Selective Inhibition of HIV-1 Reverse Transcriptase by an Antiviral Inhibitor, (R)-9-(2-Phosphonylmethoxypropyl)adenine*

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(R)-9-(2-Phosphonylmethoxypropyl)adenine (PMPA) is an acyclic nucleoside phosphonate that has been shown to be effective in the treatment of AIDS although it has a shorter separation between the adenine and phosphorus than dideoxy-AMP and dAMP. By using pre-steady state kinetic methods, we examined the incorporation of the diphosphate of PMPA, 2',3'-dideoxyadenosine 5'-triphosphate (dATP), and dATP catalyzed by wild-type human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, an exonuclease-deficient T7 DNA polymerase (T7 exo -), and wild-type rat DNA polymerase β in order to evaluate the selectivity of PMPA as an antiviral inhibitor. With a DNA/DNA or DNA/RNA 22/43-mer duplex, the diphosphate of PMPA (PMPApp) is as effective as ddATP in reactions catalyzed by HIV-1 reverse transcriptase in that both analogs have similar steady state kinetic forms as ddATP and dATP. Similarly, PMPApp is 800-fold less inhibitory toward polymerase β with the DNA/DNA 22/43-mer duplex, whereas in studies with a single nucleotide gapped DNA (22-20/43-mer) PMPApp is 13-fold less inhibitory than ddATP. Although parallel studies will need to be performed using appropriate human polymerases, these results begin to define the mechanistic basis for the reported lower toxicity of PMPA in the treatment of AIDS.

The human immunodeficiency virus (HIV) is a retrovirus and a causative agent of the Acquired Immunodeficiency Syn-

drome (AIDS) (1–3). During replication, virally encoded reverse transcriptase (RT) copies the single-stranded viral RNA genome into a minus strand of DNA and then uses the resultant cDNA to synthesize a plus strand DNA to allow the double-stranded viral genome to be integrated into the DNA of the host. RT has been a key target in chemotherapy due to its essential role during the virus life cycle and due to the absence of a proofreading exonuclease that affords selective inhibition by chain terminators. Currently, five anti-RT nucleoside analogs, 3'-azido-2',3'-dideoxythymidine (AZT), 2',3'-dideoxycytidine (ddC), 2',3'-dideoxyinosine (ddI), 2',3'-didehydro-2',3'- dideoxythymidine (ddT), and (−)β-β',3'-dideoxy-3'-thiacytidine (3TC), have been approved for the treatment of AIDS (4). These nucleoside analogs are converted to their triphosphate forms in cellu

lar kinases where they compete with natural 2',3'-dideoxynucleoside 5'-triphosphates (dNTPs) for uptake by RT for incorporation into viral DNA (5). Once incorporated into viral DNA, these nucleotide analogs terminate the DNA synthesis due to the lack of a 3'-hydroxy group. A combination therapy of two nucleoside analogs and one protease inhibitor has been used with remarkable success in the clinical treatment of AIDS (6). These potent three-drug mixtures can significantly drive down the viral load of HIV in the blood, increase levels of CD4 cells, and in many cases slow progression and prolong survival (6). However, long term usage of nucleoside analogs leads to selection of mutants of RT showing drug resistance (7). It has been shown that the quadruple mutation on RT (D67N, K70R, T215F or T215Y, and K219Q) confers more than 100-fold decrease in virus sensitivity to AZT (8). Moreover, nucleoside analogs also inhibit human DNA replication, particularly mitochondrial DNA synthesis, which leads to the toxicity of these nucleoside analogs. The inhibition of human mitochondrial DNA polymerase by AZT and ddC has been implicated as the basis for the toxicity of the two nucleoside analogs (9, 10). Drug resistance and toxicity problems challenge us to develop new anti-HIV drugs that are more powerful and less toxic.

PMPA, 9-(2-phosphonylmethoxypropyl) adenine, is an acyclic nucleoside phosphonate (Fig. 1). PMPA has been reported to effect

ively prevent infection of simian immunodeficiency virus (SIV), a relative of HIV, in all tested long-tailed macaques for up to 56 weeks without adverse side effects after they were exposed to SIV up to 24 h (11). PMPA has also been shown to have potent anti-HIV activity in vitro and in vivo (12); and particularly, Gilead Sciences recently reported (13, 14) in Phase I/II clinical evaluation without significant side effects. Like nucleoside analogs, PMPA is phosphorylated to its triphosphate form by cellular kinases in vivo (15). The antiviral activity of PMPA is believed to be due to its incorporation into viral DNA which terminates DNA elongation due to its lack of a

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1 The abbreviations used are: HIV-1, human immunodeficiency virus type 1; AZT, 3'-azido-2',3'-dideoxythymidine; ddATP, 2',3'-dideoxyadenosine 5'-triphosphate; ddC, 2',3'-dideoxycytidine; ddI, 2',3'-dideoxyinosine; ddT, dithirotetrol; PMPA, (R)-9-(2-phosphonylmethoxypropyl)adenine; PMPApp, diphosphate of PMPA; Pol, rat DNA polymerase β; RT, reverse transcriptase; SIV, simian immunodeficiency virus; T7 exo−, an exonuclease-deficient T7 DNA polymerase with D3A and ETA double-point mutations; ddC, 2',3'-dideoxycytidine; ddI, 2',3'-dideoxyinosine; ddT, 2',3'-didehydro-2',3'-dideoxythymidine; 3TC, (−)β-β',3'-dideoxy-3'-thiacytidine.

2 Gilead Sciences on-line address is as follows: www.gilead.com.
Escherichia coli son, WI). The exonuclease-deficient mutant (D5A and E7A) of T7 DNA alkaline phosphatase and T4 polynucleotide kinase, New England Bio-
acetylated bovine serum albumin, Life Technologies, Inc.; calf intestine effectiveness and toxicity of PMPA as an antiviral drug. Therefore, these data provide a kinetic basis to assess the ddATP. Since the structure of the ribose and not the nucleobase the same kinetic parameters for the incorporation of dATP and polymerase (pol)
the inhibition by antiviral nucleoside analogs than DNA polymerases β and γ (16). For comparison, we determined the same kinetic parameters for the incorporation of dATP and dDATP. Since the structure of the ribose and not the nucleobase was the determinant of inhibitory effects of nucleotide analogs (16), in our studies dDATP represents other nucleotide analogs. Therefore, these data provide a kinetic basis to assess the effectiveness and toxicity of PMPA as an antiviral drug.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chemicals and where they were purchased are as follows: γ-32P]ATP, ICN (Costa Mesa, CA); dATP and ddATP, Sigma; acetylated bovine serum albumin, Life Technologies, Inc.; calf intestine alkaline phosphatase and T4 polynucleotide kinase, New England Biolabs (Beverly, MA); polyacrylamide and Bissip columns, Bio-Rad. PM-PApp was obtained from Gilead Sciences (Foster City, CA).

