essentially equivalent at both 0.5 and 6 mM Mg\(^{2+}\) while \(\alpha\)-carba was a weak inhibitor under both conditions. The 3′-F analogue was the weakest inhibitor among the chain terminators and was also incorporated the slowest in pre-steady-state reactions (Table 1, note that 3′-O-CH\(_3\)) produced no inhibition in this.

**Figure 3.** Pre-steady-state determination of the equilibrium dissociation constants (\(K_D\)) and the maximum incorporation rates (\(k_{\text{pol}}\)) for dTTP, AZTTP, and d4TTP. Assays were conducted as described in Materials and Methods with a 20-nt 5′-32P end-labeled DNA primer bound to a 36-nt DNA template using rapid quench analysis. Data points are from independent experiments at various concentrations of dTTP (A), AZTTP (B), or d4TTP (C) (see Figure 1 for an example). Points from several experiments (typically 2–3, while only 1 point was used for the highest d4TTP concentration) were plotted and fit to a hyperbolic equation for ligand binding with single site saturation to determine values as described in Materials and Methods. The indicated values for \(K_D\) and \(k_{\text{pol}}\) are derived from the curve fit program. Values are ± standard errors as determined by the program.

**Table 2.** Pre-Steady State Kinetic Data for HIV RT Incorporation of TTP, AZTTP, and D4TTP

| nucleotide | [Mg\(^{2+}\)] (mM) | \(k_{\text{pol}}\) (s\(^{-1}\))^a | \(K_D\) (μM)^a | \(k_{\text{pol}}/K_D\) | efficiency decline^b |
|-----------|---------------------|-----------------|---------------|----------------|---------------------|
| dTTP      | 0.5                 | 14.3 ± 0.8      | 3.3 ± 0.6     | 4.3            | 1                   |
| AZTTP     | 0.5                 | 3.3 ± 0.3       | 5.5 ± 1.7     | 0.6            | 7.2                 |
| d4TTP     | 0.5                 | 4.9 ± 0.5       | 7.6 ± 2.2     | 0.64           | 6.7                 |
| dTTP      | 6                   | 25.0 ± 1.3      | 4.2 ± 0.7     | 6.0            | 1                   |
| AZTTP     | 6                   | 17.0 ± 0.6      | 3.9 ± 0.6     | 3.9            | 1.5                 |
| d4TTP     | 6                   | 22.3 ± 1.7      | 11.6 ± 2.3    | 1.9            | 3.2                 |

Values were derived from graphs shown in Figure 2 and are predicted values based on curve fitting ± standard error (as determined by the fitting program). See Table 1 and Materials and Methods for a description of the primer template used. Numbers represent the fold decline in enzyme efficiency relative to dTTP at the same \(Mg^{2+}\) concentration. Enzyme catalytic efficiency was taken as \(k_{\text{pol}}/K_D\).
OH with Mg\(^{2+}\). The translocation inhibitor 4'-C-ethyl dTTP (4'-OH) with Mg\(^{2+}\). This is consistent with previous experiments showing


theresults in Table 1 and those in the inhibition assay would not necessarily be expected. Finally, results in Table 1 were performed using a single sequence position on a primer-template while the experiments in Figure 4 use a long template with positions for thymidine incorporation presented in several sequence contexts.

## DISCUSSION

In this report, several chain-terminating 3'-OH substitutions of dTTP were tested for incorporation by HIV RT. All the analogues with 3' alterations were incorporated, and several (e.g., AZT, ddTTP, and d4TTP) were incorporated with only modestly slower rates (~2-fold) than dTTP in 6 mM Mg\(^{2+}\) (Table 1). In contrast, the rate of incorporation compared to dTTP dropped to ~4.5–16-fold slower (excluding 3'-O-CH\(_3\), which was much slower at both high and low Mg\(^{2+}\)) in 0.5 mM Mg\(^{2+}\). This is consistent with previous experiments showing that NRTIs are better inhibitors with higher Mg\(^{2+}\) concentrations in vitro,\(^{19}\) an observation that was also shown for analogues tested here (Figure 4). Overall, the results support a previously unreported interaction, either direct or indirect, between Mg\(^{2+}\) and the 3'-OH of incoming nucleotides. Further, the results suggest that this interaction plays a role in catalysis rather than nucleotide binding affinity (see below).

