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Short Communication

KINETIC ANALYSIS OF THE INTERACTION BETWEEN THE DIPHOSPHATE OF (*S*)-1-(3-HYDROXY-2-PHOSPHONYLMETHOXYPROPYL)CYTOSINE, ddCTP, AZTTP, AND FIAUTP WITH HUMAN DNA POLYMERASES β AND γ

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Abstract—The inhibitory effects of the diphosphate of (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPCpp) toward human DNA polymerases β and γ were studied. The K_i values of HPMPCpp were compared with the K_i values of the triphosphates of 3′-azidothymidine (AZTTP), 2′,3′-dideoxycytidine (ddCTP) and 5-iodo-2′-fluoroarabinosyluridine (FIAUTP). The K_i values toward DNA polymerase β in increasing order were 1.32, 1.43, 140, and 520 μM for ddCTP, FIAUTP, AZTTP and HPMPCpp, respectively. The K_i values toward DNA polymerase γ in increasing order were 0.034, 0.031, 18.3 and 299 μM for ddCTP, FIAUTP, AZTTP and HPMPCpp, respectively. Therefore, HPMPC would be expected to have less inhibitory effects on DNA repair (DNA polymerase β) and mitochondrial DNA synthesis (DNA polymerase γ) than ddC, FIAU or AZT.

Key words: antiviral; DNA polymerase β; DNA polymerase γ; enzyme inhibition; DNA synthesis; mechanism of action

HPMPC†, an acyclic cytosine nucleotide analog with potent *in vitro* and *in vivo* activity against a broad spectrum of herpes viruses [1, 2], has been shown to exert a dose-dependent anti-CMV effect, as measured in the urine and semen of advanced AIDS patients [3]. HPMPC is also in clinical trials for treating topical HSV and HPV infections. HPMPC is a cytosine nucleoside monophosphate analog; unlike ACV and GCV, HPMPC does not require a rate-limiting step of phosphorylation by viral encoded kinases. Many strains of HSV mutants resistant to ACV (thymidine kinase-altered or -deficient mutants) [2, 4] and CMV mutants resistant to GCV [5] remain sensitive to HPMPC. Similar to other antiviral nucleoside analogs, the mechanism of cytotoxic activity of HPMPC is believed to be the inhibition of human DNA polymerases by HPMPCpp. The inhibition of human DNA polymerase α and DNA polymerases from HSV 1 and 2 by HPMPCpp has been reported [6]. This manuscript reports the kinetic analyses of HPMPCpp with human DNA polymerases β and γ. AZTTP, ddCTP and FIAUTP were included for comparison purposes.

Materials and Methods

Materials. Radioactive dNTPs were purchased from New England Nuclear, Inc. (Boston, MA). dNTPs, ddCTP and activated calf thymus DNA were purchased from Pharmacia (Piscataway, NJ). BSA, dithiothreitol, KCl, $MgCl_2$, glycerol and Tris–HCl were from Sigma (St. Louis, MO). DE-52 cellulose was obtained from Whatman, Inc. (Clifton, NJ).

Synthesis of HPMPCpp. The title compound was prepared using a modified method of Ho *et al.* [6]. The following modifications were used in the synthesis of HPMPCpp. Pyrophosphate was removed after the conversion of (*S*)-1-[3-benzyloxy-2-(phosphonomethoxy)-propyl]cytosine to the diphosphate by passing through a Prep-Sep® C-8 reversed-phase column using a water-acetonitrile gradient. ^{31}P-NMR analysis showed that this method was effective in removing a majority of the excess pyrophosphate. The resulting benzyloxy-HPMPCpp was then deprotected and purified. Benzyloxy-HPMPCpp (37 mg, 31 μmol, 1.0 eq) in 1 mL of D_2O was treated with ammonium formate (18 mg, 0.29 mmol, 9.4 eq) and palladium on carbon (18 mg). This mixture was immersed in a 50° oil bath and stirred for 85 min. *It is noteworthy that higher reaction temperatures led to sizable formation of the over-reduced product, dihydro-HPMPCpp, as identified by ^{1}H-NMR, which was not distinguishable from the desired product by HPLC.* The mixture was again passed through a Prep-Sep® C-8 reversed-phase column using a water-acetonitrile gradient. This removed all of the unreacted benzyloxy-HPMPCpp. The material was then passed through a DEAE Trisacryl® column using a water to 500 mM triethylammonium bicarbonate gradient at pH 8.3. The identity and purity of the final product were confirmed by HPLC and ^{13}C-, ^{31}P-, and ^{1}H-NMR analysis.