**Proteins**—Wild-type HIV-1 RT was prepared as described previously (17). Escherichia coli thioredoxin was purchased from Promega (Madison, WI). The exonuclease-deficient mutant (D5A and E7A) of T7 DNA polymerase (T7 exo-), and wild-type rat DNA polymerase β (pol β). T7 DNA polymerase and rat DNA polymerase β were selected because they are considered to be good models of human DNA polymerase β and γ, respectively (see “Discussion”), and current preparations of the human enzymes are not yet sufficiently robust for these studies. Nuclear replicative polymerases α, δ, and ε were not studied because they were less sensitive to inhibition by antiviral nucleoside analogs than DNA polymerases β and γ (16). For comparison, we determined the same kinetic parameters for the incorporation of dATP and ddATP. Since the structure of the ribose and not the nucleobase was the determinant of inhibitory effects of nucleotide analogs (16), in our studies ddATP represents other nucleotide analogs. Therefore, these data provide a kinetic basis to assess the effectiveness and toxicity of PMPA as an antiviral drug.

**Rapid Quench Experiments**—Rapid quench experiments were carried out in an apparatus designed by Johnson (21) and built by KinTek Corp. (State College, PA). Typically, the experiments were carried out by allowing enzyme and DNA to preincubate in buffer in the absence or presence of Mg2+.

**Data Analysis**—The equilibrium dissociation constant of dNTP, Kd, and the maximum rate for incorporation of dNTP, kcat, were determined by nonlinear regression using the program GraFit. The data were fitted to a hyperbola (see “Data Analyses”) to give the observed rate of nucleotide incorporation. Then, the observed rates abstracted from a series of time courses of product formation were plotted against the concentrations of Mg2+-dNTP, and the data were fitted to a hyperbola (see “Data Analysis”) to give the equilibrium dissociation constant of dNTP, Kd, and the maximum rate for incorporation of dNTP, kcat.

**DNA Analysis**—The DNA products were analyzed by sequencing gel electrophoresis (16% acrylamide, 8 μ urea, 1 X TBE running buffer) and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**KinTek Corp.**

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**Fig. 1. Structures of dAMP, ddAMP, and PMPA.** The nucleobase adenine and the phosphorus of α-phosphate in dAMP and ddAMP are separated by N—C, C—C, 2 C—O, and C—P bonds. In contrast, the base adenine is separated from the phosphorus of phosphate in PMPA by N—C, C—C, 2 C—O, and C—P bonds.

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was dephosphorylated with calf intestine alkaline phosphatase and purified by electrophoresis through 10% acrylamide, 8 μ urea, and TBE (89 mM Tris, 89 mM borate, 2 mM EDTA). The full-length template was electrophoresed from the excised gel, precipitated, and resuspended in RNA-free Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.0). The purified RNA was 5'-32P-labeled, electrophoresed through a sequence gel, and autoradiographed to determine purity. The concentration of purified RNA was measured spectrophotometrically at 260 nm, with an extinction coefficient of 473,480 M⁻¹ cm⁻¹ (20), in the presence of 8 μ urea.

DNA primer of 22-mer, 5'-phosphorylated 20-mer, and DNA template listed in Table I were purchased from Integrated DNA Technologies, Inc. (Corvalis, IA). They were purified by denaturing polyacrylamide gel electrophoresis, and concentrations were determined by UV absorbance at 260 nm.

5'-32P Labeling of 22/43-Mer—Before annealing, the DNA primer 22-mer was 5'-32P-labeled with T4 polynucleotide kinase according to manufacturer’s instructions. Unincorporated nucleotides were removed using a Bissip-30 column.

Annealing—The duplex DNA/DNA or DNA/RNA 22/43-mer was formed by annealing 5'-32P-labeled 22-mer and 43-mer at the molar ratio of 1.0:1.1. The gapped DNA 22–20/43-mer was formed by annealing 5'-32P-labeled 22-mer, 5'-phosphorylated 20-mer, and 43-mer at the molar ratio of 1.0:0.5/1.0:5. Prior to annealing, mixtures were denatured at 85 °C for 5 min and then cooled slowly to room temperature in 2 h. To ensure that annealing had taken place the duplex mixtures were analyzed by nondenaturing polyacrylamide gel electrophoresis.

Buffers—HIV-1 RT was preincubated with DNA/DNA and DNA/RNA substrates in RT buffer containing 50 mM Tris acetate (pH 7.5 at 37 °C), 100 mM potassium acetate, 0.1 mM EDTA. All reactions using RT were carried out at 37 °C in RT buffer containing 10 mM magnesium acetate.

T7 exo- was preincubated with DNA 22/40-mer, E. coli thioredoxin, and DTT in T7 buffer containing 40 mM Tris chloride (pH 7.5 at 20 °C), 50 mM sodium chloride, 1 mM EDTA, 1 mM DTT, and 0.1 mg/ml bovine serum albumin as described (18). All reactions using T7 exo- were carried out at 20 °C in the T7 buffer containing 12.5 mM magnesium chloride additionally.

All experiments using rat DNA polymerase β were carried out at 37 °C in pol β buffer containing 50 mM Tris chloride (pH 8.0 at 37 °C), 50 mM potassium chloride, 0.2 mg/ml bovine serum albumin, 1 mM DTT, 10 mM magnesium chloride, 0.1 mM EDTA, and 5% glycerol (v/v).

**Rapid Quench Experiments**—Rapid quench experiments were carried out in an apparatus designed by Johnson (21) and built by KinTek Corp. (State College, PA). Typically, the experiments were carried out by allowing enzyme and DNA to preincubate in buffer in the absence or presence of Mg2+.

An aliquot of this solution (15 μl) was rapidly mixed with an equal volume of solution containing nucleotide and Mg2+ loaded in the second loop of tubing. The reactions were quenched with 90 μl of 0.3 M EDTA (final concentration) after time intervals ranging from 5 ms to seconds. All concentrations reported in this paper refer to concentrations during reaction after mixing.

