Selective Action of 4′-Azidothymidine Triphosphate on Reverse Transcriptase of Human Immunodeficiency Virus Type 1 and Human DNA Polymerases α and β†

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ABSTRACT: 4′-Azidothymidine (ADRT) is a novel nucleoside analogue that exhibits potent inhibitory activity against the replication of human immunodeficiency virus (HIV) in lymphocytes. The mechanisms by which ADRT inhibits HIV reverse transcriptase (HIV-RT) as ADRT 5′-triphosphate (ADRT-TP), the active intracellular metabolite of ADRT, and as the ADRT-MP molecule incorporated into DNA were examined and compared to their effects on human DNA polymerases α and β. Inhibition of HIV-RT by ADRT-TP is competitive against TTP and is more potent against RNA to DNA synthesis (K_i = 0.009 μM versus K_m = 3.3 μM for TTP) than it is against DNA to DNA synthesis (K_i = 0.95 μM versus K_m = 16.3 μM for TTP). ADRT-TP is also a more potent inhibitor for primer elongation on RNA template than on DNA template. ADRT-TP is a poor inhibitor of human DNA polymerases α (K_i = 62.5 μM) and β (K_i = 150 μM) (Chen et al., 1992). The consequences of ADRT incorporation into DNA are strikingly different for the HIV-RT and for human DNA polymerases α and β. DNA polymerases α and β incorporate a single ADRT-MP molecule into nascent DNA at a very slow rate and continue to elongate. They are unable to incorporate a second consecutive ADRT-MP. However, HIV-RT is able to efficiently incorporate two consecutive ADRT molecules. Incorporation of two consecutive ADRT-MP molecules by HIV-RT prevents further DNA chain elongation. Incorporation of two ADRT-MP molecules separated by one deoxyribonucleoside monophosphate (dAMP, dCMP, or dGMP) also abolishes DNA chain elongation by HIV-RT.

HIV-1[1] reverse transcriptase (EC 2.7.7.49) has been widely targeted for chemotherapy to combat AIDS and advanced AIDS-related complex (ARC). In addition to the three clinically approved drugs, zidovudine (AZT), ddI, and ddC (Mitsuya et al., 1985, 1986; Balzarini et al., 1986), many 2′,3′-dideoxynucleoside analogues (ddNs) [2′,3′-didehydro-2′,3′-dideoxycytidine, 2′,3′-didehydro-2′,3′-dideoxythymidine, 3′-azido-2′,3′-dideoxyuridine, and carbovir] (Lin et al., 1987; Schinazi et al., 1987; Vince et al., 1990) have demonstrated potent inhibitory activity against the replication of HIV-1 *in vitro*. The anti-HIV nucleoside analogues must be phosphorylated by cellular kinases to their respective 5′-triphosphate derivatives (ddNTPs) in order to inhibit DNA replication catalyzed by HIV-1 reverse transcriptase. The ddNTPs interfere with DNA replication of HIV by inhibiting HIV-1 reverse transcriptase via two mechanisms. These ddNTPs competitively inhibit against normal substrates for DNA polymerization and serve as DNA chain terminators after incorporation into the nascent 3′-terminus by HIV-RT (St. Clair et al., 1987; Furman et al., 1986; Parker et al., 1991). Chain termination is believed to be the primary mechanism by which AZT and the other ddNs exert their anti-HIV activities. However, despite the inhibitory effect of these agents on HIV DNA synthesis, prolonged treatment with AZT has been accompanied by the rising incidence of AZT-resistant strains of HIV-1 in immuno-compromised patients (Larder et al., 1989; St. Clair et al., 1991; Larder & Kemp, 1989). Furthermore, albeit with lower efficiency, the ddNTPs could also be utilized by human DNA polymerases and in prolonged treatment could exert adverse effects on the human genome (Copeland et al., 1992). Thus, there is a need to explore and develop new antiviral agents that can discriminate between HIV-RT and host DNA polymerases.

4′-Azidothymidine (4-azido-2-deoxyribose-1-thymine, ADRT) has recently demonstrated potent and selective inhibition of the replication of HIV-1 (Maag et al., 1992). ADRT is also active against clinical isolates of HIV-1 that are resistant to AZT (Maag et al., 1992). Unlike AZT and ddNs, ADRT retains the 3′-hydroxy group and has been shown not to be a DNA chain terminator in human T-cells (Chen et al., 1992). In addition, ADRT and its phosphorylated metabolites do not inhibit the enzymes involved in the *de novo* synthesis of TTP, and they appear to be poor inhibitors of human DNA polymerases α and β (Chen et al., 1992). ADRT is not a classical DNA chain terminator and contradicts the accepted dogma that the absence of a 3′-hydroxy group is required for an effective nucleoside analogue against HIV. The selective activity of ADRT against wild-type and AZT-resistant strains of HIV-1 suggests that it has an unusual mechanism of action and makes it a valuable probe to study

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[1] Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; ddNs, 2′,3′-dideoxynucleoside analogues; carbovir, the carbocyclic analogue of 2′,3′-didehydro-2′,3′-dideoxyguanosine; ddNTPs, triphosphate derivatives of ddNs; AZT, 3′-azidothymidine; AZT-TP, AZT triphosphate; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; ADRT, 4-azido-2-deoxyribose-1-thymine or 4′-azidothymidine; ADRT-MP, ADRT monophosphate; ADRT-TP, ADRT triphosphate; RT, reverse transcriptase.

[2] H. Maag, E. J. Prisbe, and J. P. Verheyden (Syntex Research), unpublished results.

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the biochemical properties of DNA polymerases. Here we describe the mechanism of the *in vitro* inhibitory effect of 4'-azidothymidine triphosphate (ADRT-TP) on both the RNA to DNA and DNA to DNA replication catalyzed by HIV-1 reverse transcriptase, and we compare its inhibitory effect on the two major human DNA polymerases, α and β.

EXPERIMENTAL PROCEDURES

Chemicals. [γ-^{35}S]ATP (1256 Ci/mmol) and [^{3}H]TTP (4.1 Ci/mmol) were obtained from NEN-Du Pont. Poly(A) and poly(dA) were from Sigma. Oligo(dT)$_{12-18}$, dATP, dGTP, and TTP were from Pharmacia. Acrylamide/BIS 29:1 (3.3%), acrylamide/BIS 19:1 (5%), TEMED, and ammonium persulfate were from Bio-Rad. The primer and DNA templates used in the primer elongation studies were prepared by the solid-phase β-cyanoethyl *N,N*-diisopropylphosphoramidite method in an automated DNA synthesizer (Milligen/Biosearch 8700) and were purified by electrophoresis in 7 M urea/20% polyacrylamide gel (Sinha et al., 1984). ADRT triphosphate was synthesized by established methods (Hoard & Ott, 1965; Imai et al., 1969). All other chemicals were of the highest grades commercially available.

