ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2002, p. 1610–1613 Vol. 46, No. 5
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.5.1610–1613.2002
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Tenofovir Diphosphate Is a Poor Substrate and a Weak Inhibitor of Rat DNA Polymerases α, δ, and ε*

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Received 30 August 2001/Returned for modification 4 December 2001/Accepted 4 February 2002

Tenofovir diphosphate (PMPApp) is a weak inhibitor of DNA polymerases (pol) α, δ, and ε*, with values for the K_i for PMPApp ($^{PMPApp}K_i$) relative to the K_m for dATP ($^{dATP}K_m$) of 10.2, 10.2, and 15.2, respectively. Its incorporation into DNA was about 1,000-fold less efficient than that of dATP, with $^{PMPApp}K_m$ values 350-, 2,155-, and 187-fold higher than $^{dATP}K_m$ values for pol α, δ, and ε*, respectively.

Tenofovir {9-*R*-[2-(phosphonomethoxy)propyl]adenine; PMPA} is an acyclic nucleoside phosphonate (ANP) (12, 14) whose lipophilic prodrug, tenofovir disoproxil fumarate (Viread), was recently approved (18) for the treatment of human immunodeficiency virus (HIV) infection. In cells, PMPA is phosphorylated by AMP kinase (23, 28) and subsequently by nucleoside diphosphate kinase to tenofovir diphosphate (PMPApp) (Fig. 1). PMPApp is a competitive inhibitor (with respect to dATP) and a substrate of HIV type 1 (HIV-1) reverse transcriptase (RT) (9, 30). Diphosphates of several ANPs have been identified as good substrates as well as potent inhibitors of cellular replicative DNA polymerases (pol) in vitro (5, 6, 19–21). This activity correlates well with the cytostatic effects of their parental compounds (1, 2, 14, 25, 31), suggesting that the interaction of phosphorylated analogs with cellular DNA synthesis may significantly contribute to their cellular toxicity.[2]

In this study, we examined the substrate affinity and inhibitory efficiency of PMPApp with respect to rat pol α, δ, and ε*. PMPApp was synthesized from PMPA (16) by the standard procedure (17). The enzymes were isolated from Sprague-Dawley rat compact transplantable lymphomas by previously described procedures (7, 22) but omitting glycerol gradient centrifugation. The human recombinant proliferating cell nuclear antigen was purified from BL21(DE3) cells by using a published protocol (15). Enzyme activities on the template-primer $DNA_{40/18}$ (5′-GAGATCTCCTAGGGGCCC, 3′-CTC TAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAAT C-5′; molar ratio, 1:1.5) were measured under the following reaction conditions: (i) for pol α, 40 mM HEPES-KOH (pH 7.0), 25 mM KCl, and 10 mM $MgCl_2$; (ii) for pol δ, 40 mM HEPES-KOH (pH 7.0), 50 mM KCl, 10 mM $MgCl_2$, and proliferating cell nuclear antigen (18 μg ml^{-1}); and (iii) for pol ε*, 40 mM HEPES-KOH (pH 7.5), 100 mM KCl, and 10 mM $MgCl_2$. All reaction mixtures also contained 1 mM dithiothreitol, 200 μg of BSA ml^{-1}, and 10% glycerol. The experiments using poly(dT)-oligo$(dA)_{12–18}$ as a template primer were conducted as previously published (21). One unit of DNA pol activity is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of dATP into the template primer poly(dT)-oligo$(dA)_{12–18}$ after 30 min of incubation under previously described reaction conditions (21).

Efficiencies of PMPApp and dATP incorporation catalyzed by examined DNA pol were compared by measurement of kinetic constants (K_m for PMPApp [$^{PMPApp}K_m$], $^{PMPApp}V_{max}$, $^{dATP}K_m$, $^{dATP}V_{max}$) using the template-primer $DNA_{40/18}$ with 0.5 μM ^{32}P-labeled primer. The experiments using the reaction mixture (25 μl) were carried out at 37°C in the presence of eight different concentrations of either dATP or PMPApp by using the reaction conditions described above. After incubation over four different time intervals, the products were processed as described previously (6) and separated by polyacrylamide gel electrophoresis. The amounts of extended and unextended primer were assessed by using a PhosphoImager (Molecular Dynamics, Sunnyvale, Calif.). Kinetic experiments to determine $^{PMPApp}K_i$ and $^{dATP}K_m$ values for the enzymes on the poly(dT)-oligo$(dA)_{12–18}$ template-primer were performed as outlined previously by Kramata et al. (21).

