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Articles

Mechanism of HIV Reverse Transcriptase: Enzyme–Primer Interaction As Revealed through Studies of a dNTP Analogue, 3′-Azido-dTTP†

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ABSTRACT: Primer and dNTP recognition by purified HIV reverse transcriptase have been investigated. Earlier kinetic studies suggested that the reaction pathway for DNA synthesis is ordered, with template-primer and free enzyme combining to form the first complex in the reaction sequence [Majumdar et al. (1988) *J. Biol. Chem. 263*, 15657-15665], and through use of a particularly high affinity template-primer analogue [$r(I)_n$·$Sd(C)_{28}$], rate values for formation of the first complex were calculated [Majumdar et al. (1989) *Biochemistry 28*, 1340-1346]. We now report rate values for first complex formation in the usual model replication system with poly[r(A)]·oligo [d(T)] as template-primer. We find that 3′-azido-dTTP (AZTTP) is a linear competitive inhibitor of DNA synthesis against the substrate dNTP (dTTP) in the poly[r-(A)]·oligo[d(T)] replication system. This suggests that 3′-azido-dTTP and dTTP combine with the same form of the enzyme in the reaction scheme, i.e., the enzyme–primer complex. This is not trivial, since a second analogue, 3′-amino-dTTP, also is an inhibitor against dTTP, but the mechanism in this case is linear noncompetitive. Because the inhibition by 3′-azido-dTTP is linear competitive, the K_D for physical binding to the enzyme is assumed to be the same as the K_i for inhibition (20 nM). Substrate kinetic studies of DNA synthesis using 3′-azido-dTTP as substrate revealed that the Michaelis constant is 3 μM. Therefore, the K_m for this substrate analogue is 100-fold higher than the K_D for binding of the analogue to the enzyme–primer complex. This difference is consistent with the proposed kinetic scheme and is due primarily to the effect of enzyme–primer complex dissociation on the value of K_m. The results enable calculation of rate values for the enzyme–poly[r(A)]·oligo[d(T)] association (k_{on} = 2.6 × 10^8 M^{-1} s^{-1}) and dissociation (k_{off} = 39 s^{-1}) and a K_D value for dTTP binding (~180 nM).

The HIV[1] reverse transcriptase catalyzes both RNA-directed DNA synthesis and DNA-directed DNA synthesis in the cytoplasm shortly after retrovirus infection of a cell. In the first step of this process, the enzyme is capable of recognizing its natural primer, Lys-tRNA subspecies 3. The enzyme then conducts the DNA synthesis, template switching, and RNase H steps necessary for replication of the complete viral genome (Gilboa et al., 1979). The enzymatic mechanism of these events is the subject of current investigation.

As a means of understanding the mechanism of the HIV reverse transcriptase, Majumdar et al. (1988, 1989) applied steady-state kinetic and processivity analysis to obtain a kinetic scheme for the overall DNA synthetic reaction. The data suggested that the free enzyme interacts with the template-primer substrate in the initial phase of the DNA synthesis

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[1] Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZTTP or 3′-azido-dTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; NH_2T, 3′-amino-3′-deoxythymidine; NH_2TTP or 3′-amino-dTTP, 3′-amino-3′-deoxythymidine 5′-triphosphate; DTT, dithiothreitol; dNMP, deoxynucleoside monophosphate; dNTP, deoxynucleoside triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; HIV-1, human immunodeficiency virus, type 1; oligo[d(T)], oligomer of deoxythymidylate; poly[r(A)], polyriboadenylate; RT, reverse transcriptase; $Sd(C)_{28}$, 28-residue oligodeoxycytidylate with sulfur substituted at a nonbridge oxygen of each phosphate atom; T-P, template-primer; TEAB, triethylammonium bicarbonate. Polynucleotide subscripts indicate precise chain length unless otherwise noted. The kinetic nomenclature and constants are according to Cleland (1963a,b) and Fersht (1985).

reaction pathway. These studies were extended here by identifying a dNTP analogue with precise and high-affinity binding to the enzyme–primer complex. We then made use of this analogue to determine its Michaelis constant (K_m) for DNA synthesis, and mathematical treatment of the constant then enabled calculation of the off-rate for interaction between free enzyme and template-primer. The results are discussed in the context of a kinetic model for the DNA polymerase activity of HIV reverse transcriptase and of the use of high-affinity substrate analogues as antiviral agents.

Experimental Procedures

Materials

Poly[r(A)$_{810}$], of average chain length 810, was obtained from Miles Laboratories. [^{3}H]dTTP was from New England Nuclear. Unlabeled dTTP, ddTTP, and oligo[d(T)] were obtained from Pharmacia LKB Biotechnology Inc. [γ-^{32}P]-ATP was obtained from ICN Radiochemicals. Electrophoresis grade acrylamide and bis(acrylamide) were obtained from Bio-Rad Laboratories. Formamide and urea were obtained from Bethesda Research Laboratories.

HIV-1 reverse transcriptase, expressed in *Escherichia coli* from a plasmid, was obtained from Genetics Institute. The expression plasmid contains the coding sequence of HIV-1 reverse transcriptase with the carboxyl-terminal Leu omitted (Huber et al., 1989). The final fraction of the expressed enzyme is >90% pure, and analysis by sodium dodecyl sulfate–polyacrylamide gel of the final fraction displays two major bands of approximately equal intensity at 66 and 51 kDa, similar to the immunoaffinity-purified HIV enzyme (di Marzo Veronese et al., 1986). Specific activity of the expressed enzyme was 1000 nmol h^{-1} mg^{-1} at 37 °C on a poly[r(A)]-oligo[d(T)] template-primer. We also obtained HIV-1 reverse transcriptase immunoaffinity purified from virions as a generous gift from Dr. M. G. Sarngadharan, Bionetics Research. We have found that the recombinant Genetics Institute enzyme and the Bionetics enzyme show the same behavior on a poly[r(A)] template (data not shown) by conducting processive DNA synthesis and showing a termination frequency after insertion of the first nucleotide that is an order of magnitude greater than termination for processive synthesis (Majumdar et al., 1988). We have also indicated the similarities of the recombinant and natural enzymes under conditions described in Figures 2 and 3. Unless otherwise indicated, experiments were conducted with the Genetics Institute enzyme.

Methods

Synthesis of AZT and NH_2T 5′-Triphosphates. AZT was purchased from Sigma (St. Louis). The NH_2T was synthesized from AZT by the method described in the literature (Lin & Prusoff, 1978, 1986).