The importance of the 3'-OH was supported by experiments with translocation-type inhibitors (4'-C-methyl and d-carba dTTP), which retain the 3'-OH and behaved more like dTTP in that their rates of incorporation were less affected by low Mg\(^{2+}\) (Table 1). However, 4'-C-ethyl dTTP was an exception, showing a 3.1-fold lower rate of incorporation in low Mg\(^{2+}\) than in high Mg\(^{2+}\). As was noted, this analogue terminates further extension by RT, in contrast to 4'-C-methyl and d-carba dTTP, which delay extension.\(^{23}\) This suggests that the 3'-OH may be in the wrong position for catalysis, and this position may also weaken the proposed interactions of the 3'-OH with Mg\(^{2+}\). The translocation inhibitor 4'-ethynyl-2-fluoro-2'-deoxyadenosine triphosphate (EFdATP) is currently in clinical trials. It also binds strongly to HIV RT as the ethynyl group can access a hydrophobic pocket in RT, which may stabilize binding.\(^{7,29}\) This and favorable kinetics lead to EFdATP showing a competitive advantage over the natural dATP nucleotide.\(^{30}\) EFdATP inhibition is also insensitive to Mg\(^{2+}\) concentration,\(^{19}\) and incorporated EFdA can accept additional nucleotides at a higher reduced rate.\(^{30}\) This suggests that translocation inhibitors with the 3'-OH positioned for nucleotide addition behave more like natural nucleotides with respect to Mg\(^{2+}\) interactions.

It was interesting that the 4'-C-ethyl dTTP was clearly a more effective inhibitor than 4'-C-methyl or d-carba dTTP at 6 mM Mg\(^{2+}\) during extension on the long template (Figure 4). Even at 0.5 mM Mg\(^{2+}\), where 4'-C-ethyl dTTP was incorporated much more slowly than 4'-C-methyl dTTP and at a rate similar to d-carba dTTP (Table 1), it was still more modestly more effective than 4'-C-methyl dTTP and much more effective than d-carba dTTP in the template inhibition assay. This may be due to two factors. First, 4'-C-ethyl dTTP, as stated above, behaves like a chain terminator and does not allow the addition of other nucleotides after incorporation.\(^{23}\) Additional nucleotides can be added with delayed kinetics with 4'-C-methyl and d-carba dTTP\(^{23,31}\) and for these analogues, extended products in the reactions in Figure 4 may include DNA chains containing more than one inhibitor nucleoside. Second, the K\(_D\) values for 4'-C-ethyl, 4'-C-methyl, and d-carba dTTP binding to HIV RT were measured at 6 mM Mg\(^{2+}\) in a separate analysis on a different template (manuscript in preparation). The K\(_D\) value for d-carba dTTP was ~4–8-fold higher than the others. This helps explain why d-carba dTTP is by far the weakest inhibitor among both translocation and chain terminating inhibitors in the template extension inhibition assay (Figure 4). It binds more weakly to RT than the other translocation inhibitors, and this, coupled with a relatively low incorporation rate (Table 1), weakens inhibition.