Our results show a very poor capability of all three enzymes for incorporation of the analog into DNA (Fig. 2 and 3); the incorporation efficiency (f_{ins}) (8) was approximately 3 orders of magnitude lower for PMPApp than for dATP, with $^{PMPApp}K_m$ values being 350-, 2,155-, and 187-fold higher than those for

TABLE 1. Incorporation of PMPApp into template-primer $DNA_{40/18}$ catalyzed by DNA pol α, δ, and ε*[a]

DNA pol	$^{dATP}K_m$ (μM)	$^{dATP}V_{max}$ (pmol min^{-1} $μg^{-1}$)	$^{PMPApp}K_m$ (μM)	$^{PMPApp}V_{max}$ (pmol min^{-1} $μg^{-1}$)	% f_{inc}[b]
pol α	0.69 ± 0.28	8.5 ± 0.9	244 ± 87	3.2 ± 0.6	0.106
pol δ + PCNA	0.29 ± 0.05	0.58 ± 0.05	625 ± 73	0.34 ± 0.05	0.027
pol ε*	5.1 ± 1.1	1.3 ± 0.2	955 ± 203	0.20 ± 0.01	0.08

[a] Reaction procedures were performed as described in the text. Kinetic constants are given as the means ± standard deviations from at least three independent experiments.
[b] f_{inc} (%) = 100 × ($^{PMPApp}V_{max}$ × $^{dATP}K_m$)/($^{dATP}V_{max}$ × $^{PMPApp}K_m$) (8).

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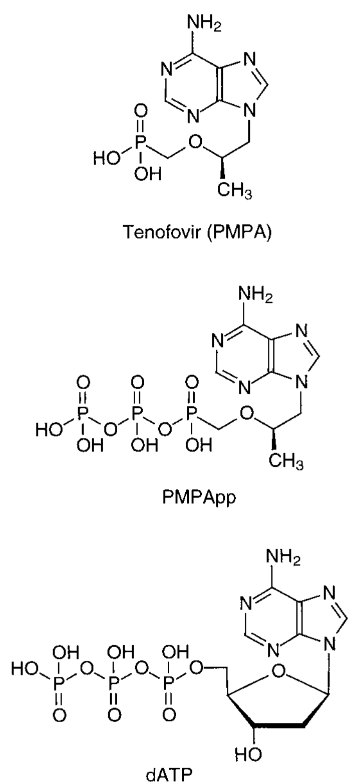

FIG. 1. Structures of tenofovir, PMPApp, and dATP.

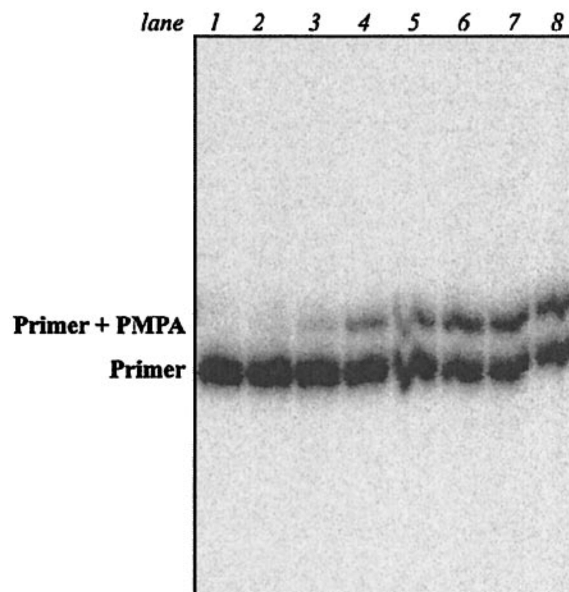

FIG. 3. Kinetics of PMPA incorporation catalyzed by DNA pol α. Reaction mixtures containing pol α (4 U/ml) and PMPApp at various concentrations (left to right, lanes 2 to 8: 5, 25, 125, 250, 500, 750, and 1,500 μM) were incubated in the presence of the template-primer $DNA_{40/18}$ for 32 min at 37°C. Lane 1: primer only.