Measurement of Equilibrium Dissociation Constant of Next Correct Nucleotide to the E-DNA Complex—A preincubulated solution of enzyme and DNA or RNA at fixed concentrations (vary with different enzymes, see “Results”) was mixed with varying concentrations of Mg2+-dNTP (2–400 μM) in reaction buffer to start the reaction at 37 (for RT and pol β) or 20 °C (for T7 exo-). The reaction at each concentration of Mg2+-dNTP was terminated by 0.3 mM EDTA at different times from milliseconds to seconds. The reaction products were analyzed by sequencing gel analysis. The time course of product formation was fitted to a single exponential equation for each concentration of Mg2+-dNTP (see “Data Analysis”) to give the observed rate of nucleotide incorporation. Then, the observed rates abstracted from a series of time courses of product formation were plotted against the concentrations of Mg2+-dNTP, and the data were fitted to a hyperbola (see “Data Analyses”) to give the equilibrium dissociation constant of dNTP, Kd, and the maximum rate for incorporation of dNTP, kcat.

**Product Analysis**—The DNA products were analyzed by sequencing gel electrophoresis (16% acrylamide, 8 μ urea, 1 X TBE running buffer) and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**DNA Analysis**—Data were fitted by nonlinear regression using the program GraFit (Erithacus Software). Data from burst experiments were fitted to a burst equation: [product] = E11 — exp(—k1 t) + k2 t, where E represents the active enzyme concentration based on the
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**Results**

Synthesis of an RNA Template of 43 Nucleotides—An RNA template of 43 nucleotides (Table I) was derived from nucleotide 1623 to nucleotide 1665 of the HIV-1 RNA genome (22). This RNA 43-mer was synthesized by runoff transcription by T7 RNA polymerase (see “Experimental Procedures”), yielding more than 100 full-length RNA molecules per template molecule. The sequence of the single-stranded template portion of the RNA/RNA 22/43-mer duplex (Table I) was computationally examined using an RNA folding program, Mfold (17), and no RNA secondary structure was located. Thus, the kinetics of polymerization on 22/43-mer will not be affected by any RNA secondary structure (17). According to the template sequence, the next correct nucleotide for incorporation on the DNA or RNA 22/43-mer duplexes and the gapped DNA 22–20/43-mer is dATP (Table I).

Kinetic Pathway of Nucleotide Incorporation—We studied the kinetics for incorporation of dATP, ddATP, and PMPApp catalyzed by T7 exonuclease (18), and rat DNA polymerase (24, 25) follows the kinetic pathway shown in Equation 1, where $k_1$ is the observed rate of nucleotide incorporation, $k_p$ is the maximum rate of nucleotide incorporation, and $K_d$ represents the nucleotide equilibrium dissociation constant in the ground state. The $K_d$ measurement was performed by reacting a preincubated solution of enzyme and primer/template (DNA or RNA template) with varying concentrations of Mg$^{2+}$-dNTP and monitoring product formation over time. The observed rates of single nucleotide incorporation ($E^{-}22/43$-mer or $E^{-}22-20/43$-mer) were plotted against nucleotide concentrations. The data fit a hyperbola supporting the above pathway and gave values of $k_p$ and $K_d$. The substrate specificity constant, $k_p/K_d$, was then calculated from the values of $K_d$ and $k_p$.

| Oligonucleotides | RNA 22/43-mer | DNA 22/43-mer | Gapped DNA 22–20/43-mer$^a$ |
|------------------|---------------|---------------|-----------------------------|
| Sequence         | 5′-CCCTCCCTTGAGGAAAGGCCGAG-3′ | 5′-GGAGGGAAACAUCCCCGGCGCGUAGAAGGUAUUUUAACUCC-5′ | 5′-GGAGGGAAACATTCCTCTGGCTTAGAGGGATTTTTTAATTCG-5′ |
| 22/43-mer        | 5′-GGAGGGAAACAUCCCCGGCGCGUAGAAGGUAUUUUAACUCC-3′ | 5′-GGAGGGAAACATTCCTCTGGCTTAGAGGGATTTTTTAATTCG-3′ |

$^a$ The 20-mer of the gapped DNA was 5′-phosphorylated.

The kinetics of incorporation of dATP and ddATP to DNA 22/43-mer catalyzed by RT were measured in the same manner as for PMPApp, and the kinetic data are reported in Table II. As expected, the values of $K_d$ and $k_p$ of dATP are similar to the corresponding kinetic data obtained by Kim et al. (19) using RNA 25/45-mer. The specificity constants for the three substrates, $k_p/K_d$, were calculated and are also summarized in Table II.

Measurement of Binding Affinity of PMPApp, dATP, and ddATP to the HIV-1 RT-RNA 22/43-Mer Complex at 37 °C—The binding affinity of PMPApp to the HIV-1 RT-RNA 22/43-mer complex (RNA template, DNA primer) was determined as above. At a fixed RT-RNA 22/43-mer concentration (59:3:200 nM), the single turnover rates of PMPApp incorporation increased with increasing concentrations of PMPApp (Fig. 4A). The rates were plotted against the PMPApp concentrations, and the data were fitted to a hysteric (Fig. 4B). The hyperbolic plot gave a PMPApp $K_d$ of 54 ± 4 μM and a $k_p$ of 22 ± 1 s$^{-1}$ (Table III).

The kinetics of incorporation of dATP and ddATP to RNA 22/43-mer catalyzed by RT were measured similarly, and the data are summarized in Table III. Again, the values of $K_d$ and $k_p$ of dATP are similar to the ones measured by Kim et al. (19) using RNA 25/45-mer. The substrate specificity constants for dATP, ddATP, and PMPApp were calculated and summarized in Table III.

Measurement of Binding Affinity of PMPApp, dATP, and ddATP to the T7 exonuclease-DNA 22/43-Mer Complex at 20 °C—The binding affinity and maximum rates of incorporation of dATP and ddATP catalyzed by T7 exonuclease at 20 °C were measured in the similar manner as above, and the data were listed in Table IV. The kinetics of dATP incorporation were similar to those previously observed by Patel et al. (18) using a DNA 25/36-mer. However, incorporation of Mg$^{2+}$-PMPApp was much slower based on the pre-steady state kinetic analysis (data not shown).
Kinetic constants with DNA 22/43-mer and HIV-1 RT at 37 °C

|        | \( h_p \) | \( K_d \) | \( h_p/K_d \) |
|--------|-----------|-----------|--------------|
| dATP   | 41.3 ± 0.6| 8.1 ± 0.9 | 5.10         |
| ddATP  | 55 ± 5    | 54 ± 11   | 1.02         |
| PMPApp | 49 ± 5    | 58 ± 11   | 0.84         |