Although positioning of the 3'-OH of an incoming nucleotide is coordinated through many interactions with HIV RT, glutamine 151 (Q151) presumably interacts directly with this group through a proposed hydrogen bond between the amide oxygen from Q151 and the 3'-OH position of dTTP. However, binding relative to dTTP improves with this group through a proposed hydrogen bond between Q151 and the 3'-OH of dTTP. Q151N, which has weakened association with the 3'-OH, as the advantage of a 3'-OH group can access a hydrophobic pocket in RT, which may stabilize binding.\(^{7,29}\) This indicates that Q151 likely plays a key role in stabilizing dNTP binding. Dideoxy dTTP, which lacks a 3'-OH, demonstrated weaker binding to HIV RT than dTTP. However, binding relative to dTTP improves with Q151N, which has weakened association with the 3'-OH. This again predicts a role for Q151 in stabilizing binding of dNTPs through interactions with the 3'-OH, as the advantage of a 3'-OH for binding is mitigated when Q151 is altered to a noninteracting amino acid.

High divalent cation concentrations can also improve incorporation of analogues without 3'-OH groups with other polymerases, similar to what was found here. The Klenow fragment of E. coli polymerase I (KF) discriminates strongly against ddNTPs relative to dNTPs. However, ddTTP incorporation improves in high Mg\(^{2+}\) showing an optimum of 25 mM, which is several-fold greater than the optimal concentration for incorporation of dTTP (~2 mM).\(^{32}\) Like Q151N for HIV RT, an E710A (glutamic acid to alanine) KF mutation reduced ddNTP/dNTP discrimination, consistent with a role for this amino acid in interactions with the 3'-OH.
The authors hypothesize that Mg$^{2+}$ may bridge an interaction between KF E710 and the 3'-OH group of incoming nucleotides, although more complex explanations where E710 interacts with Mg$^{2+}$ via repositioning of other active site residues could not be ruled out by the data. It was also not clear if the Mg$^{2+}$ ion in question was metal ion A and/or B, the putative metal ions involved in nucleotide catalysis at polymerase active sites, or another, as yet undescribed metal ion. In this regard, it is interesting that a third metal ion has been proposed to be involved in polymerase nucleotide catalysis and may play a role in these interactions.33-37 Interestingly, unlike Q151N, E710A had a larger effect on $k_{cat}$ rather than on binding, leading the authors to postulate that its interaction with the 3'-OH does not contribute to ground-state binding. Instead, the data was more consistent for E710-Mg$^{2+}$ interactions having a role in the transition state occurring after nucleotide binding but before catalysis.32 Our work indicates that lowering the concentration of Mg$^{2+}$ disproportionately depresses the rate of catalysis for all thymidine analogues lacking a 3'-OH without strongly affecting affinity (based on $K_D$ values) for a limited set (dTTP, AZTTP, and ddTTP) of tested nucleotides (Figure 3 and Tables 1 and 2). This is consistent with the observations with the KF and suggests that Mg$^{2+}$ may interact with the 3'-OH, either directly or indirectly, in a step after nucleotide binding during the transition state or the chemistry step (the latter was ruled for the KF by demonstrating a lack of a sulfur elemental effect32 but we did not test this possibility).

It was noteworthy that HIV RT was able to incorporate all nine analogues that were tested. Only 3'-O-CH$_3$ dTTP showed very poor incorporation. For chain terminating analogues, there was not a clear consistent chemical property that explained the different rates of incorporation of the analogues. However, electronnegative groups that, unlike a 3'-OH, could not donate a H atom for hydrogen bonding (e.g., 3'-F and 3'-O-CH$_3$) were incorporated the slowest. Groups with potential for positive charge or hydrogen bond formation (e.g., 3'-NH$_2$ and 3'-N$_2$) and small groups (e.g., 3'-H (dddTTP) or ddTTP (no group)) were better substrates, although all were less efficiently incorporated than dTTP, especially in low Mg$^{2+}$ (Tables 1 and 2). It is possible that 3'-NH$_2$ and 3'-N$_2$ groups may interact with Q151 through charge interactions or H-bonding, and this may help compensate for the loss of the 3'-OH. Both ddTTP and ddTTP could not establish these interactions, but the small size of the 3' substitution and the noncharge nature could minimize repulsive interactions. Also, ddTTP is different from the other tested chain terminators as the 2',3'-didehydro configuration of the sugar moiety alters the structure of the sugar. This makes it more difficult to directly compare it to the other 3'-modified analogues.