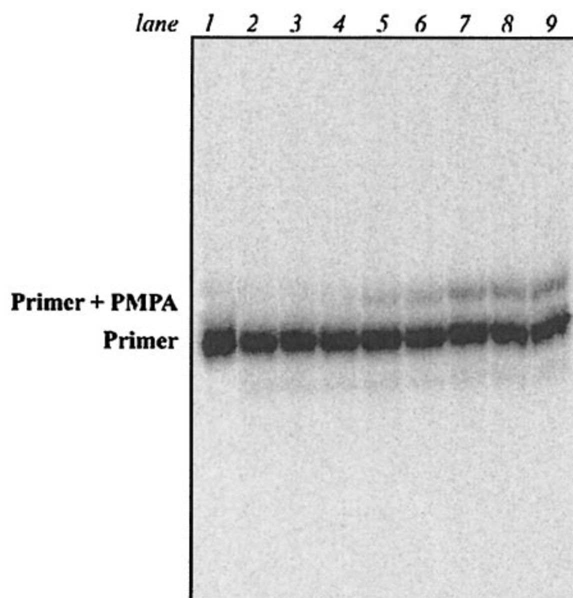

FIG. 2. Kinetics of PMPA incorporation catalyzed by DNA pol δ. Reaction mixtures containing pol δ (0.6 U/ml) and PMPApp at various concentrations (left to right, lanes 2 to 9: 1, 5, 25, 125, 250, 500, 750, and 2,500 μM) were incubated in the presence of the template-primer $DNA_{40/18}$ for 18 min at 37°C. Activity of pol δ-associated 3′-5′-exonuclease is detectable at all concentrations of PMPApp. Lane 1: primer only.

${}^{dATP}K_m$ for pol α, δ, and ε*, respectively (Table 1). In contrast, HIV RT incorporates PMPApp with only sixfold lower efficiency than dATP (30). The relative incorporation efficiencies for several other ANPs, as determined in our earlier studies for pol α, δ, and ε*, were in the range of 0.35 to 120% (5, 6, 19, 20) (Table 2). The values for PMPApp as measured in this study (0.03 to 0.11%; Table 1) clearly demonstrate a significantly lower substrate affinity of this analog toward examined DNA pol compared to those of other ANPs. Relative incorporation efficiency values determined for PMPApp and human DNA pol α by Cihlar and Chen (13) were approximately 10-fold higher than our values, owing to different ${}^{PMPApp}K_m$ values in the two studies. This discrepancy might reflect differences in reaction conditions, template-primer, and/or biological origin of the enzymes used.

In addition to being recognized as a substrate, PMPApp may also act as an inhibitor of DNA pol. To analyze its inhibitory activity toward pol α, δ, and ε*, we used poly(dT)-oligo $(dA)_{12-18}$ as a template-primer. Kinetic analysis of the experiments showed that PMPApp was a very weak competitive (for pol δ and pol ε*) or uncompetitive (for pol α) inhibitor, with K_i values more than 10-fold higher than K_m values for dATP (Table 3). On the other hand, HIV RT is relatively strongly inhibited by PMPApp, with a ${}^{PMPApp}K_i/{}^{dATP}K_m$ ratio equal to 0.34 (9). Our data correlate with the observation that PMPApp exhibits little inhibitory effect on the in vitro model of eukaryotic DNA replication at concentrations as high as 1 mM (27). When compared to the results of a previous study (performed under identical conditions), PMPApp seems to be an at least 10-fold-weaker inhibitor of DNA pol than PMEApp, the active form of another antiretroviral drug, adefovir (21). Moreover, it

TABLE 2. Comparisons of ANP cytotoxicities to their interactions with DNA pol α, δ, and ε*