DNA polymerases. Recombinant human DNA polymerase β was purchased from Molecular Biology Resources (Milwaukee, WI) and was cloned and purified by methods described previously [7, 8]. DNA polymerase γ was purified from A3.01 cells. (A3.01 is a human T-cell hypoxanthine/aminopterin/thymidine-sensitive derivative of CEM cells

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† Abbreviations: ACV, acyclovir; AZTTP, 3′-azidothymidine triphosphate; CMV, cytomegalovirus; ddCTP, 2′,3′-dideoxycytidine triphosphate; dNTPs, deoxynucleoside triphosphates; d4T, 2′,3′-dideoxy-2′,3′-didehydrothymidine; FIAUTP, 5-iodo-2′-fluoroarabinosyluridine triphosphate; GCV, ganciclovir; HPMPC, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; HPMPCpp, HPMPC diphosphate; HPV, human papillomavirus; and HSV, herpes simplex virus.

Table 1. Kinetic constants for human DNA polymerases β and γ*

DNA polymerase	K_m (μM)		K_i (μM)			
	dCTP	TTP	HPMPCpp	ddCTP	AZTTP	FIAUTP
Beta	4.3	4.7	520	1.32	140	1.43
Gamma	0.21	0.54	299	0.034	18.3	0.031

* All inhibitions showed competitive kinetics.

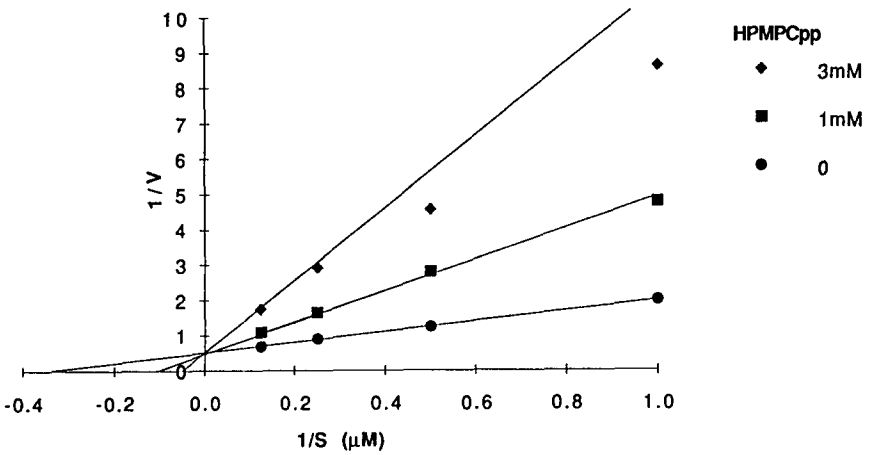

Fig. 1. Lineweaver–Burk plot showing competitive inhibition of human DNA polymerase β by HPMPCpp. The enzyme assay is described in Materials and Methods. Velocities are shown in the absence of HPMPCpp and in the presence of 1 and 3 mM HPMPCpp. The plot was generated by the KinetAsyst program, which fit the data into competitive, non-competitive, and uncompetitive inhibition patterns. The data were found to have the best fit with a competitive inhibition pattern.

