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Kinetic Analysis of the Interaction of Cidofovir Diphosphate with Human Cytomegalovirus DNA Polymerase

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ABSTRACT. Cidofovir [CDV, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, HPMPC] is an acyclic cytosine nucleoside phosphonate analog with potent *in vitro* and *in vivo* activity against a broad spectrum of herpesviruses. CDV diphosphate (CDVpp), the putative antiviral metabolite of CDV, is a competitive inhibitor of dCTP and an alternate substrate for human cytomegalovirus (HCMV) DNA polymerase. HCMV DNA polymerase used a synthetic DNA primer–template with a K_m value of 90 ± 8 nM and incorporated dCTP approximately 42 times more efficiently than CDVpp. HCMV DNA polymerase also utilized a synthetic DNA primer containing a single molecule of CDV at the 3′-terminus. The K_m value for this DNA primer–template was 165 ± 42 nM and incorporation of dCTP was approximately 17 times more efficient than that of CDVpp. The slower rate of incorporation of CDVpp was due mostly to the higher K_m value of CDVpp toward the enzyme–primer–template complexes. These data demonstrate that incorporation of a single CDV into DNA by HCMV DNA polymerase does not lead to chain termination. BIOCHEM PHARMACOL 51;11:1563–1567, 1996.

KEY WORDS. antiviral; mechanism; DNA; polymerase; cytomegalovirus; enzymology

HCMV§ is a member of the herpesviruses and can cause life- and sight-threatening infections in immunocompromised patients. The herpesvirus-encoded enzymes have been the targets of successful antiviral therapy. For example, kinases encoded by herpes simplex virus (HSV), varicella zoster virus (VZV), or cytomegalovirus (CMV) selectively phosphorylate antiviral nucleoside analogs such as acyclovir and ganciclovir [1]. Additional phosphorylations of antiviral nucleoside monophosphates by cellular kinases to their respective triphosphate derivatives are required to inhibit the viral polymerases and viral DNA synthesis [2]. Acyclovir {9-[(2-hydroxyethoxy)methyl]guanine} is a potent inhibitor of HSV replication *in vitro* and *in vivo* [3, 4]. Another acyclic nucleoside analog, ganciclovir {9-[(1,3-dihydroxy-2-propoxy)methyl]guanine}, is a potent inhibitor of HCMV replication [5]. The interactions of the triphosphates of acyclovir and ganciclovir with human DNA polymerases α and β, and with HSV-1 DNA polymerase have been studied [2, 6]. Acyclovir is an obligatory DNA chain terminator after its incorporation into DNA. The formation of a tight ternary complex between acyclovir monophosphate-terminated primer–template, HSV-1 DNA polymerase, and the next incoming deoxynucleoside triphosphate was shown to be the mechanism of anti-HSV activity of acyclovir [7]. Ganciclovir is a non-obligatory DNA chain terminator after its incorporation into DNA, catalyzed by both HSV-1 DNA polymerase and DNA polymerase α [2, 8]. The incorporation of ganciclovir monophosphate into DNA by HSV-1 DNA polymerase does not cause chain termination but results in a significant reduction in V_{max} for subsequent elongation [6].

CDV (structure shown below) is an acyclic phosphonate analog of deoxycytidine monophosphate. CDV is resistant to phosphatases and does not require virus-encoded kinases for phosphorylation [9]. CDV has been shown to exert a dose-dependent anti-CMV effect as measured by viral titers in the urine and semen of advanced AIDS patients. CDV inhibits CMV DNA synthesis at the level of the HCMV encoded DNA polymerase. CDVpp inhibits HSV-1 and HSV-2 DNA polymerases with K_i values of 1–2 μM. To further define the mechanism of antiviral activity, we investigated the kinetics of CDVpp interaction with HCMV DNA polymerase using activated calf thymus DNA, a synthetic primer–template system, and a synthetic primer–template containing a single molecule of CDV at the 3′-terminus of the primer.

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§ *Abbreviations:* HCMV, human cytomegalovirus; CDV, Cidofovir, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, HPMPC; CDVpp, CDV diphosphate; DTT, dithiothreitol; and MOI, multiplicity of infection.