ANP	$^{ANPpp}K_i/^{dNTP}K_m$ [h]			% f_{inc} [e,i]			CC_{50} [f]				
	pol α	pol δ	pol ε*[g]	pol α	pol δ	pol ε*[g]	MT4[j] (μM)	CEM[k] (μM)	F-PBMC[k] (μM)	hRPTEC[l] (μM)	HEL[m] (μg/ml)
PMEG[a]	0.03	ND	0.004[r]	6.4	40.2	5.9[h]	11	≥0.8	0.23	ND	2.5
PMEDAP[b]	0.3	0.1	1.3[r]	2.6	20.5	4.2[h]	18	ND	ND	ND	40
(*S*)-HPMPA[c]	2.3	0.3	0.1[r]	7.6	8.7	18.4[r]	52; 26	ND	ND	ND	20
PMEA[d]	1.2	0.6	0.9[r]	0.7	4.4	2.0[r]	67	64	24	500	100
(*R*)-PMPA	10.2	10.2	15.6[r]	0.11	0.03	0.08[r]	>300	≥500	165	>2,000	ND

[a] PMEG, 9-[(2-phosphonomethoxy)ethyl]guanine.
[b] PMEDAP, 9-[(2-phosphomethoxy)ethyl]-2,6-diaminopurine.
[c] (*S*)-HPMPA, (*S*)-9-[(3-hydroxy-2-phosphonomethoxy)propyl]adenine.
[d] PMEA, 9-[(2-phosphonomethoxy)ethyl]adenine.
[e] Percent $f_{inc} = 100 \times (^{ANPpp}V_{max} \times {}^{dNTP}K_m)/(^{dNTP}V_{max} \times {}^{ANPpp}K_m)$ (see reference 8).
[f] CC_{50}, 50% cytotoxic concentration; ND, not determined.
[g] h, human DNA polymerases; r, rat DNA polymerases.
[h] See reference 21.
[i] See references 5, 6, and 19.
[j] See references 1 and 26.
[k] See reference 1.
[l] See reference 11.
[m] See reference 2.

has been shown that PMPApp is also a poor inhibitor and substrate of DNA pol β as well as of mitochondrial DNA pol γ (9, 13). This feature distinguishes PMPApp from several nucleoside analogs presently used in anti-HIV therapies, e.g., didanosine, zalcitabine, and stavudine (4, 9, 13, 24).

Taken together, PMPApp was found to be an exceptionally poor substrate and a weak inhibitor for all three replicative DNA pol. These observations indicate that PMPApp may minimally interfere with nuclear DNA synthesis and at least partially explain the low toxicity of tenofovir in a number of cell culture models (1, 10, 11, 29) as well as the favorable safety profile in treatment of HIV-infected patients with its oral prodrug, tenofovir disoproxil fumarate (see reference 3 and R. Schooley, R. Myers, P. Ruane, G. Beall, H. Lampiris, M. Miller, R. Mills, and I. McGowan, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 692, 2000).

We thank M. Otmar for the synthesis of PMPA diphosphate, L. Tovcigrecko for excellent assistance, and T. Cihlar from Gilead Sciences for comments and review of the manuscript.

This work was supported by the program of key projects no. S4055109 of the Academy of Sciences of the Czech Republic.

TABLE 3. Inhibition of DNA pol α, δ, and ε* with PMPApp on template-primer poly(dT)-oligo(dA)$_{12-18}$ [a]

DNA pol	Kinetic constant value[d]		$^{PMPApp}K_i/^{dATP}K_m$
	$^{dATP}K_m$	$^{PMPApp}K_i$	
pol α	10.0 ± 1.1	102.0 ± 9.5[c]	10.2
pol δ + PCNA	0.71 ± 0.08	7.2 ± 1.3[b]	10.2
pol ε*	6.1 ± 2.3	95.3 ± 13.5[b]	15.6

[a] Reaction procedures were performed as described in the text. Kinetic constants are given as the means ± standard deviations from three independent experiments.
[b] Competitive inhibition.
[c] Uncompetitive inhibition.
[d] Micromolar.

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