Kinetic constants with DNA/RNA 22/43-mer and HIV-1 RT at 37 °C

|        | \( h_p \) | \( K_d \) | \( h_p/K_d \) |
|--------|-----------|-----------|--------------|
| dATP   | 74 ± 5    | 16 ± 4    | 4.63         |
| ddATP  | 53 ± 6    | 51 ± 13   | 1.04         |
| PMPApp | 22 ± 1    | 34 ± 4    | 0.65         |

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and required longer time courses to compensate for the slower rates. In order to eliminate the complication resulting from steady state formation of products in long reaction times, the measurements of \( K_d \) and \( h_p \) for PMPApp incorporation should be performed with T7 exonuclease in excess over DNA (26). However, we used slightly more DNA than enzyme in the experiments for incorporation of PMPApp in order to keep the same reaction conditions as for incorporation of ddATP and dATP. Thus, the observed single turnover rates of PMPApp incorporation in Fig. 5 may be slightly underestimated (maximum 2-fold). Fig. 5A represents a series of time courses for the incorporation of Mg\(^{2+}\)-PMPApp at a fixed concentration of T7 exonuclease 22/43-mer (100:200 nM) and varying PMPApp concentrations (2–200 μM). The time courses were fit to first-order processes individually to obtain observed rates. The incorporation rates were plotted against the concentrations of PMPApp in Fig. 5B. The data fit a hyperbola (solid line) to yield a \( K_d \) value of 34 ± 4 μM for PMPApp dissociation and a maximum rate of incorporation of 22 ± 1 s\(^{-1}\).

![FIG. 2. Pre-steady state kinetics of incorporation of PMPApp into 22/43-mer DNA/DNA duplex by wild-type HIV-1 RT at 37 °C. A preincubated solution of HIV-1 RT (68.3 nM, active site concentration) and 5'-\( ^{32P} \)P-labeled 22/43-mer (200 nM) was mixed with a solution of PMPApp (150 μM) in RT buffer containing 10 mM Mg\(^{2+}\) to start the reactions. The data (●) were fitted to a burst equation (solid line) with a burst rate of 35 ± 4 s\(^{-1}\) and a steady state rate of 0.28 ± 0.04 s\(^{-1}\).](image)

![FIG. 3. PMPApp concentration dependence of the pre-steady state rate at 37 °C with wild-type HIV-1 RT and 22/43-mer DNA/DNA duplex. A, a preincubated solution of HIV-1 RT (52.9 nM, active site concentration) and 5'-\( ^{32P} \)P-labeled 22/43-mer (200 nM) was mixed with increasing concentrations of PMPApp in RT buffer containing 10 mM Mg\(^{2+}\) to start the reactions. The reactions were quenched at indicated times and analyzed by sequencing gel electrophoresis. The PMPApp concentrations were 4 μM (●), 10 μM (○), 20 μM (■), 30 μM (□), 50 μM (▲), 75 μM (■), and 110 μM (○). The solid lines represent the best fit of data to first-order processes. B, the first-order rates (●) measured from the time courses in A were plotted against the PMPApp concentrations. The data were fitted to a hyperbola (solid line) to yield a \( K_d \) value of 58 ± 11 μM for PMPApp dissociation and a maximum rate of incorporation of 49 ± 5 s\(^{-1}\).](image)

![FIG. 4. PMPApp concentration dependence of the pre-steady state rate at 37 °C with wild-type HIV-1 RT and 22/43-mer DNA/RNA heteroduplex. A, a preincubated solution of HIV-1 RT (59.3 nM, active site concentration) and 5'-\( ^{32P} \)P-labeled 22/43-mer (200 nM) was mixed with increasing concentrations of PMPApp in RT buffer containing 10 mM Mg\(^{2+}\) to start the reactions. The reactions were quenched at indicated times and analyzed by sequencing gel electrophoresis. The PMPApp concentrations were 4 μM (●), 10 μM (○), 15 μM (■), 25 μM (□), 45 μM (▲), 75 μM (■), and 110 μM (○). The solid lines represent the best fit of data to first-order processes. B, the first-order rates (●) measured from the time courses in A were plotted against the PMPApp concentrations. The data were fitted to a hyperbola (solid line) to yield a \( K_d \) value of 34 ± 4 μM for PMPApp dissociation and a maximum rate of incorporation of 22 ± 1 s\(^{-1}\).](image)
we used a 4:1 molar ratio of active enzyme to DNA to compensate for the slow dissociation of the DNA/DNA duplex. In order to follow a single turnover of nucleotide incorporation, the reaction rates were plotted versus the PMPApp concentrations and a fit of the experimental data was used to determine the best estimate of the dissociation constant of PMPApp to the rat DNA polymerase. The solid line represents the best fit of data to the first-order processes. The first-order rates measured from the time courses in A were plotted against the PMPApp concentrations and a fit of the experimental data was used to determine the best estimate of the dissociation constant of PMPApp to the rat DNA polymerase. The solid line represents the best fit of data to the first-order processes. The first-order rates measured from the time courses in A were plotted against the PMPApp concentrations and a fit of the experimental data was used to determine the best estimate of the dissociation constant of PMPApp to the rat DNA polymerase.

| Kinetic constants with DNA 22/43-mer and T7 DNA polymerase at 20 °C | $k_p$ | $K_d$ | $k_p/K_d$ |
|---|---|---|---|
| dATP | $156 \pm 8$ | $8 \pm 2$ | 19.5 |
| ddATP | $88 \pm 3$ | $6 \pm 1$ | 14.5 |
| PMPApp | $0.096 \pm 0.009$ | $268 \pm 39$ | $3.6 \times 10^{-4}$ |

In Table IV, the rate constants for PMPApp incorporation into the DNA 22/43-mer are presented. The rates were plotted against the PMPApp concentrations and a fit of the experimental data was used to determine the best estimate of the dissociation constant of PMPApp to the rat DNA polymerase. The solid line represents the best fit of data to the first-order processes. The first-order rates measured from the time courses in A were plotted against the PMPApp concentrations and a fit of the experimental data was used to determine the best estimate of the dissociation constant of PMPApp to the rat DNA polymerase.

there is little curvature in Fig. 6B, we could not perform experiments at higher concentrations of PMPApp. Nonetheless, the quality of the data allows a sufficiently precise estimate of the $K_d$ and maximum rates within reasonable limits of error. The $K_d$ and $k_p$ values for incorporation of dATP and ddATP were measured similarly except that shorter time courses were used due to faster incorporation rates. The data are summarized in Table V. Previous kinetic measurement for dATP incorporation to the rat DNA pol β DNA 25/45-mer gave values of $K_d$ and $k_p$ of $52 \pm 6$ μM and $24.1 \pm 0.9$ s⁻¹, respectively (27). These are slightly different from our data (Table V). The differences could be due to different DNA sequences and different reaction conditions. The substrate specificity constants for dATP, ddATP, and PMPApp were calculated and summarized in Table V.