**MATERIALS AND METHODS**

**Materials.** 3'-Fluor (F) and 3'-O-methyl (O-CH$_3$) dTTP were synthesized by AX Molecules Inc. (San Marcos, CA). 3'-Amino (NH$_2$) dTTP and dideoxy TTP (dddTTP) were from TriLink Bio Technologies (San Diego, CA). AZTTP and ddTTP were from Sierra Bioresearch (Tucson, AZ). 4'-C-Methyl, 4'-C-ethyl, and d-carba dTTP23,31 were a generous gift from Dr. Stephen Hughes (National Institutes of Health). All other nucleotides were from Roche Diagnostics Corporation (Indianapolis, IN). All nucleotides had purity levels >95% (as determined by mass spectroscopy and TLC analysis). T4 polynucleotide kinase (PNK) was from New England Biolabs (Ipswich, MA). Radiolabeled compounds were from PerkinElmer (Waltham, MA). DNA oligonucleotides were from Integrated DNA Technologies (IDT) (Coralville, IA). G-25 spin columns were from Harvard Apparatus (Holliston, MA). All other chemicals were obtained from Thermo Fisher Scientific (Waltham, MA), VWR International (Radnor, PA), or Sigma-Aldrich (St. Louis, MO).

**Preparation of HIV Reverse Transcriptase (HIV RT).** The expression plasmids (pRT66 and pRT51) for HIV-1 RT (HXB2 sequence) were used to produce HIV RT.38 The enzyme, which is a nontagged heterodimer consisting of a 1:1 molar ratio of p66 and p51, was prepared as described.39

**Preparation of Primer-Templates for Pre-Steady-State Kinetics and Inhibitor Analysis on the 100-nt Template.** For pre-steady-state kinetics, the 5'-TTCCCGTACGCCATCTGGTCTAAGGCAAGGGAATTTTCTTCAGAGCAGACCAGAGCCAA-CAGGGAATTTTCTTCAGACAGACCAGACAGCCAAGCGCCCCACACACCAGAAGAGCTTCAGGTTTGGGGAA-GAGCACAAAC-3' (F) and 3'-Fluor (F) and 3'-32P-labeled primer (5'-TTCCCGTACGCCATCTGGTCTAAGGCAAGGGAATTTTCTTCAGAGCAGACCAGAGCCAA-CAGGGAATTTTCTTCAGACAGACCAGACAGCCAAGCGCCCCACACACCAGAAGAGCTTCAGGTTTGGGGAA-GAGCACAAAC-3') were mixed at a molar ratio of 1:1.25 (primer/template) in a buffer containing 50 mM Tris-HCl (pH 8), 80 mM KCl, and 1 mM DTT. For inhibitor analysis, during primer extension on a long template, the 5'-2'-32P-labeled 20-nt primer (5'-TTGTTTGTCTCTTCCCCAAAAC-3') and 36-nt template (5'-ATCCTCGTACCTGAGCTGCTGACAGGGAAATTGTAT-3') were mixed at a molar ratio of 1:1.25 (primer/template) in a buffer containing 50 mM Tris-HCl (pH 8), 80 mM KCl, and 1 mM DTT. For inhibitor analysis, during primer extension on a long template, the 5'-2'-32P-labeled 20-nt primer (5'-TTGTTTGTCTCTTCCCCAAAAC-3') and 36-nt template (5'-ATCCTCGTACCTGAGCTGCTGACAGGGAAATTGTAT-3') were mixed at a molar ratio of 1:1.5 (primer/template).