The 5′-triphosphates were prepared from the corresponding unprotected nucleosides according to an approach published by Kovács and Ötvös (1988). A known amount of dry nucleoside (0.3 mmol) was stirred in dry trimethyl phosphate (0.75 mL) with powdered 1,8-bis(dimethylamino)naphthalene (Proton Sponge, Aldrich, 0.45 mmol) at 0 °C. Distilled $POCl_3$ (60 μL) was added, and the mixture was stirred at 0–4 °C. After 2 h, a mixture of 0.5 M bis(tri-*n*-butylammonium) pyrophosphate in anhydrous DMF (3 mL, 5 equiv) and tri-*n*-butylamine (0.3 mL) was quickly added to the reaction mixture under vigorous stirring at 0 °C. After 1 min, 0.2 M aqueous triethylammonium bicarbonate (TEAB) buffer, pH 7.5 (30 mL), was poured into the reaction mixture; the mixture was stirred for 30 min at room temperature and then evaporated to dryness. The residue was taken up in water and applied to a DEAE-Sephadex A-25 (Pharmacia) column, which was subsequently eluted with a linear gradient of TEAB buffer, pH 7.5, from 0 to 400 mM (800–800 mL) at 4 °C. The appropriate fractions were collected and evaporated to dryness, and the 5′-triphosphates were converted into their sodium salts by sodium–iodide precipitation from acetone. On this scale, isolated yields were 45% of theoretical for AZTTP and 43% for NH_2TTP from the corresponding nucleoside.

For the enzyme kinetic studies the 5′-triphosphates were further purified by HPLC using a Beckman Ultrasphere ODS column (10 mm × 25 cm) and a gradient from 15% to 100% solvent B in solvent A [B, methanol–water = 75:25 v/v, with 5 mM PIC A (tetrabutylammonium phosphate) (Waters); A, 0.06 M KH_2PO_4 with 5 mM PIC A (Waters, final pH 5.0)] over an elapsed time of 30 min and a 2 mL/min flow rate. Retention times were as follows: AZTTP, 28 min; NH_2TTP, 22 min. After the HPLC purification, the 5′-triphosphates were desalted on a DEAE-Sephadex A-25 column (1 × 10 cm) by using a linear gradient of TEAB buffer, pH 7.5, from 200 to 400 mM (50–50 mL) at 4 °C. After evaporation and freeze-drying, the 5′-triphosphates were used for enzyme studies as triethylammonium salts.

The structures of AZTTP and NH_2TTP were confirmed by enzymatic hydrolysis and NMR data. For enzyme digestion 1 OD unit of each 5′-triphosphate was incubated with snake venom phosphodiesterase (0.1 unit, Cooper Biochemical) in the presence of 10 mM Tris-HCl, pH 8.5, and 50 mM $MgCl_2$ at 37 °C overnight, followed by the addition of 0.5 unit of bacterial alkaline phosphatase (Sigma). After a further 2 h of incubation, the reaction mixture was analyzed by HPLC. In both cases the enzymatic degradation gave only one product, which was identical with the starting AZT and NH_2T nucleoside, respectively. NMR data were determined in D_2O solution in the presence of 5 mM sodium phosphate buffer (pH 7.0) as internal standard. For AZTTP: ^{1}H NMR (D_2O) δ 7.75 (s, 1 H, H-6), 6.28 (t, 1 H, H-1′), 4.59 (m, 1 H, H-3′), 4.21 (d, 3 H, H-4′, 5′, 5″), 2.47 (q, 2 H, H-2′ and 2″), 1.91 (s, 3 H, 5-CH_3); ^{31}P NMR (D_2O) δ −9.9 (d, γP), −12.8 (d, αP), −23.7 (t, βP). For 3′-NH_2dTTP: ^{1}H NMR (D_2O) δ 7.68 (s, 1 H, H-6), 6.31 (t, 1 H, H-1′), 4.46–4.24 (m, 4 H, H-3′, 4′, 5′, and 5″), 2.64 (q, 2 H, H-2′ and 2″), 1.90 (s, 3 H, 5-CH_3); ^{31}P NMR (D_2O) δ −9.0 (d, γP), −12.6 (d, αP), −23.4 (t, βP).

For studies on the kinetics of reduction of AZTTP by DTT, aliquots were removed from reaction mixtures at indicated times and analyzed by HPLC (Ultrasphere ODS, Altex, 4.6 × 250 mL column, flow rate 1 mL/min, UV detection at 270 nm). The elution program was 15% buffer B in buffer A for 2 min followed by a linear gradient of 15–100% buffer B in 30 min, where buffer A was 60 mM KH_2PO_4–5 mM tetrabutylammonium phosphate (PIC A, Waters), pH 5.0, and buffer B was 75% methanol in 5 mM PIC A. The following retention times were observed: AZTTP, 20 min; NH_2TTP, 11 min; dTTP, 16 min.

DNA Polymerase Assay. Reaction mixtures in a final volume of 12 μL contained 20 mM Tris-HCl, pH 8.3; 6 mM $MgCl_2$; 8 μM [^{3}H]dTTP (2000–3000 dpm/pmol); 22 μg/mL poly[r(A)] hybridized to 11 μg/mL dT_{12-18}; and 56 ng (480 fmol) of HIV-1 reverse transcriptase. Incubation was for 20 min at 37 °C, and enzyme activity was linear with time to 20 min. Incorporation was measured by acid-precipitable counts collected on nitrocellulose filters (Schrier & Wilson, 1973).

Preparation of Labeled Primers. Synthetic dT_{14} primers were 5′ end labeled with ^{32}P in accordance with the procedure

of Maxam and Gilbert (1980). DNA was extracted with phenol; residual phenol was removed with ether, and the DNA was precipitated with ethanol.

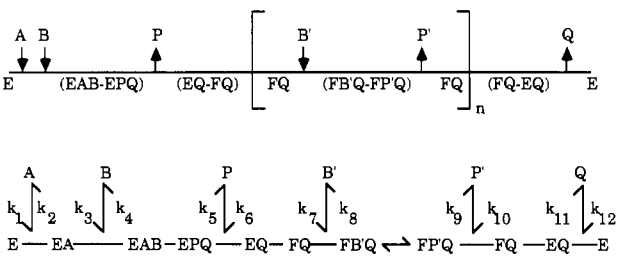
In Vitro DNA Synthesis with Labeled Primers. ^{32}P-Labeled primers were hybridized to poly[r(A)] at a 1:1 molar ratio of primer–template by heating to 100 °C and slowly cooling to room temperature. DNA synthesis reactions (10 μL) contained 20 mM Tris-HCl, pH 8.0; 6 mM $MgCl_2$; 10 mM dithiothreitol (DTT); 100 fmol of hybridized primer–template; 10–25 fmol HIV reverse transcriptase; and dNTPs as indicated. Reaction mixtures were incubated at 30 °C, and reactions were stopped by adding EDTA to a final concentration of 18 mM. A dye mixture in deionized formamide was then added to a total volume of 15 μL; 5-μL portions were loaded for gel electrophoresis. We found with these reactions that omission of DTT had no significant effect on enzyme activity; however, when the HIV RT was stored at −20 °C at concentrations lower than 14 μg/mL in buffer without DTT, the enzyme lost nearly all activity after a few days (data not shown). Experiments with this system with DTT omitted were therefore carried out by diluting enzyme on the day of the reaction.