Synthesis of RNA Templates for Primer Elongation Study. Three RNA templates which contained the same coding sequences as the templates (36-mer, templates containing a single A, two consecutive A's, and three consecutive A's) used in the DNA to DNA synthesis were synthesized as follows: 50 pmol of each of the three DNA templates was mixed with 50 pmol of the primer (12-mer, the primer used in all of the primer elongation studies). These mixtures were lyophilized and resuspended in 7 μL of H_2O and 1 μL of 10× restriction buffer A (Boehringer Mannheim; 1× = 33 mM Tris–acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, and 0.5 mM DTT). After a brief heating to 65 °C, samples were allowed to anneal for 10 min at room temperature before the addition of 1 μL of 5 mM dNTPs and 1 μL of Klenow (Boehringer, 5 units/μL) and incubation for an additional 30 min at room temperature. Meanwhile, pGEM3Z was digested with an excess of *Sma*I. Half of the extension/fill reaction was added to 200 ng of the *Sma*I cut vector in a total volume of 20 μL of 1× ligase buffer with 1 unit of T4 DNA ligase (BRL). Ligation was carried out for 4 h at room temperature. The ligase was inactivated by heating to 70 °C for 10 min. The volume was increased to 50 μL with 30 μL of 1× restriction buffer A containing 20 units of *Sma*I. After 1 h at 25 °C (to digest non-recombinants), the reactions were phenol/chloroform extracted, ethanol precipitated, dried, and resuspended in 10 μL of TE (10 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA), and 4 μL was used to transform competent XL1-Blue MCRF′ *Escherichia coli* (Stratagene). Ampicillin-resistant colonies were selected and grown up (5 mL total). Mini-prep DNAs were analyzed for the presence of a single insert by digestion with *Pvu*II. One of each set was selected, and the remaining mini-prep DNA was purified by ultracentrifugation in CsCl in the TLA100 table-top ultracentrifuge (Beckman). DNA was recovered from the gradients, extracted with ethidium bromide, and following two rounds of ethanol precipitation, a portion of each was sequenced using the M13 universal (–40) primer and the sequenase 2.0 kit (USB). This served to confirm the sequence of the inserts and to determine the orientation. A further portion of each CsCl preparation was digested with *Eco*RI and the linear form was gel isolated. Approximately 2–4 μg of each template was incubated with SP6 RNA polymerase (Pharmacia) in a scaled-up transcription reaction (100 μL each of 1 mM ATP, CTP, GTP, and UTP). After 90 min at 37 °C, 2 μL of RQ1 DNAase (Promega) was added and incubation continued for an additional 15 min at 37 °C. RNA was recovered by phenol/chloroform extraction and two rounds of ethanol precipitation and then quantitated by A_{260}.

Cell Cultures and HIV Virions. The A3.01 cell line is a human T-cell HAT-sensitive derivative of CEM (obtained from Dr. Thomas Folks of NIAID, now at the Centers for Disease Control, Atlanta, GA). A3.01 cells in log phase (2 × 10^5/mL in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 μg of gentamicin/mL) were pelleted by low-speed centrifugation (3000*g*, 15 min). The cells were suspended in fresh medium to a density of 2 × 10^6/mL, and supernatant from an 8-day HIV-1 (LAV strain) infected culture was added (1:4 infected/uninfected (by volume)). The cells were incubated at 37 °C with agitation for 3 h, diluted with an equal volume of medium, and cultured at 37 °C. The cell density was monitored daily and maintained at 10^6/mL by the addition of fresh medium. Viral replication was monitored daily by assays of reverse transcriptase activity in the supernatant. When peak reverse transcriptase activity was achieved (ca. day 8 post infection), the medium was cleared of cells by centrifugation (3000*g*, 15 min), followed by filtration through a 0.45-μm filter. The virus in the medium was pelleted by centrifugation at 110000*g* (30 000 rpm, 60 min, Beckman SW 41 rotor). These virus pellets were used to purify HIV virions and HIV reverse transcriptase.

Purification of HIV-1 Virions. Virus pellets were resuspended in 0.1 M NaCl and 0.01 M Tris-HCl (pH 7.4) and applied to a discontinuous sucrose gradient composed of 2 mL of 10%, 2 mL of 20%, 5 mL of 35%, and 1 mL of 60% sucrose in 0.1 M NaCl and 0.01 M Tris-HCl (pH 7.4). The gradients were centrifuged at 33 000 rpm for 80 min in a Beckman SW 41 rotor. The visible band which contained virions was collected from the gradient, and the virions were lysed by the addition of 0.5% NP-40. The viral lysates were stored at –70 °C until use.

Purification of HIV-1 Reverse Transcriptase. The virus pellets obtained as described in the above section were lysed with buffer A (1% Triton X-100, 10% glycerol, 10 mM Tris-HCl (pH 8.0), 1 mg/mL BSA, and 5 mM DTT) and used for the purification of HIV-1 reverse transcriptase. A virus pellet from 250 mL of medium of HIV culture was lysed with 2.5 mL of buffer A and diluted with buffer B (5 mL of 20 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, and 20% glycerol). This enzyme solution was applied to a DE-52 column (1 × 9 cm) equilibrated with buffer B. The enzyme was eluted from the column with a linear gradient of 0–0.5 M NaCl (total volume = 80 mL) in buffer B. The fractions were collected into tubes containing BSA (1 mg/mL final concentration). The fractions containing enzyme activities were pooled and dialyzed against buffer C (20 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, and 20% glycerol). The enzyme solution was then applied to a Heparin-5PW column (TSK-GEL, 5 cm × 5 mm i.d.) and eluted with a linear gradient (FPLC) of 0–0.5 M NaCl (total volume = 60 mL). The fractions were collected into tubes containing BSA (1 mg/mL final concentration) to stabilize the purified reverse transcriptase. The fractions containing enzyme activities were pooled, concentrated, and stored at –70 °C until use.

Human DNA Polymerases α and β. The four-subunit human DNA polymerase α/primase complex was immunopurified as described (Wang et al., 1984; Wong et al., 1986). The single-subunit human DNA polymerase α produced by recombinant baculovirus was purified as described (Copeland & Wang, 1991). The recombinant human DNA polymerase

β produced by *E. coli* was a generous gift from Dr. S. H. Wilson of the University of Texas at Galveston (Abbotts et al., 1988; Kumar et al., 1990).

Enzyme Assays. HIV-1 reverse transcriptase activity measured the incorporation of tritiated thymidine triphosphate into an acid-precipitable form as described previously (Chen et al., 1984; Chen & Oshana, 1987). Kinetic analyses were as described previously (Chen et al., 1979; Chen & Prusoff, 1977). Human DNA polymerases α and β were also assayed as previously described (Wang et al., 1977, 1984; Copeland & Wang, 1991).