Measurement of Binding Affinity of PMPApp, dATP, and ddATP to the Rat DNA Polymerase β-DNA 22/43-mer Complex at 37 °C—Pre-steady state kinetic analysis revealed slow incorporation of PMPApp into DNA 22/43-mer catalyzed by pol β (data not shown). DNA was previously found to dissociate fast from the rat DNA pol β/DNA complex at a rate of 3–2 s⁻¹ (27). In order to follow a single turnover of nucleotide incorporation, the reaction rates were plotted versus the PMPApp concentrations and a fit of the experimental data was used to determine the best estimate of the dissociation constant of PMPApp to the rat DNA polymerase. The solid line represents the best fit of data to the first-order processes. The first-order rates measured from the time courses in A were plotted against the PMPApp concentrations and a fit of the experimental data was used to determine the best estimate of the dissociation constant of PMPApp to the rat DNA polymerase. The solid line represents the best fit of data to the first-order processes. The first-order rates measured from the time courses in A were plotted against the PMPApp concentrations and a fit of the experimental data was used to determine the best estimate of the dissociation constant of PMPApp to the rat DNA polymerase. The solid line represents the best fit of data to the first-order processes. The first-order rates measured from the time courses in A were plotted against the PMPApp concentrations and a fit of the experimental data was used to determine the best estimate of the dissociation constant of PMPApp to the rat DNA polymerase.

FIG. 5. PMPApp concentration dependence of the pre-steady state rate at 20 °C with T7 exo– and 22/43-mer DNA/DNA duplex. A, a preincubated solution of T7 exo– (100 nM), E. coli thioredoxin (2.0 μM), 5'-32P-labeled 22/43-mer (200 nm), and DTT (10 mM) was mixed with increasing concentrations of PMPApp in T7 buffer containing 12.5 mM Mg2⁺ to start the reactions. The reactions were quenched at indicated times and analyzed by sequencing gel electrophoresis. The PMPApp concentrations were 2 μM (●), 7 μM (○), 15 μM (■), 30 μM (▲), 60 μM (▲), 100 μM (□), and 200 μM (●). The solid lines represent the best fit of data to first-order processes. B, the first-order rates measured from the time courses in A were plotted against the PMPApp concentrations. The data were fitted to a hyperbola (solid line) to yield a $K_d$ value of 1033 ± 120 μM for PMPApp dissociation and a maximum rate of incorporation of 1.0 ± 0.1 s⁻¹.

FIG. 6. PMPApp concentration dependence of the pre-steady state rate at 37 °C with wild-type rat DNA polymerase β and 22/43-mer DNA/DNA duplex. A, a preincubated solution of rat DNA polymerase β (200 nM, active site concentration), 5'-32P-labeled 22/43-mer (50 nM), and Mg2⁺ (10 mM) was mixed with increasing concentrations of PMPApp in pol β buffer containing 10 mM Mg2⁺ to start the reactions. The reactions were quenched at indicated times and analyzed by sequencing gel electrophoresis. The PMPApp concentrations were 10 μM (●), 25 μM (■), 50 μM (○), 100 μM (▲), 200 μM (□), and 400 μM (●). The solid lines represent the best fit of data to first-order processes. B, the first-order rates measured from A were plotted against the PMPApp concentrations. The data were fitted to a hyperbola (solid line) to yield a $K_d$ value of 1033 ± 120 μM for PMPApp dissociation and a maximum rate of incorporation of 1.0 ± 0.1 s⁻¹.

summarized in Table IV.

Measurement of Binding Affinity of PMPApp, dATP, and ddATP to the Rat DNA Polymerase β-DNA 22/43-mer Complex at 37 °C—Pre-steady state kinetics revealed slow incorporation of PMPApp into DNA 22/43-mer catalyzed by pol β (data not shown). DNA was previously found to dissociate fast from the rat DNA pol β/DNA complex at a rate of 3–2 s⁻¹ (27). In order to follow a single turnover of nucleotide incorporation, we used a 4:1 molar ratio of active enzyme to DNA to compensate for the fast DNA dissociation from pol β (28). The measurement of the PMPApp equilibrium dissociation constant was carried out at a fixed concentration of pol β (22/43-mer (200:50 nm) and varying PMPApp concentrations (10–400 μM). As shown in Fig. 6A, the single turnover rates of PMPApp incorporation increased with increasing concentrations of PMPApp. The rates were plotted versus the PMPApp concentrations (Fig. 6B), and the data fit a hyperbola with a $K_d$ of 1033 ± 120 μM and a maximum rate of incorporation of 1.0 ± 0.1 s⁻¹. Although there is little curvature in Fig. 6B, we could not perform experiments at higher concentrations of PMPApp. Nonetheless, the quality of the data allows a sufficiently precise estimate of the $K_d$ and maximum rates within reasonable limits of error. The $K_d$ and $k_p$ values for incorporation of dATP and ddATP were measured similarly except that shorter time courses were used due to faster incorporation rates. The data are summarized in Table V. Previous kinetic measurement for dATP incorporation to the rat DNA pol β DNA 25/45-mer gave values of $K_d$ and $k_p$ of 52 ± 6 μM and 24.1 ± 0.9 s⁻¹, respectively (27). These are slightly different from our data (Table V). The differences could be due to different DNA sequences and different reaction conditions. The substrate specificity constants for dATP, ddATP, and PMPApp were calculated and summarized in Table V.