Analysis of Products of DNA Synthesis. Products were analyzed by gel electrophoresis as previously described (Abbotts et al., 1988). Electrophoresis was conducted on a 12% polyacrylamide, 7 M urea gel. The gel was prerun for 1 h at 40–45 W without cooling, and electrophoresis was performed at 40–45 W. After electrophoresis, the gel was transferred to Whatman 3MM paper, covered with Saran Wrap, and dried in a Bio-Rad gel dryer. Products were visualized by autoradiography with Kodak XAR-5 film at room temperature. Some exposed films were evaluated for band intensity with a Zeineh soft laser scanning densitometer (Biomed Instruments). Areas under peaks were determined by cutting and weighing. Cutting regions of the gel corresponding to individual bands and determining radioactive counts in a liquid scintillation counter gave data similar to those determined by cutting and weighing peaks from densitometer tracings (data not shown).

Synthesis on an M13 Template. Experiments were also carried out with an M13mp2 DNA template by using an oligodeoxynucleotide primer, 15 nucleotides long, with its 5′ hydroxyl at position 115 in the *lacZ* region; template and primer were generous gifts from Dr. T. A. Kunkel (National Institute of Environmental Health Sciences). In the absence of dCTP, synthesis can proceed on this template-primer for 12 nucleotides; dTMP is inserted at the 1st, 5th, 6th, and 10th positions (Bebenek et al., 1989). Labeling of primer and hybridization to template were carried out as described above. Reaction mixtures (10 μL) contained 20 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 2 mM DTT; 10 μM dATP, dGTP, and dTTP as indicated; 60 fmol of template-primer; and 10 fmol of HIV reverse transcriptase. Incubations were at 37 °C for 20 min. Products of DNA synthesis were displayed by gel electrophoresis and autoradiography as described above. Gel lanes were cut into pieces corresponding to positions for each dTMP incorporation and longer products. Pieces of gel were placed in scintillation vials, covered with 10 mL of Econofluor (Du Pont-New England Nuclear), and radioactive counts were measured in a Beckman LS-9000 liquid scintillation counter. Background counts were determined from corresponding gel lane positions from reactions containing no dTTP.

Theory

The proposed kinetic scheme for the HIV reverse transcriptase (Majumdar et al., 1988) is



The steps inside brackets represent processive synthesis. E and F are stable forms of the enzyme. A is the template-primer complex. B and B′ are, respectively, the first and all subsequent dNTP added to the enzyme during one cycle of free enzyme binding to A, processive synthesis, and dissociation from Q (i.e., termination). P and P′ are the first and all subsequent pyrophosphate molecules released, respectively. Q is the polynucleotide product (extended primer). The initial rate data are analyzed by using the following rate equation for the above scheme with the additional assumption that the free enzyme does not form an enzyme–product complex with the template-primer

$$v = \frac{[A][B]V_{max}}{K_mBK_mA + K_mA[B] + K_mB'[A] + [A][B]} \quad (1)$$

where the constants are

$$K_mB = \frac{k_2(k_5 + k_4)}{k_3k_5} \quad (2)$$

$$K_mA = \frac{k_5k_9k_{11}}{k_1(k_9k_{11} + k_5k_{11} + k_5k_9)} \quad (3)$$

$$K_mB' = \frac{k_{11}(k_5k_7k_9 + k_7k_9k_4 + k_3k_5k_9 + k_3k_5k_8)}{k_3k_7(k_9k_{11} + k_5k_{11} + k_5k_9)} \quad (4)$$

and

$$V_{max} = \frac{k_5k_9k_{11}E_t}{k_9k_{11} + k_5k_{11} + k_5k_9} \quad (5)$$

K_mB represents the Michaelis constant for addition of the first dNMP residue to the primer. K_mB' represents a consensus Michaelis constant for subsequent dNMP additions during each cycle of enzyme binding to A, processive polymerization, and termination.

For the present study, a simplified representation of the scheme above is

$$E + d(T)_{14} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{-}d(T)_{14} + dTTP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E \begin{matrix} \xrightarrow{d(T)_{14}} \\ \xrightarrow{dTTP} \end{matrix} \xrightarrow{k_{cat}} E + \text{products}$$

where the first two complexes proposed are illustrated. Steps subsequent to dTTP addition are summarized as k_{cat}, the overall steady-state rate constant. The Michaelis constant for dTTP is

$$K_m\text{dTTP} = \frac{k_{off}(k_{cat} + k_4)}{k_{cat}k_3}$$

When k_{cat} is much less than k_4, as is the case here (k_{cat} = 2–4 s^{-1} vs $k_4 \cong 35$ s^{-1}), the relationship can be simplified by omitting k_{cat} from the numerator. Therefore

$$K_m\text{dTTP} \cong \frac{k_{off}k_4}{k_{cat}k_3}$$

or

$$k_{cat}K_m\text{dTTP} = k_{off}(k_4/k_3)$$

Since k_4/k_3 is K_D, the relationship

$$d(T)_{14}k_{off} = k_{cat}K_m\text{dTTP}/K_D$$

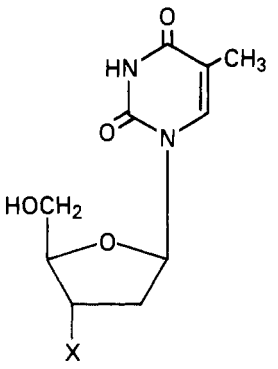


FIGURE 1: Structure of AZT and NH_2T. These thymidine analogues differ only at the position indicated by X. For 3′-azido-3′-deoxythymidine (AZT), X = N_3; for 3′-amino-3′-deoxythymidine (NH_2T), X = NH_2.

can be used to calculate $d(T)_{14}$–enzyme dissociation.