**Pre-Steady-State Kinetic Parameters for dTTP or dTTP Analogue Incorporation.** Experiments were performed using a QFM-4000 from Bio-Logic Scientific Instruments. All experiments were carried out at 37 °C. For each
reaction, 20 μL of solution containing 50 nM (in primer) primer—template prepared as described above (final concentration 25 nM in reactions) and 50 nM HIV-1 RT (HXB2) (final concentration 25 nM in reactions) in buffer 1 (50 mM Tris—HCl (pH 8), 80 mM KCl, 1 mM DTT, and 0.5 or 6 mM MgCl₂) were loaded into one loop of the apparatus. A second loop contained 20 μL of a solution with various concentrations of dTTP or specific dTTP analogues in the same buffer. Concentrations used ranged from 1 to 100 μM (final concentrations in reaction) for experiments to determine Kᵦ and kᵦ,fb (depending on the nucleotide) and were 5 μM for pre-steady-state rate constant (kᵦ,fb) determinations with various analogues. For reactions with higher nucleotide concentrations, the starting Mg²⁺ concentration in the 0.5 mM Mg²⁺ reactions was adjusted to keep the free Mg²⁺ concentration at 0.5 mM. For adjustments, the dissociation constants (Kᵦ) for dNTP and Mg²⁺ were assumed to be the same as that of ATP (Kᵦ = 89.1 × 10⁻⁶ M). A third loop contained 20 μL of a solution containing 0.5 M EDTA (pH 8). Reactions were initiated by rapidly mixing the solutions in loops 1 and 2 and then terminated by mixing with loop 3 at time points ranging from 5 ms to 2 s. Specific time points depended on the incorporation rates of the individual analogues. The products were mixed with an equal volume of 2X loading buffer (90% formamide, 0.025% each bromophenol blue, and xylene cyanol) and resolved on 20% polyacrylamide—7 M urea denaturing PAGE gels as described. Gels were dried and then exposed to phosphorimager screens. Quantification was performed using a Fujifilm FLA7000 phosphorimager and Fuji ImageQuant software. First-order-rate constants for incorporation (kᵦ,obs) were determined using SigmaPlot by plotting the concentration of the extended starting material versus time and fitting the data to a single exponential equation:

\[ y = a(1 - e^{-kt}) + C \]

where \( a \) is the amplitude, \( k \) (kᵦ,obs in this case) is the rate, and \( C \) is the end point. In some cases, especially for analogues with slower incorporation rates, the data fit better to a simpler equation:

\[ y = a(1 - e^{-kt}) \]

For some analogues, rate constants at different analogue concentrations were used to determine the equilibrium dissociation constant and maximum rate of nucleotide addition (Kᵦ and kᵦ,obs respectively) by plotting kᵦ,obs versus the concentration of the nucleotide and fitting to a hyperbolic equation for ligand binding with single site saturation:

\[ y = \frac{B_{\text{max}}(x)}{K_\text{D} + x} \]

where \( B_{\text{max}} \) corresponds to kᵦ,obs. Standard error values for Kᵦ and kᵦ,obs were generated by the program.

**Inhibitor Analysis during Primer Extension on a Long Template.** Primer extension reactions were performed to study the inhibition of extension by thymidine analogues as described previously. Briefly, the 15 nM 5'-32P-labeled primer was hybridized with the 22.5 nM template at a molar ratio of 1:1.5 as described above. Hybrids were preincubated for 3 min at 37 °C in 8.5 μL of buffer 1, all 4 dNTPs, and 1 of the analogues (5 μM each and 3 μM, respectively (final concentrations in reactions)). Extension was initiated by adding 4 μL of HIV RT (final concentration 100 nM) in buffer 1. After extension for 30 min, the reactions were terminated by adding 12.5 μL of 2X gel loading buffer and samples were resolved on an 8% denaturing urea gel and processed as described above.

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**Notes**

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health [grant numbers R01AI150480]. The sponsor was not involved in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. We thank Dr. Stephen Hughes (National Institutes of Health) for 4′-C-methyl, 4′-C-ethyl, and α-carba dTTP.

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