Measurement of Binding Affinity of PMPApp, dATP, and ddATP to the Rat DNA Polymerase β-Gapped DNA 22–20/43-Mer Complex at 37 °C—Pre-steady state kinetic analysis revealed that incorporation of PMPApp into gapped 22–20/43-mer is much faster than incorporation into non-gapped 22/43-mer (data not shown). Therefore, the measurement of the binding affinity of PMPApp to the pol β 22–20/43-mer was carried out at a fixed concentration of pol β 22–20/43-mer (200:50 nm) and varying PMPApp concentrations (10–400 μM) in a shorter time scale than in Fig. 6A. Fig. 7A shows that the single turnover rates of PMPApp incorporation increased with increasing concentrations of PMPApp. Fig. 7B shows a plot of the rates versus the PMPApp concentrations and a fit of the
The inhibition of DNA polymerization by PMPApp and its analogs as well as two nucleotide analogs AZTTP and ddCTP on HIV-1 RT, human DNA polymerases α, β, and γ has been studied previously using steady state kinetic methods (28). The inhibitory constants \((K_i)\) of PMPApp, AZTTP, and ddCTP to human polymerase α were 5.2, 258, and 87.1 \(\mu M\), respectively, to human polymerase β were 81.7, 140, 1.32 \(\mu M\), respectively, and to human polymerase γ were 59.5, 18.3, 0.034 \(\mu M\), respectively (28). PMPApp was then concluded to be more inhibitory to human polymerase α than AZTTP and ddCTP, less inhibitory to human polymerase β than ddCTP, and less inhibitory to human polymerase γ than AZTTP and ddCTP (28). However, due to the limitations of the steady state kinetic method, especially as applied to DNA polymerases with chain terminating analogs (28), these steady state kinetic analyses do not provide rigorous kinetic information to explain how PMPApp could be a highly selective and effective inhibitor.

Steady state kinetic analysis follows the rate-limiting step of multi-step reactions over multiple turnovers. In studies of DNA polymerases, the rate-limiting step is often the dissociation of the enzyme from the DNA following a rapid extension of primer. Considering the further complications that arise due to irreversible inhibition by chain terminating analogs, steady state analysis of DNA polymerization in the presence of chain terminators such as PMPApp is difficult to interpret. Thus, although steady state kinetic studies are useful for preliminary screens of activity, pre-steady state kinetic studies are needed for a rigorous analysis.

Previous mechanistic studies based upon single nucleotide incorporation catalyzed by HIV-1 RT have shown that the rate-limiting step in polymerization is a protein conformational change prior to phosphodiester bond formation, and in single nucleotide incorporation assays subsequent turnovers are limited by the dissociation of the E-DNA complex (19, 23). In this paper, we used transient kinetic methods to follow polymerization at the enzyme active site in the first turnover and to determine the true substrate dissociation constants \((K_d)\) and incorporation rates \((k_p)\) for PMPApp, dATP, and ddATP. The probability of primer extension \(versus\) reaction termination is then determined by the relative rates of incorporation of a regular nucleotide \(versus\) an inhibitor. This probability is a function of the substrate specificity constant, \(k_p/K_d\), and substrate concentration as defined by Equation 2 (29). Neither \(K_d\) nor \(k_p\) individually is enough to assess the selectivity of PMPApp as an inhibitor. The substrate specificity constant,

\[
\text{Relative rates} = \frac{[dNTP]}{[inhibitor]} \frac{k_p}{k_p/K_d} \frac{K_d}{[dNTP]} \frac{k_p}{[K_d]} \frac{K_d}{[inhibitor]} = k_p/K_d
\]

\(k_p/K_d\) is a measure of how selectively an enzyme incorporates two different types of molecules that compete for the same substrate-binding site when present in the same reaction mixture and at the same concentration.

Structural Difference of PMPA, dAMP, and ddAMP—As shown in Fig. 1, ddAMP lacks only the ribose 3’-hydroxyl in dAMP. PMPA has a very different structure from dAMP or ddAMP. The base adenine is separated by only 5 bond lengths \((N—C—C—O—C—P)\) from the phosphorus of PMPA, which is the target of the primer’s nucleophilic attack during the nucleotidyl transfer reaction. In contrast, the adenine and the phos-

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### Table V

| dNTP   | \(k_p\) | \(K_d\) | \(k_p/K_d\) |
|--------|---------|---------|-------------|
| dATP   | 79 ± 5  | 97 ± 25 | 0.81        |
| ddATP  | 62 ± 5  | 79 ± 16 | 0.78        |
| PMPApp | 1.0 ± 0.1| 1033 ± 120 | 0.97 × 10^{-3} |

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### Table VI

| dNTP  | \(k_p\) | \(K_d\) | \(k_p/K_d\) |
|-------|---------|---------|-------------|
| dATP  | 77 ± 5  | 11 ± 2  | 7.0         |
| ddATP | 89 ± 6  | 12 ± 2  | 7.4         |
| PMPApp| 82 ± 6  | 145 ± 24| 0.57        |
phorus of α-phosphate of dAMP or ddAMP are separated by 6 bond lengths (N—C—O—C—C—O—P). Thus, the separation between the adenine and phosphorus in PMPA is shorter by one C—O bond (1.4 Å) than in dAMP or ddAMP. Although the spatial arrangement of these bonds is different in PMPA and dAMP and an O—P bond (1.76 Å) in dAMP is slightly shorter than a C—P bond (1.87 Å) in PMPA (30), the overall distance between the adenine and phosphorus is shorter by approximately 1 Å in PMPA than in dAMP or ddAMP. This shorter distance, the phosphate, and very different structure will significantly affect the incorporation of PMPApp as catalyzed by the three different enzymes examined. Given these structural differences and the well established fidelity of DNA polymerases, it is surprising that the PMPApp is as effective as it is.

**PMPApp Is an Effective Inhibitor of HIV-1 RT**—As shown in Table II, PMPApp binds to the RT-DNA complex as tight as ddATP, and its $K_d$ value is 7-fold lower than that of dTATP. PMPApp binds to the RT-RNA complex slightly tighter than ddATP, and its $K_v$ value is only 2-fold lower than that of dATP (Table III). These analyses indicate that significant structural changes of the nucleotide from regular dATP to ddATP and to PMPApp did not dramatically influence substrate affinity. The data suggest that the catalytic interaction between the aspartic acid triad (Asp-185, -110, and -186) of RT through two active data suggest that the catalytic interaction between the aspartic acid triad (Asp-185, -110, and -186) of RT through two active

The binding of PMPApp to the T7 exo-DNA complex is 34-fold weaker than dATP, and incorporation of PMPApp is 1625-fold slower than dATP (Table IV). Thus the substrate specificity constant for PMPApp is 5.4 $\times$ 10$^4$-fold lower than that of dATP (Table IV) and is only 4-fold higher than for a misincorporation of dGTP opposite a template base A (29). This indicates that PMPApp is a very weak inhibitor of T7 DNA polymerase, and its incorporation by T7 DNA polymerase will be tremendously slow and will be further attenuated by the proofreading exonuclease (38). Interestingly, the excision of incorporated PMPApp by the 3′→5′-exonuclease of T7 DNA polymerase could involve breakage of the C→P bond and produce an unstable leaving carbanion based on a general kinetic mechanism for exonuclease-catalyzed hydrolysis (39). The structural and phosphorylation sites of PMPApp discussed above. For example, the hydrogen bonding between the hydroxyl of Tyr-26 and the β-phosphate of nucleotide (31) was not disrupted by the ribose structural change from ddATP to PMPApp.