In Vitro DNA Synthesis with 5'-Labeled Primer. Synthetic 12-mer (5'-TGA-CCA-TGT-AAC-3') was phosphorylated with [γ-^{35}S]ATP using T4 polynucleotide kinase (New England Biolabs). The end-labeled primer was purified by passage through a quick-spin column (G-25 Sephadex, Boehringer Mannheim Biochemicals, Inc.). [^{35}S]-labeled primer was hybridized to different templates at a 1:3 molar ratio of primer to template at room temperature. The HIV-1 reverse transcriptase reaction mixture (12.5 μL) contained 20 mM Tris-HCl (pH 8.0), 6.4 mM $MgCl_2$, 0.4 mM DTT, 40 mM KCl, 7.3 pmol of [^{35}S]-labeled primer, 21.9 pmol of template, 7.2 μM dATP, 7.2 μM dGTP, and 7.7 μM TTP or 7.3 μM ADRT-TP, and 5.5×10^{-3} unit of HIV-1 reverse transcriptase (1 unit is the amount of enzyme that incorporates 1 nmol of TMP into oligo(dT)/min at 37 °C). The human DNA polymerase α reaction mixture (11.5 μL) contained 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.4 mg/mL BSA, 2% glycerol, 7.3 pmol of [^{35}S]-labeled primer, 21.9 pmol of template, 3.1 μM dATP, 3.1 μM dGTP, 3.5 μM TTP or 15.1 μM ADRT-TP, and 3.15 units of human DNA polymerase α (1 unit is the amount of enzyme that incorporates 1 nmol of TMP into acid-insoluble product/h at 37 °C). The human DNA polymerase β reaction mixture (10.6 μL) contained 40 mM Tris-HCl (pH 8.9), 8 mM $MgCl_2$, 0.06 mM β-mercaptoethanol, 79 mM KCl, 0.6 mg/mL BSA, 4.5% glycerol, 7.3 pmol of [^{35}S]-labeled primer, 21.9 pmol of template, 3.4 μM dATP, 3.4 μM dGTP, 3.8 μM TTP or 16.4 μM ADRT-TP, and 2.1 units of human DNA polymerase β (same unit definition as DNA polymerase α). Reaction mixtures were incubated at 37 °C, and samples were removed at various times and put into an equal volume of stop solution (United States Biochemicals). The products of DNA synthesis were analyzed by gel electrophoresis. Electrophoresis was conducted on a 15% polyacrylamide/8 M urea gel. The gel was prerun for 1 h at 55 W without cooling, and electrophoresis was performed at 55 W. After electrophoresis, the gel was dried and the products were visualized by autoradiography with Kodak XAR-2 film at −20 °C.

RESULTS

Purification of HIV-1 Reverse Transcriptase. A rapid purification protocol was developed to isolate reverse transcriptase from HIV-1 virions. To avoid contaminating DNase activities from cell lysates and to remove intact cells and cell debris from HIV-1 virions, infected cell culture media were first filtered through a 0.45-μm filter prior to the ultracentrifugation step to obtain a virus pellet. The chromatographic profiles of HIV-RT on DE-52 and Heparin-5PW columns are shown in Figure 1A,B, respectively. The purified HIV-RT is free of DNase and gives linear initial rates in kinetic analyses.

Inhibition of Reverse Transcriptase by ADRT-TP. The kinetics of inhibition of ADRT-TP against the purified HIV-1 reverse transcriptase was determined as described previously (Chen & Oshana, 1987; Wu et al., 1988). The inhibition constants of ADRT-TP toward HIV-1 reverse transcriptase are presented in Table I.

Effects of ADRT-TP on Primer Elongation. Specific templates were designed to investigate the effects of ADRT-TP on the rate of DNA chain elongation by HIV-RT and human DNA polymerases. Most of the templates were designed to contain no GMP or dGMP to avoid the obligatory incorporation of dCMP, since dCTP may contain minute contaminating amounts of dUTP or undergo spontaneous deamination during incubation to generate dUTP, which can substitute efficiently for TTP.

Primer Elongation on RNA Templates Catalyzed by HIV-RT. Figure 2 shows the effects of single and consecutive ADRT-MP incorporation into the primer on the elongation rate of DNA catalyzed by HIV-RT. Samples 3, 6, and 9, control reactions in which TTP and ADRT-TP are omitted, show a major pause at the 18-mer prior to the incorporation of TMP or ADRT-MP. Accumulation of 40-mer polynucleotide and polynucleotides larger than the 40-mer are also observed in sample 3 (one AMP at position 19 on the template), indicating mis-incorporation of nucleotides by HIV-RT. The RNA templates used in samples 6 and 9 contain two and three consecutive AMPs, respectively. Sample 6 shows a major accumulation of 18-mer and a minor accumulation of 19-mer. A minute amount of 40-mer and polynucleotides larger than the 40-mer is also detected in sample 6, indicating that HIV-RT can elongate a primer end containing two consecutive mis-incorporated nucleotides. Sample 9 shows a minute accumulation of 19-mer in addition to the major accumulation of 18-mer. Sample 9 does not generate polynucleotides larger than the 19-mer. For all three templates, TTP incorporation in the absence of inhibitor is very efficient (samples 4, 7, and 10), since mainly the accumulation of 40-mer and polynucleotides larger than the 40-mer are detected. Incorporation of a single ADRT-MP molecule into the DNA (sample 2) has a profound effect on the rate of further DNA chain elongation; accumulation of 19-, 20-, 21-, and 22-mers is observed. A small amount of 40-mer is also observed in sample 2. The incorporation of two consecutive ADRT-MP molecules results in the accumulation of 19-mer and 20-mer (sample 5). Sample 8 can potentially incorporate three consecutive ADRT-MP molecules, and accumulation of 19-, 20-, and 21-mers is observed.

Primer Elongation on DNA Templates Catalyzed by HIV-RT. The effects of ADRT-MP incorporation upon subsequent chain elongation by HIV-1 reverse transcriptase using DNA templates were analyzed (Figures 3 and 4). Figure 3 shows the effects of single and consecutive ADRT-MP incorporation into the DNA primer on the rate of DNA elongation catalyzed by HIV-RT. Samples 3, 6, and 9 do not contain either TTP or ADRT-TP, and the elongation of the primers stopped at the 18-mer prior to the incorporation of either TTP or ADRT-TP. Incorporation of one ADRT-MP molecule into the DNA (sample 2) has a nominal effect on the rate of further DNA chain elongation; 36-mer full-length products are formed under these conditions, with a modest observable pause at the 21-mer (compare sample 2 to sample 4). The incorporation of two consecutive ADRT-MP molecules resulted in the accumulation of a 20-mer (two consecutive ADRT-MP molecules incorporated at the 3'-end, sample 5). After prolonged incubation (lane b of sample 5), a small amount of a 21-mer can also be detected. Sample 8 potentially can incorporate three consecutive ADRT-MP molecules; however, the pattern