obtained from Dr. Thomas Folks of the National Institute of Allergy and Infectious Diseases. Dr. Folks is now at the Centers for Disease Control.) One unit of DNA polymerase β or γ is designated as the amount of enzyme activity that incorporates 1 nmol of total nucleotide into an acid-insoluble form in 60 min at 37°. Briefly, mitochondria were purified from freshly harvested A3.01 cells by disrupting with a Dounce homogenizer and differential centrifugation. The purified mitochondria were disrupted, and the DNA polymerase γ was purified by DEAE and heparin chromatographies. (Purified DNA polymerase γ has a specific activity of 3.8 U/mg protein, while the supernatant of the mitochondria homogenate has a specific activity of 0.081 U/mg protein.) This purified DNA polymerase γ was found to be free of nuclease activity.

Assay for DNA polymerases β and γ. Assays were carried out as previously described [9, 10]. Reaction mixtures for DNA polymerase β contained 50 mM Tris (pH 8.9), 5% glycerol, 1 mM dithiothreitol, 500 μg/mL BSA, 5 mM $MgCl_2$, 100 mM KCl, 200 μg/mL activated calf thymus DNA, 60 μM each dATP, dGTP, and TTP, and various concentrations of [^{3}H]dCTP (3 Ci/mmol). HPMPCpp or ddCTP was present at various concentrations. Reaction mixtures contained 0.15 U of DNA polymerase β in a total volume of 60 μL. At various times, 15-μL samples of the reaction mixture were spotted onto Whatman 3 MM filters. The filters were washed three times in 5% trichloroacetic acid containing 1% sodium pyrophosphate, once in ethanol, then dried and counted in 4 mL of Beckman Ready Safe scintillation fluid. DNA polymerase γ assays were carried out in the same buffer as described above for DNA polymerase β except at pH 8.0. HPMPCpp or ddCTP was present at various concentrations. Reaction mixtures contained 0.01 U of DNA polymerase γ in a total volume of 60 μL. Samples (15 μL) were taken at various times and processed as above. [^{3}H]TTP was the variable substrate when AZTTP or FIAUTP was the inhibitor. Kinetic constants were determined by fitting the initial rate data to Lineweaver–Burk plots using the KinetAsyst program (Think Technologies, based on the algorithms described in Ref. 11).

Results and Discussion

The inhibition constants of HPMPCpp with respect to DNA polymerases from HSV-1, HSV-2, and human DNA polymerase α were reported to be 0.86, 1.4, and 51 μM, respectively [6]. The K_i/K_m (HPMPCpp/dCTP) value for DNA polymerase α was reported to be 10.8 [6]. These data indicate that HPMPCpp selectively inhibits HSV-1 and 2 DNA polymerases rather than human DNA polymerase α. Table 1 shows the inhibition constants of the compounds against human DNA polymerases β and γ. The K_i/K_m (HPMPCpp/dCTP) values for DNA polymerases β and γ (Table 1) were 121 and 1424, respectively. These K_i/K_m values indicate that HPMPCpp is also a poor inhibitor of human DNA polymerases β and γ. At the concentrations needed to effect inhibition of herpes simplex viral DNA polymerases, the inhibition of

the human enzymes will be small. Figure 1 shows the Lineweaver–Burk plot for interaction of DNA polymerase β with HPMPCpp, and is interpreted as competitive inhibition. A similar inhibition pattern was obtained for DNA polymerase γ.

The data presented in Table 1 demonstrate that HPMPCpp is much less inhibitory to human DNA polymerases β and γ than ddCTP, FIAUTP, or AZTTP. The previous reported inhibition constants of ddCTP [12] toward human DNA polymerases β and γ were 2.6 and 0.016 μM, respectively, in agreement with our data. The K_i values of 2′,3′-dideoxy-2′,3′-didehydrothymidine triphosphate (d4TTP) toward human DNA polymerases β and γ were reported to be 1.2 and 0.048 μM, respectively.* These data suggest that HPMPC would be expected to have less inhibitory effects on DNA repair and mitochondrial DNA synthesis than ddC, FIAU, AZT, and d4T.

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