Surprisingly, the polymerization rates for incorporation of the three inhibitors are similar with the DNA 22/43-mer duplex (Table II). This suggests that the significant structural change in the ribose of the nucleotide is tolerated by RT which is apparently more flexible in the requirement of base pairing geometry at its active site affording a lower fidelity (29). The incorporation of PMPApp to the RT-RNA complex is 2–3-fold slower than ddATP and dATP (Table III). This indicates that the change from α-phosphate of ddATP or ddATP to α-phosphate of PMPApp slightly slows down the rate-limiting protein conformational change prior to the chemistry step of nucleotide incorporation with the RNA template (19). The higher sensitivity to the change in nucleotide ribose observed with the RNA template may be related to the helix type because DNA/RNA duplexes are A-type and DNA/DNA duplexes are B-type.

As shown in Tables II and III, the substrate specificity constant for PMPApp is close to that of ddATP and is 6–7-fold lower than that of dATP with either the DNA or RNA template. This suggests that PMPApp is as potent as ddATP in inhibition of HIV-1 RT confirming steady state results (28) but with a more rigorous kinetic basis. Since HIV-1 RT has no proofreading exonuclease, DNA synthesis is terminated once PMPApp is incorporated. These data provide the mechanistic basis for the effectiveness of PMPA as an antiviral drug as previously established by reports of its anti-SIV (11, 32) and anti-HIV activities both in vitro and in vivo (12, 13).

**PMPApp Is a Very Poor Inhibitor of T7 DNA Polymerase**—As shown in Table IV, ddATP and dATP have similar binding affinity, incorporation of ddATP is 2-fold slower than dATP, and the substrate specificity constant for ddATP is slightly lower than that for dATP. These kinetic data suggest that ddATP is an effective inhibitor for T7 DNA polymerase and provide a kinetic basis for the previous observation that T7 DNA polymerase does not discriminate against dideoxynucleotides (33). The efficient incorporation of ddNTPs by T7 DNA polymerase is due to the hydroxyl of Tyr-526 in T7 DNA polymerase which replaces the missing 3′-hydroxyl of a ddNTP to hydrogen bond with the pro-(S)-oxygen of the β-phosphate of ddNTP (34). A phenylalanine mutation of Tyr-26 increases discrimination against ddNTPs several thousand-fold (35). Interestingly, all mitochondrial DNA polymerases have a corresponding tyrosine located in the polymerase motif B (human, Tyr-951) (36). Like T7 DNA polymerase, these tyrosines may lead to the high sensitivity of mitochondrial DNA polymerases to dideoxynucleotides. Mitochondrial toxicity has been observed using nucleoside analogs in the treatment of AIDS and hepatitis B (9, 37). Although the mitochondrial polymerase has a proofreading exonuclease that could remove chain terminators, the efficacy of the proofreading function has not been established.

The binding of PMPApp to the T7 exo-DNA complex is 34-fold weaker than dATP, and incorporation of PMPApp is 1625-fold slower than dATP (Table IV). Thus the substrate specificity constant for PMPApp is 5.4 $\times$ 10$^4$-fold lower than that of dATP (Table IV) and is only 4-fold higher than for a misincorporation of dGTP opposite a template base A (29). This indicates that PMPApp is a very weak inhibitor of T7 DNA polymerase, and its incorporation by T7 DNA polymerase will be tremendously slow and will be further attenuated by the proofreading exonuclease (38). Interestingly, the excision of incorporated PMPApp by the 3′→5′-exonuclease of T7 DNA polymerase could involve breakage of the C→P bond and produce an unstable leaving carbanion based on a general kinetic mechanism for exonuclease-catalyzed hydrolysis (39), or involve breakage of the P→O bond and produce a stable leaving PMPA in a non-traditional kinetic pathway. The crystal structure of T7 DNA polymerase complexed with E. coli thioredoxin, DNA 22/26-mer duplex, and next correct nucleotide has revealed that the ribose and triphosphate moieties of the nucleotide are contacted extensively by a number of conserved residues (Asp-475, Glu-480, His-506, Arg-518, Tyr-526, Lys-533, and Asp-654) and by two metal ions at the polymerase active site (34). These interactions could be affected significantly by the very different structure of PMPApp discussed above. For example, the hydrogen bonding between the hydroxyl of Tyr-526 and the β-phosphate of nucleotide could be disrupted by the phosphate in PMPApp, resulting in low affinity of PMPApp to the T7 exo-DNA complex. Further studies on the excision of DNA containing a 3′ terminal PMPA are needed to address these questions.

The very low incorporation rate of PMPApp (0.096 s$^{-1}$) is possibly due to a rate-limiting chemistry step, or a rate-limiting protein conformational change prior to the chemistry step (18), or both. In contrast to HIV-1 RT, T7 DNA polymerase is a highly selective enzyme that is extremely sensitive to proper base pair geometry (29). The lack of a ribose and the shorter distance between the Watson-Crick base-paired adenine and the phosphorus by 1 Å in PMPApp compared with dATP could lead to misalignment of the primer 3′-hydroxyl against the phosphate of PMPApp resulting in either slower protein conformational change step, or slower chemistry step, or both.

**PMPApp Is a Poor Inhibitor of Rat DNA Polymerase β and Very Likely Human DNA Polymerase β**—With a non-gapped DNA 22/43-mer duplex, the binding and incorporation of ddATP are very similar to dATP (Table V). The specificity constants for ddATP and dATP are almost identical. These data indicate that ddATP is a very effective inhibitor of rat DNA polymerase β complexed with a non-gapped DNA. However, the binding of PMPApp to the pol β/22/43-mer complex is 10-fold weaker, and the incorporation of PMPApp is 79-fold slower than dATP (Table V). These differences suggest that significant structural alteration in PMPApp compared with dATP dramatically affects nucleotide binding and incorporation. The crystal structure has shown that the phosphorus of α-phosphate of incoming dNTP in the open conformation is only...
1 Å further away from the primer 3’ terminus than in the closed conformation of human DNA polymerase β complexed with a gapped DNA (40). As discussed above with T7 DNA polymerase, the unique structure of PMPApp, particularly the 1 Å shorter distance between the adenine and phosphorus in PMPApp compared with regular dATP, could significantly affect the alignment and distance between the primer 3’ terminus and the phosphorus, leading to both slower protein conformational change and/or chemistry. The substrate specificity constant for PMPApp is 1.2 × 10^4-fold lower than for dATP (Table V). This suggests that PMPApp is a poor inhibitor of rat DNA polymerase β regardless of the enzyme role in DNA replication (41, 42).