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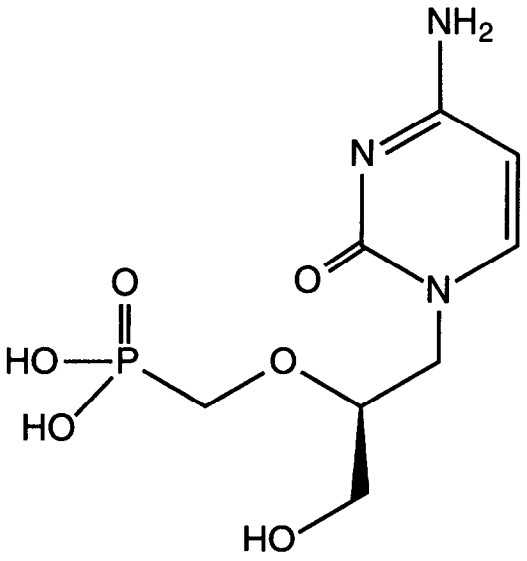

Cidofovir (HPMPC)

MATERIALS AND METHODS

Chemicals

DE52 cellulose and P-11 cellulose were from Whatman (Clifton, NJ). Heparin agarose and single-stranded DNA agarose were from GIBCO-BRL (Grand Island, NY). [γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham (Arlington Heights, IL). [^{3}H]TTP (81 Ci/mmol) and [^{3}H]dCTP (25 Ci/mmol) were from New England Nuclear (Boston, MA). Activated calf thymus DNA was from Pharmacia (Piscataway, NJ). CDVpp was synthesized as previously described [10]. All other chemicals were of the highest grade commercially available.

Purification of HCMV DNA Polymerase

Normal human dermal fibroblasts (NHDF) were infected with HCMV (Towne strain) at an input of 0.005 MOI and were harvested when 60% of the cells showed a cytopathic effect (approximately 4 days). HCMV DNA polymerase was purified through four successive chromatographic steps (DEAE-cellulose, phosphocellulose, heparin agarose, and single-stranded DNA agarose) as described previously [11] with the following modification. Only those fractions containing DNA polymerase activities that could be stimulated by 90 mM $(NH_4)_2SO_4$ were pooled for further purification [11]. For some preparations, in order to stabilize the enzyme column, eluates were collected into tubes containing BSA at a final concentration of 0.2 mg/mL. All buffer solutions contained 10% glycerol. The purified HCMV DNA polymerase could be activated 448% by 90 mM $(NH_4)_2SO_4$. SDS–PAGE of the purified HCMV DNA polymerase showed two protein bands corresponding to molecular weights of 140,000 and 58,000 [11]. The identity of the purified HCMV DNA polymerase was further confirmed by western blot analysis using antibody to 140 kDa subunit. The addition of BSA to the purified HCMV DNA polymerase was found to increase greatly the stability of the enzyme. The activity of the purified HCMV DNA polymerase during the assay stayed linear for at least 90 min. The purified HCMV DNA polymerase was stored at −70° until used.

Enzyme Assays

DNA polymerases from HCMV and human cells were assayed as described previously [12–14]. During enzyme purification, the DNA polymerase assays were monitored by measurement of the incorporation of a tritium-labeled nucleoside triphosphate into acid-precipitable product. Briefly, the 50-μL assay solution contained 5% glycerol, 10 mM $MgCl_2$, 0.4 mM DTT, 20 mM Tris (pH 8.0), a 100 μM concentration of each dATP, dGTP, and dCTP, 5 μM [^{3}H]TTP (4 Ci/mmol), 10 μg of activated calf thymus DNA, and, where indicated, 90 mM $(NH_4)_2SO_4$ [11]. Aliquots were taken at various time points during the incubation and spotted onto Whatman 3MM filter paper discs (2 cm in diameter). The paper discs were washed three times in 5% trichloroacetic acid, 1% pyrophosphate and once in 95% ethanol. After drying, the radioactivities on the discs were measured in a Beckman scintillation counter using Beckman Ready Safe scintillation fluid. One unit of HCMV DNA polymerase was defined as the amount of enzyme catalyzing the incorporation of 1 pmol of dTMP/min at 37°. The kinetic analysis of the CDVpp interaction with HCMV DNA polymerase was carried out as described previously [15].