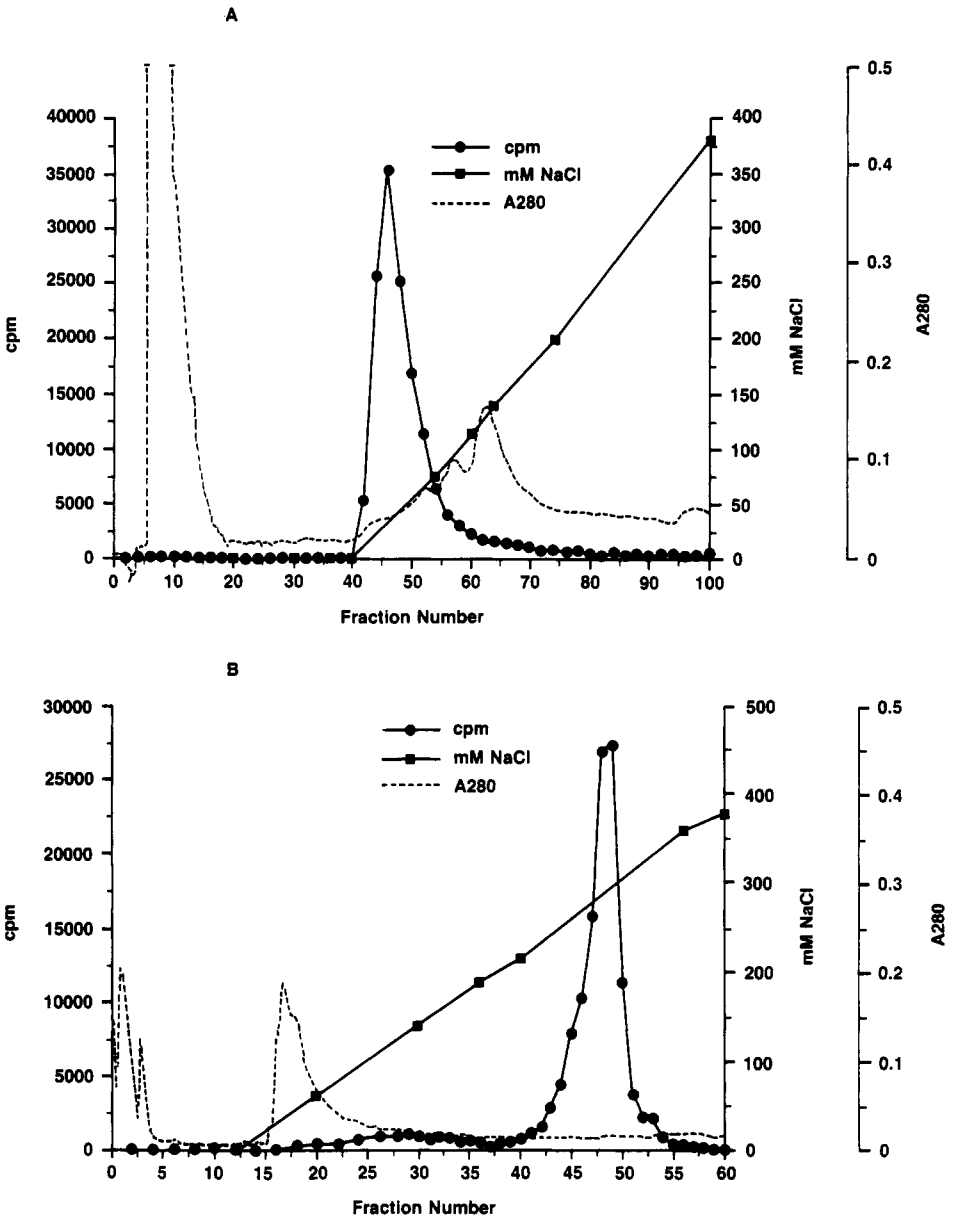

FIGURE 1: Purification of HIV-1 reverse transcriptase from virions. The experimental details are described in the Experimental Procedures. (A) Chromatogram of viral lysate on a DE-52 column (1 × 9 cm). (B) FPLC chromatogram of reverse transcriptase purified from a DE-52 column on a Heparin-5PW column (TSK-GEL, 5 cm × 5 mm i.d.). The dashed line (---) represents A_{280}; ■, concentration of NaCl in millimolar; ●, reverse transcriptase activity in counts per minute of [^{3}H]TMP incorporated in the acid-precipitable residues.

Table I: Inhibition of HIV-1 Reverse Transcriptase by ADRT-TP[a]

template/primer	K_m TTP (μM)	K_i ADRT-TP (μM)	K_i/K_m	source of HIV-RT
poly(rA)/(dT)$_{12-18}$	3.3	0.009	0.0027	lysate of purified virions
poly(rA)/(dT)$_{12-18}$	7.0	0.013	0.0019	purified enzyme
poly(dA)/(dT)$_{12-18}$	16.3	0.95	0.0583	purified enzyme

[a] Linear initial rates were obtained in the presence and absence of ADRT-TP. Inhibition of HIV-1 reverse transcriptase by ADRT-TP was competitive against TTP.

of primer elongation is similar to that of sample 5. For all three templates, the rates of TTP incorporation in the absence of inhibitor are very rapid (samples 4, 7, and 10); full-length DNAs (36-mer) are formed within 50 min (lane a).

The effects of two incorporated ADRT-MP molecules separated by one molecule of either dAMP, dGMP, or dCMP on further DNA chain elongation were analyzed (Figure 4). The elongation of the primers to full-length 36-mer in the presence of TTP was detected after 50 min (lanes a of samples 3, 6, and 9), with no accumulation of intermediate-length polynucleotides. However, the incorporation of two ADRT-MP into DNA separated by one molecule of either dAMP (sample 1), dGMP (sample 4), or dCMP (sample 7) caused the DNA elongation to pause after the incorporation of a second molecule of ADRT-MP (21-mer). The template containing the AGA motif (sample 7) also shows a slight accumulation of the 19-mer (product of the first ADRT-MP molecule), but accumulation of 19-mer is not observed with templates containing the ATA and ACA motifs (samples 1 and 4, respectively). Sample 7 also shows a low amount of read-through products (36-mer) longer than the 21-mer.

Primer Elongation Catalyzed by DNA Polymerases α and β. To elucidate the mechanisms of inhibition of human host DNA polymerases by ADRT-TP, primer templates for the incorporation of one and two consecutive ADRT-MP molecules were utilized by DNA polymerases α and β (Figure 5). The effects of ADRT-TP on DNA synthesis catalyzed by DNA polymerase α are shown in Figure 5 (samples 2–7). Samples