As shown in Table VI, the binding affinity of dATP, ddATP, and PMPApp increases by 7–9-fold to pol β complexed with a gapped DNA 22–20/43-mer than compared with a non-gapped DNA 22/43-mer. Ahn et al. (43) also found that single nucleotide gapped DNA leads to tighter binding of correct nucleotides to rat DNA polymerase β by a factor of 6–29-fold over non-gapped DNA. The higher binding affinity of nucleotide with single nucleotide gapped DNA is not due to additional base stacking interaction between the incoming nucleotide and the base at the 5’ downstream primer terminus because the gapped DNA was bound by enzyme with a 90° kink precisely at the 5’-phosphodiester link of the template residue opposite the incoming nucleotide (40). However, the kink in the gapped DNA exposed the template-dNTP base pair to the enzyme, increasing the interaction between the enzyme and DNA. The exposed base of template residue stacks with the thumb’s Lys-280 peptide plane and side chain, and the dNTP’s base contacts the methylene group of Asp-276. The nascent base pair-enzyme interactions will thereby increase binding affinity of incoming nucleotide. The binding of the HhH motif of the N-terminal 8-kDa domain to the downstream gapped DNA stabilizes an nucleotide. The binding of the HhH motif of the N-terminal 280 peptide plane and side chain, and the dNTP’s base contacts the triphosphate moiety of nucleotide and helix K of the thumb of pol β (40).

As shown in Tables V and VI, the incorporation rates of dATP and ddATP are similar with gapped or non-gapped DNA. Ahn et al. (43) also observed that the incorporation rates of correct dNTPs are the same with gapped DNA as with non-gapped DNA. However, the incorporation rate of PMPApp is 82-fold higher with 22–20/43-mer than with 22/43-mer and equals the incorporation rates of dATP and ddATP. As discussed above with HIV-1 RT, this implies that the ribose structural change from dATP or ddATP to PMPApp did not affect the base pairing geometry at the active site of pol β complexed by the gapped 22–20/43-mer. It is not clear how this tolerance occurs.

The substrate specificity constants for dATP, ddATP, and PMPApp, with the 22–20/43-mer, are 10–600-fold higher than with the 22/43-mer. This indicates that catalytic efficiency is higher with single nucleotide gapped DNA than with non-gapped DNA, an effect that has been observed by others (43, 45). This is not surprising considering that DNA polymerase β plays a central role in single nucleotide gap-filling synthesis as a part of base excision repair (46, 47). The substrate specificity constants for ddATP and dATP are similar. This confirms that DNA polymerase β is very sensitive to dideoxynucleotide inhibitors (48). However, the substrate specificity constant for PMPApp is 12-fold lower than that for dATP (Table VI). It suggests that PMPApp is a weak inhibitor of pol β in the absence of repair. If the base excision repair system can remove the incorporated PMPA, which fills a gapped substrate to generate the nicked DNA, the inhibitory effect of PMPA on pol β acting as repair enzymes will be even lower. Therefore, PMPA should be a poor inhibitor of DNA polymerase β in both replication and repair.

PMPApp Is An Effective Antiviral Drug with Low Toxicity—

The kinetic analyses with HIV-1 RT have demonstrated that PMPApp is as effective as ddATP with both DNA and RNA templates. We have also shown that in contrast to ddATP, PMPApp is a very poor inhibitor of T7 DNA polymerase. Finally, DNA polymerase β is shown to be poorly inhibited by PMPApp in both replication and base excision repair, whereas ddATP inhibits DNA polymerase β strongly. Moreover, human and rat DNA polymerases β should have very similar activities since they are 95% homologous (49). T7 DNA polymerase could be a better model for human DNA polymerase γ because they both have 3’→5’-exonuclease activities and belong to the family A DNA polymerases (50), because they are replicative polymerases, and because they are sensitive to dideoxynucleotide inhibitors (35). However, it is likely that the mitochondrial polymerase has much lower fidelity and processivity (51) although these parameters remain to be established with the complete holoenzyme.

Unlike ddATP, PMPApp could be a poor inhibitor of human DNA polymerases β and γ. Since toxicity of nucleoside analogs such as AZT and ddC in the treatment of AIDS is predominantly due to inhibition of mitochondrial DNA synthesis (9, 37), and since PMPA requires only two phosphorylation steps to reach its active metabolite, PMPApp, whereas nucleoside analogs require three phosphorylation steps, PMPA could be a less toxic but more effective anti-HIV drug than nucleoside analogs. This conclusion is consistent with the potent anti-HIV activity in Phase I/II clinical study reported recently by Gilead Sciences (11) and anti-SIV activity in macaques without toxicity.

Like nucleoside analogs, mutations in the viral genome could lead to resistance to PMPA. The study of long term therapeutic and toxic effects of PMPA in SIV-infected newborn rhesus macaques has revealed emergence of virus with 5-fold decreased susceptibility to PMPA associated with a K65R mutation and additional mutations in SIV RT (32). But all PMPA-treated macaques remained healthy for more than 13 months, and no toxic side effects were detected (32). Thus the lower toxicity, as defined by our kinetic analysis, could afford more therapeutic benefit. As the methods established in this paper, pre-steady state kinetic studies of the PMPA-resistant RT should be able to reveal the kinetic basis for PMPA resistance.

In summary, we have used pre-steady state kinetic analyses to establish a kinetic basis for PMPA as an effective and selective antiviral chain terminator. Based on the measured substrate specificity constants, PMPApp and ddATP have similar inhibitory effects on HIV-1 RT, but PMPApp is a very poor inhibitor of both T7 DNA polymerase and rat DNA polymerase β which are inhibited strongly by ddATP. In contrast to ddATP, PMPApp should be a very poor inhibitor of human DNA polymerase β and γ due to their similarity to rat DNA polymerase β and T7 DNA polymerase, respectively. These pre-steady state kinetic analyses define a kinetic basis for PMPA being an effective and selective antiviral inhibitor and will serve as a basis to screen new analogs in continued efforts to obtain less toxic drugs.

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Selectivity of an Antiviral Inhibitor PMPA

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