RESULTS

AZT and NH_2T Analogues and the Effect of Reducing Agents. 3′-Azido-3′-deoxythymidine and 3′-amino-3′-deoxythymidine (Figure 1) are closely related analogues of thymidine. A rather wide variety of conditions of pH (7.3–8.5) and DTT (1–10 mM) concentrations have been common in various studies on the ability of the 5′-triphosphate of AZT to inhibit the RT of HIV or of other retroviruses (Furman et al., 1986; Cheng et al., 1987; Eriksson et al., 1987; Matthes et al., 1987; Olsen et al., 1987; St. Clair et al., 1987; Vrang et al., 1987). While aliphatic azides, such as AZT, are stable in neutral or near neutral aqueous solutions at room temperature, they are notoriously unstable in reducing conditions such as in the presence of thiols (Patai, 1971). Ample precedent exists for the reactions of thiols with azides to produce amines (Saegusa et al., 1970; Cartwright et al., 1976; Bayley et al., 1978; Staros et al., 1978). In accordance with such reports, Handlon and Oppenheimer (1988) observed the facile reduction of AZT nucleoside with DTT. While a precise mechanism for the reduction of azides by thiols or dithiols has not been established experimentally, two reasonable mechanistic interpretations have been advanced (Cartwright et al., 1976). In both alternatives, the dithiol may add to the azide, and then a hydroxide-induced cyclization and elimination of nitrogen follows. Thus, the rate of this reaction would be influenced not only by AZT and DTT concentrations but also by pH.

We have extended the above observations to an examination of the stability of AZT 5′-triphosphate under the conditions often used in RT inhibition assays. For instance, AZTTP (100 μM) was incubated at 37 °C in a solution containing 50 mM Tris-HCl, pH 8.5, 100 mM KCl, 5 mM $MgCl_2$, 100 μM dTTP (as an internal standard for HPLC), and DTT at concentrations of 5, 7.5, or 10 mM. Under such conditions, the reduction of AZT displayed second-order kinetics with a rate constant of 0.044 mol L^{-1} s^{-1} (data not shown). In fact, since DTT is usually employed in large excess compared to AZTTP, the concentration of DTT remains practically constant, and AZTTP decomposition follows pseudo-first-order kinetics. We could calculate an AZTTP half-life of 53 min in 5 mM DTT and 266 min in 1 mM DTT. The reaction was, of course, highly pH dependent so that at pH 7.5 and a concentration of 5 mM DTT only about 4% of NH_2TTP was formed after 90 min of incubation at 37 °C. Thus, to obviate complications deriving from degradation of the azido moiety of AZTTP, the pH should be near neutrality and the concentration of DTT (or other thiol) should be as low as consistent with optimal enzyme activity.

Table I: Comparison of Inhibition of HIV Reverse Transcriptase by 3′-Azido-dTTP (AZTTP) and 3′-Amino-dTTP (NH_2TTP)

inhibitor	K_i (nM)	pattern of inhibition[a]
AZTTP	20	linear competitive
NH_2TTP	42	linear noncompetitive

[a] Versus normal system with dTTP-Mg as substrate. K_i values and patterns of inhibition were determined as described in the text.

Inhibition of HIV RT by AZTTP and NH_2TTP. From earlier kinetic studies of DNA synthesis by purified HIV reverse transcriptase, an ordered mechanism was proposed with free enzyme and template-primer combining to form the first complex in the reaction pathway (Majumdar et al., 1988). Similar results on enzyme/template-primer binding as the first step in the reaction scheme were reported recently by Huber et al. (1989). In the current study of interaction between the enzyme/template-primer complex and dNTP, we found that two analogues of the normal substrate nucleotide are strong inhibitors of the DNA synthesis reaction. These analogues, 3′-azido-3′-deoxythymidine 5′-triphosphate (AZTTP) and 3′-amino-3′-deoxythymidine 5′-triphosphate (NH_2TTP), were examined here in parallel to compare their modes of inhibition of the enzymatic reaction, although we had no reason to suspect that inhibition by the two analogues would differ. Rather, we initially chose to evaluate both compounds as inhibitors because of the observation that AZT or its triphosphate are converted to NH_2T or NH_2TTP in solutions containing a reducing agent such as DTT. Since DTT is present in the usual assay mixture for HIV reverse transcriptase, it was of interest to determine whether the inhibition seen with AZTTP addition is actually due to AZTTP itself, the reduction product NH_2TTP, or both.

The initial rate of DNA synthesis was measured with $r(A)_{810}$·$d(T)_{12-18}$ as the template-primer system and dTTP-Mg as the nucleotide substrate. We first observed that when DTT was omitted from dilution buffers and the final reaction mixture, activity of HIV RT still could be detected in the polymerase assay at 80% the normal rate (data not shown). Therefore, we examined inhibition of the RT by AZTTP and NH_2TTP using DNA polymerase assays without DTT. Both analogues are inhibitors of the HIV RT. Hill plot analysis of inhibition by each analogue revealed a simple linear pattern. The slope of the line in each case was between 0.5 and 1 (0.74 for AZTTP, 0.56 for NH_2TTP); this is consistent with molecular order of inhibition of one and with limitation of one rate-limiting step in the steady state for DNA synthesis. Fifty percent inhibition corresponded to 50 and 145 nM for AZTTP and NH_2TTP, respectively (data and plot not shown).

Kinetic Analysis of Inhibition by Substrate Analogues. To further characterize the inhibition by the two substrate analogues and to compare true K_i values, substrate kinetic studies were conducted. Fixed concentrations of AZTTP in the range 10–60 nM were used with dTTP as the variable substrate. Double-reciprocal plots at each fixed concentration of AZTTP were linear, and the patterns converged to the same point on the ordinate. The replot of slope with each fixed concentration of AZTTP also was linear. The linear slope replot with AZTTP was consistent with the result of Hill plot analysis, as was the K_i value of 20 nM (data and plots not shown). These results indicate that AZTTP blocks enzymatic activity through competitive inhibition with the normal substrate (dTTP) for binding to the primer–enzyme complex. The K_i value for AZTTP inhibition, 20 nM (Table I), is much lower

than the K_m for dTTP incorporation, 2–3 μM (Majumdar et al., 1988).

A similar kinetic study of the inhibition by NH_2TTP was conducted. In this case, however, a primary double-reciprocal plot revealed that the pattern of inhibition is noncompetitive, since the lines at different fixed concentrations of inhibitor extrapolate to different points on the ordinate. The replots of slopes and intercepts were linear also and indicated linear noncompetitive inhibition with K_i value of 42 nM (data and plots not shown). Taken together, these results with AZTTP and NH_2TTP indicate that the two analogues block DNA synthesis by interacting with different forms of the enzyme. This result was rather unexpected since the two analogues are closely related in chemical composition. Although the inhibition experiments appeared straightforward, we conducted a further, so-called "mixed inhibition" experiment to confirm that AZTTP and NH_2TTP act on different forms of the enzyme. The effect of increasing concentrations of AZTTP was examined in the presence of a fixed concentration of NH_2TTP. If the two inhibitors act on the same form of the enzyme, an identical slope is expected in the presence and absence of NH_2TTP. This result was not obtained, and instead, the presence of 10 nM NH_2TTP increased the slope for AZTTP inhibition (data and plot not shown). This is, indeed, the type of mixed inhibition pattern expected from the results with the inhibitors examined individually.