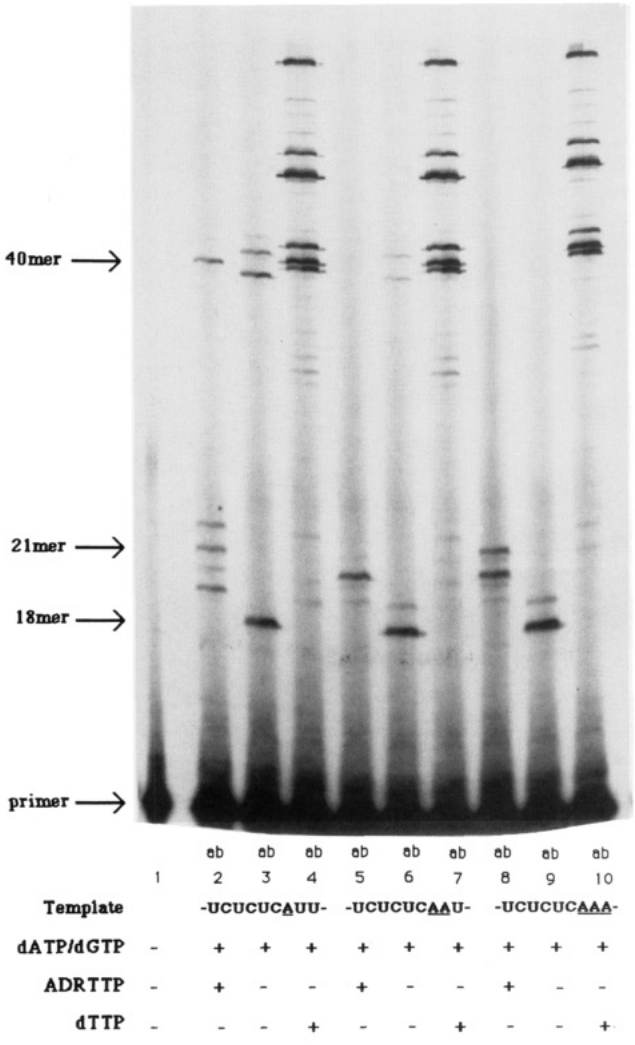

FIGURE 2: Effects of ADRT-TP on DNA strand elongation catalyzed by HIV-1 reverse transcriptase with RNA templates containing a single A, two consecutive A's, and three consecutive A's in the coding sequences. The RNA templates were synthesized as described in the Experimental Procedures. A 12-base oligodeoxyribonucleotide (3′-CAA-TGT-ACC-AGT-5′) was labeled with [^{35}S]phosphate at the 5′-end and annealed to RNA templates containing sequence A (3′-ACU-GGU-ACA-UUG-UCU-CUC-AUU-CUC-UCU-CUC-UUC-UCU-CCC-CUA-GGA-GAU-5′, samples 2–4), sequence B (3′-ACU-GGU-ACA-UUG-UCU-CUC-AAU-CUC-UCU-CUC-UUC-UCU-CCC-CUA-GGA-GAU-5′, samples 5–7), or sequence C (3′-ACU-GGU-ACA-UUG-UCU-CUC-AAA-CUC-UCU-CUC-UUC-UCU-CCC-CUA-GGA-GAU-5′, samples 8–10). The experimental details are described in the Experimental Procedures. All samples contained dATP and dGTP. Samples 2, 5, and 8 also contained ADRT-TP, while samples 4, 7, and 10 contained TTP. Two time points were taken from each sample after the addition of enzyme; lane a and lane b were taken at 30 and 60 min, respectively. Sample 1 contained primer alone.

4 and 7 show the elongation of primers in the presence of TTP to full-length DNA (36-mer) in 50 min. Samples 2–4 show chain elongation catalyzed by human DNA polymerase α with a template containing one dAMP in the coding sequence, allowing for the incorporation of one ADRT-MP molecule. Sample 2 contains ADRT-TP instead of TTP in the reaction mixture, yielding 18-mer, 19-mer, and an extremely small amount of 36-mer. The presence of ADRT-TP in the reaction caused the DNA elongation to pause at the site prior to ADRT-MP incorporation (sample 2, 18-mer) and at the site of ADRT-MP incorporation (sample 2, 19-mer). However, human DNA polymerase α was able to elongate some of the chains to full

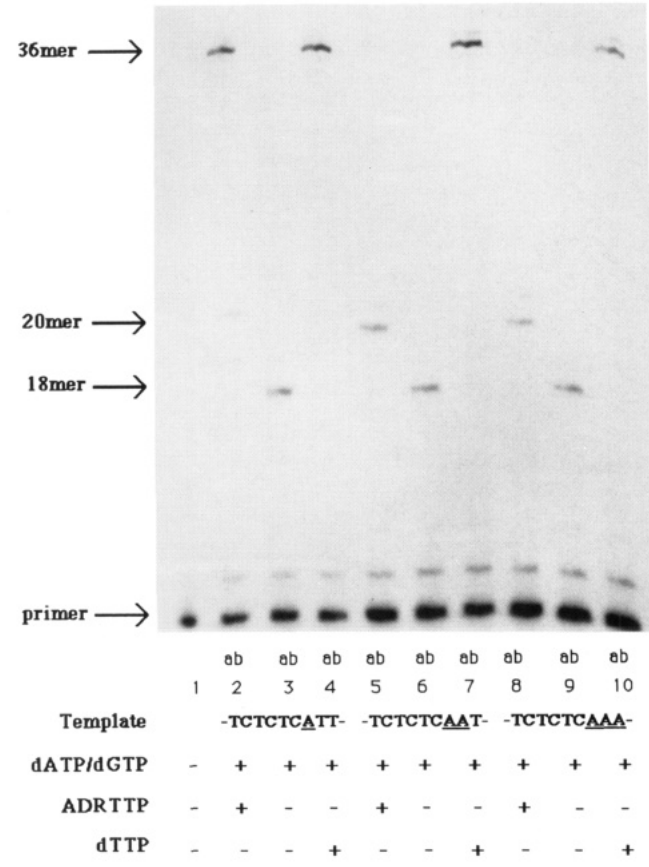

FIGURE 3: Effects of ADRT-TP on DNA strand elongation catalyzed by HIV-1 reverse transcriptase with DNA templates containing a single A, two consecutive A's, and three consecutive A's in the coding sequences. A 12-base oligodeoxyribonucleotide (3′-CAA-TGT-ACC-AGT-5′) was labeled with [^{35}S]phosphate at the 5′-end and annealed to template A (36-mer, 3′-ACT-GGT-ACA-TTG-TCT-CTC-ATT-CTC-TCT-CTC-TTC-TCT-5′, samples 2–4), template B (36-mer, 3′-ACT-GGT-ACA-TTG-TCT-CTC-AAT-CTC-TCT-CTC-TTC-TCT-5′, samples 5–7), or template C (36-mer, 3′-ACT-GGT-ACA-TTG-TCT-CTC-AAA-CTC-TCT-CTC-TTC-TCT-5′, samples 8–10). The experimental details are described in the Experimental Procedures. All samples contained dATP and dGTP. Samples 2, 5, and 8 also contained ADRT-TP, while samples 4, 7, and 10 contained TTP. Two time points were taken from each sample after the addition of enzyme; lane a and lane b were taken at 50 and 100 min, respectively. Sample 1 contained primer alone.

length (sample 2, 36-mer). Sample 3 did not contain either TTP or ADRT-TP; however, the 19-mer product was formed. With a template containing two consecutive dAMP (samples 5–7) in the coding sequence, allowing the incorporation of two consecutive ADRT-MP, the pause sites (18-mer and 19-mer) observed in sample 5 are similar to those of sample 2. However, no full-length DNA is formed in sample 5. Sample 6 shows the same pattern of accumulation of polynucleotides as sample 3.