Product Distribution in the Presence of Inhibitors. We used the technique of electrophoresis and autoradiography to examine the size distribution of the products of synthesis, poly[d(T)] molecules, when reactions are inhibited by the triphosphates of AZT or NH_2T, in the concentration range 10–50 nM. The results shown in Figure 2 indicate that as AZTTP concentration increases in the presence of 8 μM dTTP, the size distribution of products shifts toward smaller lengths, such that the average length of product molecules decreases. This is consistent with an inhibitor that acts to increase termination and is expected for a competitive inhibitor of dTTP, since reducing the effective concentration of substrate dNTP increases termination (Abbotts et al., 1988).

With NH_2TTP, as inhibitor concentration increases, one continues to see products near the top of the gel, and indeed the same overall size distribution, yet the overall amount of product, i.e., strength of signal, decreases. In addition to the concentrations in Figure 2, product size distribution also is not changed at 80 nM NH_2TTP, twice K_i (data not shown). This indicates that the chief mode of inhibition by NH_2TTP is other than chain termination and/or competition with dNTP and that this compound has the effect in a more general manner of simply reducing activity. Thus, the changes in product size distribution associated with inhibition are consistent with the kinetic data in Table I, since AZTTP is a competitive inhibitor against dTTP, whereas NH_2TTP is acting on a form of the enzyme involved perhaps in the initiation or termination of synthesis.

We find that HIV-1 reverse transcriptase purified from virions shows the same patterns of product distribution with inhibition by AZTTP and NH_2TTP as in Figure 2; we also see no significant difference in product distributions in the presence or absence of DTT (data not shown). This latter observation suggests that during a typical 20-min DNA polymerase assay the conversion of AZTTP to NH_2TTP is not significant.

Study of DNA Synthesis with Nucleotide Analogues as Substrate. Since the inhibition pattern with AZTTP is linear competitive, the K_i value is assumed to be equal or close to the K_D value for the enzyme–AZTTP interaction. It was of interest to determine if AZTTP can act as substrate for formation of the expected primer + 1 molecule, because the AZTTP Michaelis constant for the reaction can be used in combination with K_D to obtain rate values for certain steps in the DNA synthesis mechanism (see above). Figure 3 shows the results of this analysis with both AZTTP and NH_2TTP. The primer dT_{14} was labeled at the 5′ end with ^{32}P and hybridized to poly[r(A)]; dNMP incorporation then was indicated by formation of a band corresponding to primer + 1 when products of synthesis were displayed by gel electrophoresis and autoradiography. A reaction with ddTTP was included for comparison. Primer + 1 formation is seen with both AZTTP and NH_2TTP, but no products of longer chain length are detected, as expected. Certain 5′-substituted aminonucleosides can be incorporated into internucleotide bonds (Letsinger et al., 1972; Prusoff et al., 1979). In such cases, however, the amino moiety was sequestered into a phosphoramide bond in the appropriate triphosphate and thus was not directly involved in the reaction center, as would be required for the 3′-amino function of NH_2TTP. The position of migration of the primer + 1 product is different for each analogue. The primer + 3′-azido-dTMP molecule migrates slightly faster than the primer + 3′-amino-dTMP molecule, and the distinct migration properties of these product molecules are taken as evidence for incorporation of the analogue. Substrate kinetic studies of primer + 1 formation were conducted for both of the analogues by using template-primer at fixed concentration and variable concentrations of the respective analogue.

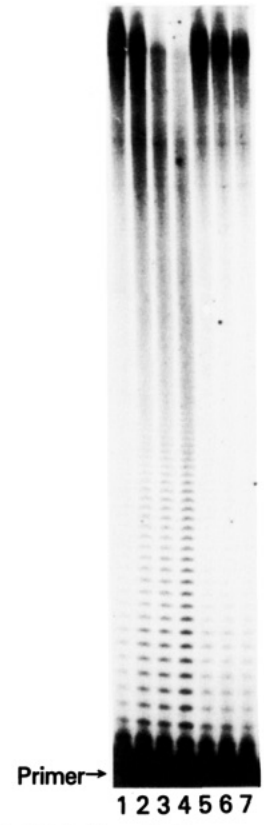


FIGURE 2: Product distributions in the presence of AZTTP and NH_2TTP. Labeling of primers with ^{32}P, in vitro DNA synthesis, and product analysis were as described under Experimental Procedures. Reaction mixtures contained 20 fmol of HIV reverse transcriptase and 8 μM dTTP; incubations were for 20 min. Inhibitor concentrations were as follows: (lane 1) none; (lane 2) 10 nM AZTTP; (lane 3) 30 nM AZTTP; (lane 4) 50 nM AZTTP; (lane 5) 10 nM NH_2TTP; (lane 6) 30 nM NH_2TTP; (lane 7) 50 nM NH_2TTP.

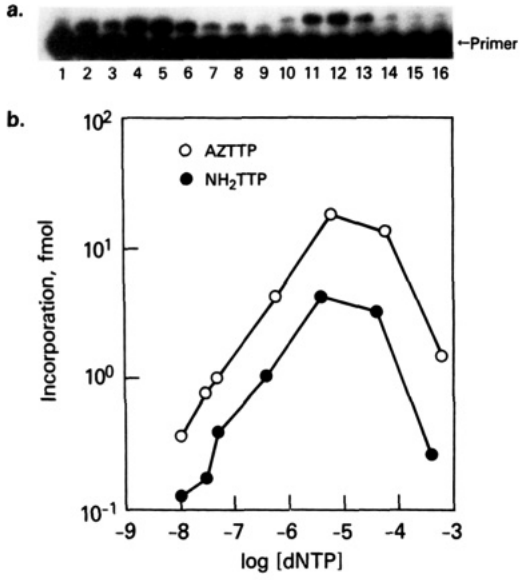


FIGURE 3: Incorporation with AZTTP and NH_2TTP. Labeling of primers with ^{32}P, in vitro DNA synthesis, and product analysis were as described in Figure 2. Reaction mixtures contained 10 fmol of (protein) HIV reverse transcriptase and 10 mM DTT; incubations were for 5 min. (Panel a) Products of reaction. Reaction mixtures contained dNTPs as follows: (lane 1) none; (lane 2) 40 μM ddTTP; (lane 3) 600 μM AZTTP; (lane 4) 60 μM AZTTP; (lane 5) 6 μM AZTTP; (lane 6) 600 nM AZTTP; (lane 7) 50 nM AZTTP; (lane 8) 30 nM AZTTP; (lane 9) 10 nM AZTTP; (lane 10) 400 μM NH_2TTP; (lane 11) 40 μM NH_2TTP; (lane 12) 4 μM NH_2TTP; (lane 13) 400 nM NH_2TTP; (lane 14) 50 nM NH_2TTP; (lane 15) 30 nM NH_2TTP; (lane 16) 10 nM NH_2TTP. (Panel b) Incorporation from the reactions shown in panel a.

Table II: Kinetic Constants for 3′-Azido-dTMP Incorporation by HIV Reverse Transcriptase[a]

kinetic parameter	value
K_m (μM)	2.9
k_{cat} (s^{-1})	0.27

[a] Template-primer was poly[r(A)]·oligo[d(T)] and product formed was primer + 1 only.

Kinetic constants for primer + 1 synthesis for each analogue were obtained by densitometer tracing of gels corresponding to incubations at appropriate analogue concentration. The results obtained from routine double-reciprocal plot analysis are summarized in Table II (data not shown). The steady-state rate of incorporation with AZTTP as substrate was about 4-fold higher than the rate of incorporation with NH_2TTP and corresponded to a k_{cat} of 0.27 nucleotide/s. This should be compared with the steady-state rate of incorporation of dTMP in the standard reaction of about 3 nucleotides/s (Majumdar et al., 1988). K_m values for AZTTP and NH_2TTP are 2.9 and 2.3 μM, respectively, and these values are a full 2 orders of magnitude greater than the values for K_i obtained from inhibition studies. This difference between K_i and K_m is consistent with the proposed kinetic scheme for the reverse transcriptase, as will be discussed below. One additional point of interest from this substrate kinetic study with each analogue is that strong inhibition was seen at higher concentrations of substrate. This is evident from inspection of the intensity of primer + 1 bands shown in Figure 3. For example, at 600 μM AZTTP, much less incorporation was observed than at 6 μM AZTTP. This secondary substrate inhibitory effect is not seen in studies with the normal substrate dTTP (Majumdar et al., 1988).

We find that HIV-1 reverse transcriptase purified from virions shows the same incorporation characteristics with the dTTP analogues as seen in Figure 3 (data not shown). We also find no significant increase in incorporation of the analogues in the presence of DTT (data not shown), suggesting again that during a typical polymerase assay conversion of AZTTP to NH_2TTP is not significant.

AZTTP Inhibition at Different dTMP Positions on an M13 Template. Under Theory, we outline how rate constants can be determined for enzyme binding to template-primer and dNTP. An assumption in calculating rate constants is that the AZTTP K_i determined for overall synthesis will also be appropriate for assuming binding of the first nucleotide incorporated. This assumption seems reasonable from inspection of Figure 3. If AZTTP inhibited first-nucleotide incorporation much differently from processive synthesis, one would expect a significant change in product at the primer + 1 position. In fact, in the presence of 50 mM AZTTP, product at primer + 1 is slightly greater than in the uninhibited reaction (Figure 3, calculations not shown). This seems consistent with increased termination and reinitiation and suggests that inhibition at the first nucleotide is not dramatically different from inhibition during processive synthesis.

To examine this issue more precisely, however, we conducted experiments with an M13mp2 DNA template-primer depicted in Figure 4. In the absence of dCTP, synthesis can proceed in this system for 12 nucleotides; dTMP is incorporated at the first position and at three subsequent positions. Incorporation was calculated by cutting gel lanes and determining counts at each dTMP position and longer products. Incorporation is plotted in this manner in Figure 4; values at position 1 represent incorporation at the first dTMP position and all longer products (this value also represents total incorporation), values at position 2 represent incorporation at the second dTMP position and all longer products, and so forth. Incorporation at each position in the absence of inhibitor is assigned a value of 1.0, and incorporation in the presence of inhibitor is expressed relative to this value. The graphs represent data with 1 and 2 μM dTTP, since inhibition was greater at these concentrations.

If AZTTP inhibition at the first incorporation were significantly different from inhibition at subsequent incorporations, one would expect to detect this from the graphs in Figure 4. What is seen instead is a gradual reduction in incorporation for products at each position and beyond. The data fit well with theoretical curves by assuming equal percentages of inhibition at each position. We conclude from these experiments that there is no significant difference in inhibition by AZTTP with nucleotide position and infer that such an assumption seems reasonable on the poly[r(A)] template.

Calculation of Rate Constants for First Complex Formation. The value of k_{off} for poly[r(A)]·oligo[d(T)]–enzyme complex formation is obtained by using results with 3′-azido-dTTP (Table II) by the relationship $k_{cat}K_m/K_D = k_{off}$, or $0.27\ s^{-1} \times 2.9\ \mu M/20\ nM = 39\ s^{-1}$. This value is then used to obtain k_{on}, in combination with the template-primer Michaelis constant obtained earlier (Majumdar et al., 1988). In this case, $K_m \cong K_D$ and is 150 nM with respect to the molecular concentration of $d(T)_{14}$. Therefore, K_D(primer) = k_{off}/k_{on} or $k_{on} = 39\ s^{-1}/150\ nM = 2.6 \times 10^8\ M^{-1}\ s^{-1}$. This k_{on} value is similar to the k_{on} value ($5.4 \times 10^8\ M^{-1}\ s^{-1}$) obtained for enzyme–$Sd(C)_{28}$–poly[r(I)] complex formation and is similar to the expected value for a diffusion-limited process involving molecules of this size (Majumdar et al., 1989).