The effect of ADRT-MP incorporation on subsequent chain elongation by DNA polymerase β is shown in samples 8-13 (Figure 5). With a template (samples 8–10) containing one dAMP in the coding sequence, DNA polymerase β can efficiently, but at a slower rate, incorporate one ADRT-MP molecule (compare sample 8 and sample 10). The pause in chain elongation occurs at sites immediately prior to ADRT-MP incorporation (sample 8, 17-mer and 18-mer). In samples 9 and 12, which did not contain TTP or ADRT-TP, accumulation of the 18-mer and a small amount of the 17-mer product is observed. With a template containing two consecutive dAMP molecules in the coding sequence (samples 11–13), DNA polymerase β could only incorporate one ADRT-

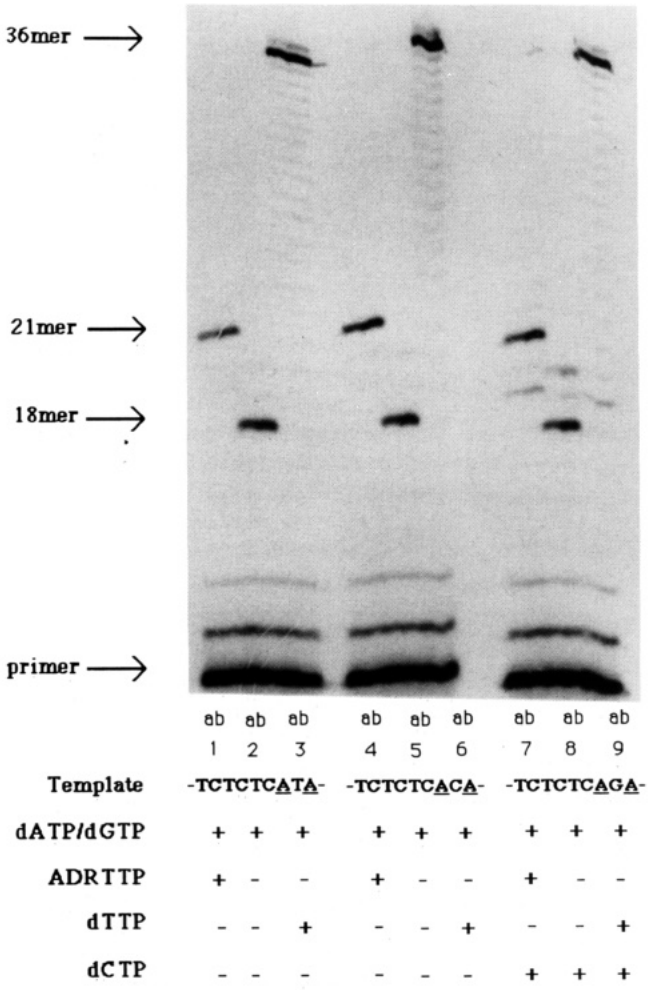

FIGURE 4: Effects of ADRT-TP on DNA strand elongation catalyzed by HIV-1 reverse transcriptase with templates containing two A's separated by either one T, one C, or one G in the coding sequences. A 12-base oligodeoxyribonucleotide (3′-CAA-TGT-ACC-AGT-5′) was labeled with [^{35}S]phosphate at the 5′-end and annealed to template A (36-mer, 3′-ACT-GGT-ACA-TTG-TCT-CTC-ATA-CTC-TCT-CTC-TTC-TCT-5′, samples 1–3), template B (36-mer, 3′-ACT-GGT-ACA-TTG-TCT-CTC-ACA-CTC-TCT-CTC-TTC-TCT-5′, samples 4–6), or template C (36-mer, 3′-ACT-GGT-ACA-TTG-TCT-CTC-AGA-CTC-TCT-CTC-TTC-TCT-5′, samples 7–9). The experimental details are described in the Experimental Procedures. All samples contained dATP and dGTP. Samples 1, 4, and 7 also contained ADRT-TP, while samples 3, 6, and 9 contained TTP. Samples 7–9 also contained 90 μM dCTP. Two time points were taken from each sample after the addition of enzyme; lane a and lane b were taken at 50 and 100 min, respectively.

MP molecule into the elongating primer (sample 11, 19-mer), and no full-length (36-mer) DNA is synthesized.

DISCUSSION

The K_i values (Table I) of ADRT-TP against TTP are very similar between the purified reverse transcriptase and the reverse transcriptase from the lysate of purified virions. This suggests that other components of the virion may not significantly influence the inhibitory effect of ADRT-TP against the reverse transcriptase. The K_i values of ADRT-TP have an order of magnitude similar to the reported K_i value of 20 nM for AZT-TP when RNA is used as the template (Kedar et al., 1990). A higher K_i value of 0.95 μM for ADRT-TP is obtained when DNA is used as the template. The data suggest that ADRT-TP is a more effective inhibitor toward HIV-RT in the replication of RNA to DNA than in the replication of DNA from DNA during HIV replication. The incorporation of a single molecule of ADRT-MP into the growing DNA chain has a greater effect on the rate of DNA elongation in using RNA template than DNA template (compare Figure 2, lane 2, to Figure 3, lane 2). This is in agreement with the inhibition constants shown in Table I where ADRT-TP is a better inhibitor of the replication of RNA to DNA than of the replication of DNA from DNA. The incorporation of a single molecule of ADRT-MP into the growing DNA chain produced marked inhibition of DNA elongation in using the RNA template, as evidenced by the absence of any polynucleotides greater than a 40-mer (compare Figure 2, lane 2, to Figure 2, lane 4).

Figure 3 indicates that HIV-RT has high fidelity in using DNA as the template (Figure 3, samples 3, 6, and 9), since DNA accumulated as an 18-mer prior to mis-incorporation. Figure 2 shows the low fidelity of HIV- RT in using RNA as the template. HIV-RT is shown to catalyze DNA elongation not only after one mis-incorporation (Figure 2, lane 3) but also after two consecutive mis-incorporations (Figure 2, lane 6). However, HIV-RT can not catalyze further DNA elongation where there are three potential consecutive mis-incorporations (Figure 2, lane 9). These data are different from the published data (Yu & Goodman, 1992) where mis-incorporations were observed in HIV-RT catalyzed DNA synthesis using both RNA and DNA templates. They also reported that HIV-RT was able to incorporate as many as three consecutive mismatches and to continue efficient elongation from mismatched primer 3′-termini without discernible pausing. The exact reason for these discrepancies is unknown. In addition to slight variations in assay conditions, a recombinant form of HIV-RT was used in the report by Yu and Goodman, while HIV-RT purified from virions is used in our experiment. The discrepancies may be due in part to the fact that recombinant forms of HIV-RT are a single population of RT. In contrast, HIV-RT purified from virions contains a mixed population of RT molecules due to the genomic variablity of HIV. Our data also suggest that HIV genetic variability may be related to the ability of HIV-RT to continue the efficient synthesis of DNA containing mismatches on RNA templates and not on DNA templates. Our data indicate that the rate of the first mis-incorporation is slow since there is a major pause at the 18-mer (Figure 2, lanes 3, 6, and 9). The elongation of the primer containing one mismatched nucleotide at the primer end is not rate-limiting since no accumulation of 19-mer is observed (Figure 2, lane 3). However, the elongation of the primer end containing one mismatched nucleotide for the addition of the next consecutive mismatched nucleotide is somewhat rate-limiting, since a slight accumulation of the 19-mer is observed (Figure 2, samples 6 and 9).