Discussion

Steps in the mechanism of DNA synthesis by the HIV reverse transcriptase have been deduced recently from

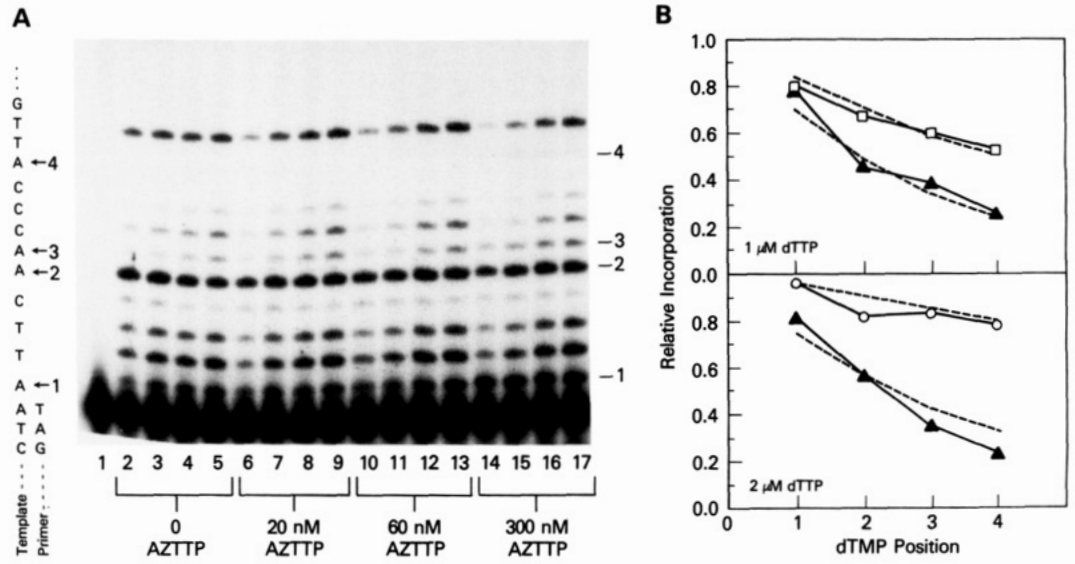
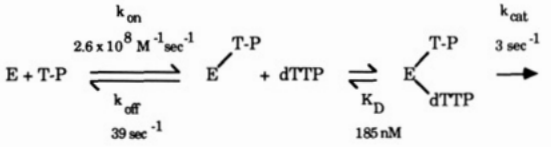


FIGURE 4: Incorporation of dTMP at four positions. (Panel A) In vitro DNA synthesis reactions were carried out on a M13mp2 template and displayed by gel electrophoresis as described under Experimental Procedures. In the absence of dCTP, synthesis can proceed on this template for 12 nucleotides. The sequence of the template strand is indicated to the left of the gel, with positions at which dTMP is inserted indicated by arrows. Concentrations of AZTTP are indicated below the gel lanes. Concentrations of dTTP were as follows: (lane 1) no dTTP; (lane 2) 1 μM; (lane 3) 2 μM; (lane 4) 4 μM; (lane 5) 8 μM; (lane 6) 1 μM; (lane 7) 2 μM; (lane 8) 4 μM; (lane 9) 8 μM; (lane 10) 1 μM; (lane 11) 2 μM; (lane 12) 4 μM; (lane 13) 8 μM; (lane 14) 1 μM; (lane 15) 2 μM; (lane 16) 4 μM; (lane 17) 8 μM. (Panel B) Incorporation was determined by cutting regions of the gel lanes in panel A, covering with scintillation fluid, and measuring counts. Incorporation was calculated for each dTMP position and longer products. That is, values at position 1 represent all incorporation at the first dTMP position and longer products (this also represents total incorporation), values at position 2 represent incorporation at the second dTMP position and all longer products, and so forth. Incorporation data in the absence of inhibitor were assigned a value of 1.0 at each position, and incorporation in the presence of inhibitors is expressed relative to uninhibited incorporation. For the upper graph, dTTP concentration in the reaction mixture was 1 μM; for the lower graph, dTTP was 2 μM. Inhibitor concentrations were as follows: 20 nM AZTTP (circles); 60 nM AZTTP (squares); 300 nM AZTTP (triangles). Theoretical curves (dotted lines) are plotted by assuming inhibition at each dTMP position of 16% (upper graph, upper line), 30% (upper graph, lower line), 5% (lower graph, upper line), and 25% (lower graph, lower line). In reactions with 20, 60, and 300 nM AZTTP alone, incorporation represented 8.5%, 12%, and 29%, respectively, of total incorporation with 1 μM dTTP and no inhibitor and represented smaller percentages of incorporation with higher dTTP concentrations (data not shown). We therefore assume that the contribution of AZTTP to incorporation in reactions with dTTP is small.

steady-state kinetic and processivity measurements (Majumdar et al., 1988; Huber et al., 1989). With this information as background, Majumdar et al. (1989) obtained individual rate constants for the first step in the reaction scheme, template-primer addition to free enzyme, in a model replication system with $Sd(C)_{28}$ as primer. This was accomplished by identification of a linear competitive inhibitor of the usual primer, which also can act as a substrate for DNA synthesis. A simple mathematical treatment of the measured K_m and K_i values then leads to calculation of rate values for enzyme/primer binding (k_{on}) and enzyme/primer dissociation (k_{off}).

One of our aims in the present study was to extend this approach by using dNTP addition to the enzyme. The theoretical basis for the approach is that the dNTP addition step in the ordered sequential pathway of synthesis depends upon prior formation of the enzyme/template-primer complex, and therefore, the amount of enzyme/template-primer complex formed can be deduced from data on the dNTP requirement for synthesis.

We first identified a linear competitive inhibitor of dNTP that can also act as a substrate for DNA synthesis. This compound, 3′-azido-dTTP is incorporated into DNA in the poly[r(A)]·oligo[d(T)] replication system with a k_{cat} about $^1/_{10}$ or less that of the normal substate. The K_m for synthesis is about 3 μM, whereas the K_D for binding to the enzyme is 20 nM as deduced from inhibition kinetics. These measured values of k_{cat}, K_m, and K_D then enable the calculation of enzyme/template-primer k_{off} as 39 s^{-1}. Since the same value also applies for the usual dTMP incorporation reaction, it can be used in combination with earlier results to obtain several constants for the usual reaction. It is important to note that k_{off} is ~10-fold higher than k_{cat} in the usual dTMP incorporation reaction; this is the case for a reaction following classical Michaelis–Menten kinetics, and, thus, the K_m for addition of this first substrate (template-primer) is $\cong K_D$ for enzyme/substrate binding. This K_m value is 150 nM molecular concentration of primer, corresponding to k_{on} of 2.6×10^8 M^{-1} s^{-1} (k_{off}/K_D(primer) = 39 s^{-1}/150 nM = k_{on}). K_D(dTTP) can then be calculated from the expression derived under Theory: $K_D(dTTP) = k_{cat}K_m dTTP/k_{off} = 3\ s^{-1} \times 2.4\ \mu M/39\ s^{-1} = 185$ nM. Values for steps in the reaction pathway can be assigned as follows:



The availability of k_{off} and k_{on} values raises interesting points for future study. For example, the k_{off} for enzyme/template-primer dissociation for the first step in the pathway of processive DNA synthesis is much greater than k_{off} during processive synthesis itself. Majumdar et al. (1988) found that the rate of processive elongation is approximately the same as the overall steady-state rate (3 s^{-1}) and that the value of k_{off} after each dNMP addition during processive synthesis is in the range of $^1/_{200}$ the rate of elongation (i.e., 0.01–0.02 s^{-1}). Therefore, k_{off} for processive synthesis is about 1000-fold lower than k_{off} during initial binding of enzyme to template-primer (39 s^{-1}). The mechanism of this change in k_{off} as synthesis proceeds must involve additional or different enzyme–nucleic acid contacts, as yet unknown, from those involved in the initial

enzyme/template-primer binding event. The k_{on} value for the enzyme/template-primer binding event is similar to the theoretical diffusion-controlled rate of collision for macromolecules in this size range, i.e., $\sim 10^9$ M^{-1} s^{-1}. The explanation of why k_{on} is close to the diffusion-controlled rate may involve so-called facilitated alignment of the substrate into the putative binding groove once collision between enzyme and substrate has occurred by random diffusion. Mechanisms of such facilitated alignment for nucleic acid binding proteins include sliding and electrostatic interactions (Record, 1988; von Hippel & Berg, 1989; Wu & Singer, 1990), but from the standpoint of amino acid residues at the surface of the protein, mechanisms are largely unknown. A frame of reference may be provided by studies of Ca^{2+} interaction with calbindin that revealed a funnel-like surface of amino acid residues providing charge–charge interactions serving to align Ca^{2+} in its binding site (Ahlström et al., 1989).

The kinetic data (Table I) indicate that AZTTP is a competitive inhibitor against the natural substrate dTTP. We find, however, that the K_i for inhibition (20 nM) is 2 orders of magnitude less than the K_m for incorporation (~ 3 μM) when AZTTP is studied as a substrate for primer + 1 formation. This may indicate that enzymatic/template/AZTTP binding is a more important factor in interfering with dTTP incorporation than actual AZTTP incorporation. Increasing concentrations of AZTTP reduce the processivity of HIV RT synthesis on a poly[r(A)] template (Figure 2), but we cannot determine from the experiment in Figure 2 whether this reduced processivity is caused by chain termination due to AZTMP incorporation or by greater dissociation of enzyme from nascent chains without AZTMP incorporation or whether both effects are important. Competitive blocking of dTTP addition to the enzyme could lead to more termination through the reduction in effective dTTP concentration.

Kinetic data (Table I) indicate that NH_2TTP is a noncompetitive inhibitor against substrate dNTP, and the pattern of product distribution (Figure 2) shows that this compound acts in a manner clearly different from AZTTP. We note that Cheng et al. (1987) have reported that NH_2TTP inhibits HIV-1 RT, but did not identify the manner of inhibition. The classical interpretation of the mechanism of a noncompetitive inhibitor is that it impedes the steady state by interacting with an enzyme form other than the form involved in dNTP binding. This could occur, for example, if NH_2TTP binds at a site different from the usual dNTP site [see, for example, Segel (1975)]. Such a mechanism cannot be ruled out with NH_2TTP, but this textbook model seems unusual for a nucleotide analogue inhibitor with low K_i, since the low K_i value may reflect hydrogen bonding with template. Alternative possibilities include models where NH_2TTP occupies the dNTP binding site but exerts its effect on enzyme forms occurring before or after usual dNTP incorporation. Thus, if NH_2TTP binding impedes an early step after binding of enzyme to template-primer or impedes dissociation of enzyme from template-primer following elongation, the results obtained here would be expected. Further experiments are in progress to test these possibilities.

The shift from competitive to noncompetitive inhibitor upon transformation of the azido group of AZTTP to an amino moiety in NH_2TTP may be related to the marked physicochemical differences between the two functional groups. The linear azido moiety is considered to be a resonance hybrid of two extreme canonical forms that result in an asymmetrical charge distribution in which the central nitrogen bears a partial positive charge and the terminal nitrogens assume a partial negative charge (Treinin, 1971). Even so, the azide group is relatively lipophilic. In fact, it is these stereoelectronic and hydrophobic properties that have been hypothesized to play a key role in the interaction of HIV RT with AZTTP (Birnbaum et al., 1987; Camerman et al., 1987; Herzyk et al., 1987; Parthasarathy & Kim, 1988). On the one hand, the amino moiety may exist at pH 8 as a predominantly positively charged protonated species with a tetrahedral structure. On the other hand, the amino group may be considered pyramidal in structure with dramatically enhanced basicity, hydrophilicity, and hydrogen-bond donor/acceptor capabilities compared to an azido function. Remarkably, the unprotonated amino group of NH_2TTP might be considered to be a closer mimic of the hydroxyl moiety of the natural substrate dTTP than is the azide group of AZTTP; nonetheless, it is the amino analogue that interacts in a fundamentally different way with the enzyme. The questions raised by these anomalies are under investigation.

In the absence of data on the metabolic fate of AZT, including conversion to NH_2T, the therapeutic implications of our findings are unclear. Larder et al. (1989) have recently reported HIV clinical isolates resistant to AZT but still sensitive to other reverse transcriptase inhibitors, including presumed chain terminators. Although in vitro mutagenesis studies have produced reverse transcriptases that were more resistant to AZTTP than wild type (Larder et al., 1987), purified RT from these AZT-resistant isolates showed no significant difference in AZTTP sensitivity (Larder et al., 1989; Larder & Kemp, 1989). Such results would be consistent with a situation where an AZT metabolite, acting by a different mechanism from AZTTP, was important for the inhibition of viral replication in cells.

These clinical results, and our findings here, provide at least a suggestion that combined therapy with agents that act upon different forms of the HIV reverse transcriptase occurring during its reaction pathway may be useful, and AZTTP and NH_2TTP may be considered prototypes for such an approach. Unfortunately, NH_2TTP may not seem suitable as a practical therapeutic candidate to test such a strategy since the nucleoside NH_2T does not inhibit HIV replication at subcytotoxic concentrations in MT-4 cells, although it is inhibitory to the replication of Moloney murine sarcoma virus in C3H/3T3 cells (J. Balzarini, E. De Clercq, T. Kovács, and P. Torrence, unpublished observations). The nucleoside NH_2T has been reported by Lin and Prusoff (1978) and Fischer et al. (1982) to be strongly cytotoxic to L1210 cells. Chen et al. (1984) ascribed this cytotoxicity to an inhibition of DNA polymerase α by the corresponding 5′-triphosphate. Thus, it may be possible that while the transformation of the azido moiety of AZT to the amino functionality of NH_2T facilitates interaction with a different form of the HIV enzyme, it may also amplify the interplay of the nucleoside with host cell DNA polymerase α, resulting in substantially enhanced toxicity.

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