Figures 2 and 3 show that the incorporation of two or more consecutive ADRT-MP molecules prevents the DNA from further elongation, regardless of whether RNA or DNA is used as the template. The primers used in these studies produce 18-mers before the incorporation of the first molecule of ADRT-MP or TMP, and termination with ADRT-MP is expected to generate 19-mers. Since no accumulation of 18-mer is observed (Figures 2–4), we conclude that the incorporation of a single ADRT-MP molecule at the growing 3′-end is not rate-limiting. In using DNA as the template, Figure 3 also shows that the rate of incorporation of the second consecutive ADRT-MP molecule is not rate-limiting since no accumulation of 19-mers are observed (lanes 2, 5, and 8).

In using DNA as the template, the incorporation of a single ADRT-MP appears to cause DNA elongation to slow slightly

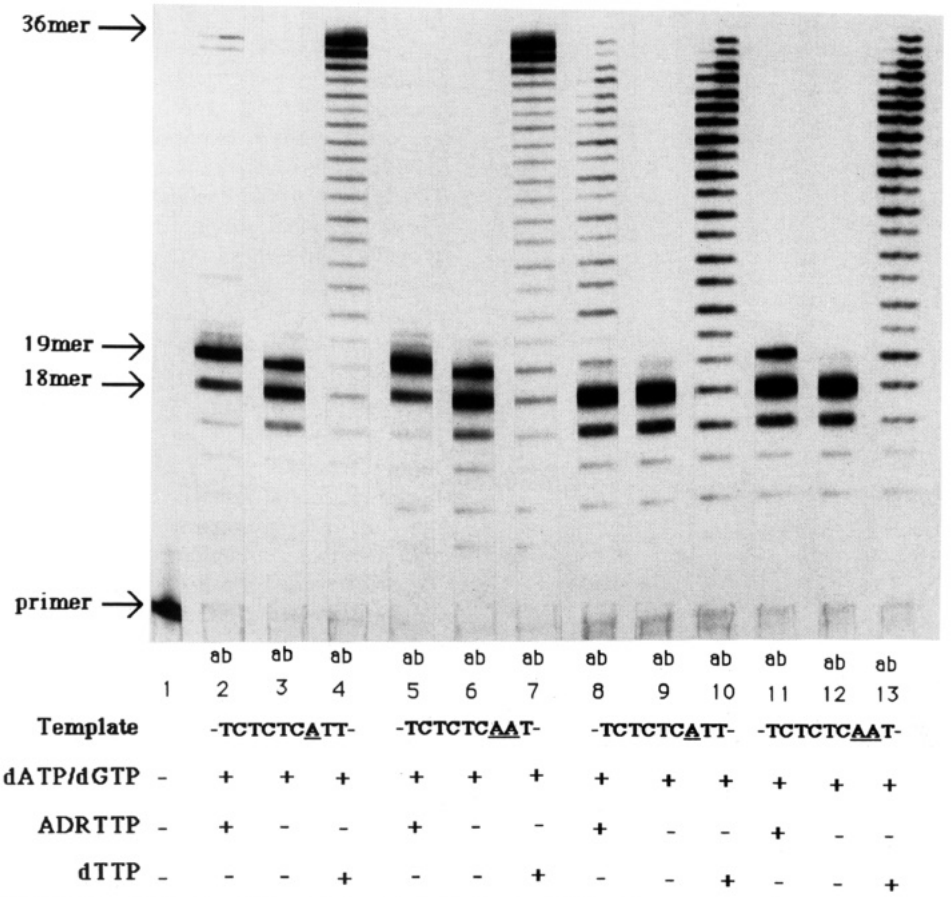

FIGURE 5: Effects of ADRT-TP on DNA strand elongation catalyzed by human DNA polymerases α and β. A 12-base oligodeoxyribonucleotide (3′-CAA-TGT-ACC-AGT-5′) was labeled with [^{35}S]phosphate at the 5′-end and then annealed to either template A (36-mer, 3′-ACT-GGT-ACA-TTG-TCT-CTC-ATT-CTC-TCT-CTC-TTC-TCT-5′, samples 2–4 and 8–10) or template B (36-mer, 3′-ACT-GGT-ACA-TTG-TCT-CTC-AAT-CTC-TCT-CTC-TTC-TCT-5′, samples 5–7 and 11–13). The experimental details are described in the Experimental Procedures. Samples 2–7 were incubated with human DNA polymerase α. Samples 8–13 were incubated with human DNA polymerase β. All samples contained dATP and dGTP. Samples 2, 5, 8, and 11 also contained ADRT-TP, while samples 4, 7, 10, and 13 contained TTP. Two time points were taken from each sample after the addition of enzyme; lane a and lane b were taken at 60 and 120 min, respectively. Sample 1 contained primer alone.

at a position two nucleotides beyond the site of ADRT-MP incorporation, leading to a slight pause at the 21-mer (Figure 3, sample 2). Observation of the 21-mer, which is two nucleotides downstream from the incorporated ADRT-MP at the 19-mer position, suggests that ADRT-MP incorporation may create a perturbation in the structure of the nascent DNA complex two nucleotide positions away from the actual site of incorporation. Further, this negative effect is greatly magnified in the event of the incorporation of two ADRT-MP molecules separated by one molecule of either dAMP, dCMP, or dGMP (Figure 4, samples 1, 4, and 7).

Figure 4 shows that the elongation of DNA stops when two incorporated ADRT-MP are separated by one molecule of another nucleotide. The templates used in samples 7–9 contain the AGA motif, which necessitated the use of dCTP (90 μM) in this set of reactions; the minute amount of 36-mer observed in sample 7 and the small amount of 20-mer and 21-mer observed in sample 8 are probably due to the presence of a minute amount of contaminating dUTP in the dCTP (even though no dUTP could be detected in dCTP by HPLC analysis, the limit of detection was 2%) or, less likely, to base mispairings.

From the data indicating that the formation of the doubly occupied ADRT-MP primer is non-rate-limiting, we believe that the affinity of ADRT-TP for the second site after a single ADRT-MP incorporation may not be adversely affected. The high affinity of ADRT-TP toward HIV-1 reverse transcriptase (a K_i value of 9 nM versus a K_m value of 3.3 μM for TTP during RNA to DNA replication, and a K_i value of 0.95 μM versus a K_m value of 16.3 μM for TTP during DNA to DNA replication; Table I) would facilitate the preferential consecutive incorporation of ADRT-MP during DNA synthesis from RNA, inhibiting further DNA elongation.

Figure 5 shows that the effects of ADRT-TP on human DNA polymerases α and β are drastically different from its effect on HIV-RT. Figure 5 shows not only that the rate of incorporation of a single ADRT-MP into DNA is slow but also that the rate of further extension of the 3′-ADRT-MP-ended primer is a slow process (Figure 5, lanes 2 and 8). Moreover, the data indicate that the incorporation of a single ADRT-MP molecule is not sufficient to stop further elongation by human DNA polymerases α and β.

It has been documented by others (Perrino & Loeb, 1989, 1990) that the extension of mis-incorporated primers by DNA polymerase α is inefficient and the rate of extension is slow. Mis-incorporation by DNA polymerase α may also partially contribute to the pause of DNA chain elongation in samples 2 and 5 (19-mer). The data from sample 5 also suggest that human DNA polymerase α is unable to incorporate a second ADRT-MP molecule following the incorporation of the first ADRT-MP molecule. It is also possible that the rate of incorporation of the second consecutive ADRT-MP molecule is a much slower process than the incorporation of the first ADRT-MP molecule. In a separate experiment, the addition of dTTP after the incorporation of one single molecule of ADRT-MP at the primer end (Figure 5, lane 5) gave rise to the formation of some full-length 36-mer and 19-mer (data

not shown). This full-length 36-mer probably contains a mixture of 36-mer with ADRT-MP at position 19 and 36-mer without ADRT-MP (elongation of 18-mer with dTMP occupied at both positions 19 and 20). These data make sense to us since the incorporation of one single molecule of ADRT-MP at the primer end causes the elonagation of DNA to drastically slow down (Figure 5, lane 2).

The data in this report demonstrate that the incorporation of a single molecule of ADRT-MP at the 3′-end of the primer greatly decreases the rate of further DNA elongation catalyzed by DNA polymerase α (Figure 5, sample 2). The singly ADRT-MP terminated 3′-end has less effect on DNA elongation catalyzed by DNA polymerase β (Figure 5, sample 8). In contrast to human DNA polymerases α and β, the singly ADRT-MP terminated 3′-end has only a nominal effect on further DNA elongation catalyzed by HIV-RT. Further differences among the polymerases are observed when we examine their abilities to catalyze multiple incorporations of ADRT-MP. Whereas human DNA polymerases α and β can incorporate only one molecule of ADRT-MP, accumulation of DNA with doubly incorporated ADRT-MP is not observed for the human enzymes (Figure 5, samples 5 and 11), HIV-RT can efficiently incorporate two consecutive ADRT-MP molecules before accumulation of terminated DNA occurs (Figures 2 and 3, samples 5 and 8). These differences in inhibition and product formation may arise from ADRT-mediated interference at specific steps of the DNA polymerization process.

There are, at minimum, four kinetically defined steps in DNA polymerization: binding of template/primer, binding of deoxynucleoside triphosphate, incorporation of nucleotide into the 3′-end of the primer, and translocation of the newly formed 3′-end primer/template at the active site of the polymerase to complete the catalytic cycle. In the polymerases studied here, at least one, and perhaps all, of these steps is affected by the faulty 3′-end generated by incorporation of the ADRT-MP molecule. The differential aspects of ADRT-MP incorporation on the three DNA polymerases suggest that the kinetic steps in the DNA polymerization cycle of these enzymes may vary substantially in their sensitivity to modulation by ADRT. The catalytic step in HIV-RT appears to be the least sensitive among the three enzymes for ADRT-MP incorporation, because HIV-RT catalyzes rapid incorporation of two ADRT-MP molecules and is the only enzyme tested that can incorporate two consecutive ADRT-MP molecules. The data suggest that the orientation of the 3′-hydroxy group in the nascent terminus in the active site, or the catalytic pocket of HIV-RT itself, is sufficiently flexible to accommodate unusual nucleotides as well as abnormal primer/template.

The low stringency in substrate binding at the active site of HIV-RT may be a direct consequence of the requirement for its reverse transcriptase activity, which necessitates recognition of both RNA–DNA and DNA–DNA double-stranded species. Flexibility at the active site of HIV-RT is likely to be related to the reported poor fidelity of this polymerase (Preston et al., 1988). The active sites of human DNA polymerases α and β do not appear to have the same flexibility. Preliminary structural analysis in the crystalline state and in solution (unpublished results) indicate that the 4′-azido-2′-deoxyribose sugar ring of ADRT has a 3′-endo configuration instead of the 3′-exo configuration normally adopted by the 2′-deoxyribose of thymidine in DNA. This abnormal configuration may alter the position of the 3′-hydroxy group of ADRT-MP at the primer 3′-end. The position of this 3′-hydroxy group may be shifted from the optimal alignment for nucleophilic attack toward the α-phosphate group of the incoming deoxynucleoside triphosphate, resulting in the inability of the entire replicative complex to extend the primer, at least not with a detectable rate in our experiments. The magnitude by which this positional shift affects the catalytic capacity of HIV-RT may be much smaller than that for the human polymerases. Moreover, the different binding affinities of ADRT-TP to the polymerases appear to influence their respective abilities to utilize this nucleotide. The only enzyme that can incorporate consecutive ADRT-MP molecules is HIV-RT, which binds ADRT-TP with a nanomolar dissociation constant during RNA to DNA replication. The other two enzymes, which do not bind ADRT-TP as well and have dissociation constant values for ADRT-TP in the 100 μM range, can catalyze only single incorporations of ADRT-MP.

ADRT-TP has been shown to be a poor inhibitor of human DNA polymerases α and β, with K_i values of 62.5 and 150 μM, respectively. In comparison, the K_m values of TTP for these two host polymerases are 2.8 and 2.7 μM, respectively (Chen et al., 1992). These differences in the affinities of both human DNA polymerases α and β for TTP and ADRT-TP strongly suggest that human DNA polymerases would preferentially incorporate TTP into DNA over ADRT-TP. This also suggests that DNA chain termination caused by ADRT-MP (Figure 5, samples 5 and 11) is not likely to occur in host DNA *in vivo*, since TTP would be preferentially incorporated over ADRT-TP by the host DNA polymerases. This conclusion is supported by the finding that DNA purified from cells labeled with ADRT contained only internally incorporated ADRT (Chen et al., 1992).

We describe here a novel and unique effect of ADRT on DNA synthesis. Incorporation of a single ADRT-MP molecule does not necessarily cause DNA chain termination in host DNA, whereas incorporation of a molecule of 2′,3′-dideoxynucleoside causes both the host and HIV DNA to terminate the chain. However, internal incorporation of ADRT-MP in DNA could lead to mutation. The selective inhibitory effect of ADRT on HIV reverse transcriptase described above is mediated by the very high affinity of ADRT-TP toward HIV-1 reverse transcriptase. ADRT is also a potent inhibitor against AZT-resistant mutants (Maag et al., 1991). In addition to the potent anti-HIV activity of ADRT, the low affinity of ADRT toward the host DNA polymerases and the differential mechanism of action of ADRT with regard to viral and host DNA polymerases make ADRT analogues potentially valuable anti-HIV nucleoside analogues in the clinic.

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