Synthesis of CDV Terminated Primer

^{32}P-End-labeled synthetic primer SX1 (5′-TGA-CCA-TGT-AAC-AGA-GAG-3′, 150 pmol) was annealed to a synthetic template SX2 (5′-TCT-CTT-CTC-TCT-CTC-TTG-CTC-TCT-GTT-ACA-TGG-TCA-3′). Incorporation of one molecule of CDV at the 3′-end of SX1 was carried out in a 60-μL reaction mixture containing 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.2 mg/mL BSA, 0.4 mM DTT, 90 mM $(NH_4)_2SO_4$, 50 μM CDVpp, and 1.2 units of HCMV DNA polymerase. After overnight incubation at 37°, the reaction mixture was loaded on a 15% polyacrylamide/8 M urea gel. After electrophoresis, the gel was visualized by autoradiography. The gel slice containing the 19 mer with one single CDV at the 3′-terminus was excised and put into 0.2 mL of gel elution buffer (0.1% SDS, 0.5 M NH_4OAc, and 10 mM $MgCl_2$). Elution was carried out at 37° for 4 hr. The gel eluate was transferred to a clean tube and extracted with *n*-butanol to reduce the volume to less than 50 μL. The concentrated gel eluate was loaded on a G-25 quick-spin column (Boehringer Mannheim) to remove salts from the sample. The CDV terminated primer

was quantitated by measuring the ^{32}P radioactivity in a scintillation counter.

Primer containing a single CDV at the 3′-terminus was used to determine the K_m of this primer to HCMV DNA polymerase, and to determine the kinetic constants for the addition of another molecule of CDVpp or dCTP.

In Vitro *DNA Synthesis with 5′-Labeled Primers*

Synthetic primers were phosphorylated with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolaboratories, Beverly, MA). The end-labeled primers were purified by passage through a quick-spin column (G-25 Sephadex, Boehringer Mannheim Biochemicals, Inc., Indianapolis, IN). ^{32}P-Labeled primers were annealed to templates at a 1:2 molar ratio of primer to template in the HCMV DNA polymerase buffer [20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.2 mg/mL BSA, 0.4 mM DTT, and 90 mM $(NH_4)_2SO_4$] by incubating at 65° for 5 min and allowing to cool to room temperature slowly. HCMV DNA polymerase reaction mixture (20 μL) contained 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.2 mg/mL BSA, 0.4 mM DTT, 90 mM $(NH_4)_2SO_4$, 0.05 μM ^{32}P-labeled primer, 0.1 μM template, and 0.096 unit of HCMV DNA polymerase. dATP, dGTP, and TTP, when present, were at 50 μM, whereas CDVpp or dCTP were present at various concentrations. Reaction mixtures were incubated at 37°. Samples were removed at various time points and put into equal volume of stop solution (95% formamide). The products of DNA synthesis were separated by gel electrophoresis on a 15% polyacrylamide/8 M urea gel. After electrophoresis, the gel was visualized by autoradiography and quantitated by an Ambis Image Acquisition and Analysis scanner.

Enzyme Kinetics

Kinetic constants were determined by fitting the initial rate data to the KinetAsyst program ([16], Think Technologies, based on the algorithms described in *Statistical Analysis of Enzyme Kinetic Data* by Dr. W. W. Cleland). All kinetic constants are the averages of at least three separate experiments.

RESULTS AND DISCUSSION

CDVpp inhibited HCMV DNA polymerase with a K_i value of 6.6 ± 0.8 μM and was competitive with respect to dCTP (K_m = 0.72 ± 0.1 μM) incorporation into activated calf thymus DNA. HCMV DNA polymerase utilized the synthetic DNA primer–template with a K_m value of 90 ± 8 nM. Table 1 shows the kinetic constants of dCTP and CDVpp toward HCMV DNA polymerase using synthetic DNA as primer–templates. The K_m values for CDVpp and dCTP using the synthetic DNA were 18.7 ± 1.4 and 0.67 ± 0.07 μM, respectively (Fig. 1). Under these conditions, the K_m value of CDVpp to HCMV DNA polymerase was approximately 28 times higher than that of dCTP. The ratio of V_{max}/K_m (catalytic efficiency) values of dCTP to CDVpp was 41.6 with synthetic DNA as the primer–template. These data indicate that HCMV DNA polymerase can incorporate dCTP about 42-fold more efficiently than CDVpp into DNA.

There are at a minimum four kinetically defined steps in DNA polymerization: (1) binding of template–primer to the enzyme, (2) binding of deoxynucleoside triphosphate to the enzyme–template–primer complex, (3) incorporation of nucleotide into the 3′-end of the primer and release of pyrophosphate from the complex, and (4) translocation of the newly formed 3′-end primer–template at the active site of the polymerase to complete the catalytic cycle. The V_{max} values for incorporating dCTP and CDVpp (0.39 ± 0.08 and 0.27 ± 0.09 pmol/min/unit, respectively) were not significantly different. This indicates that the rates of nucleophilic attack of the 3′-OH group of the elongating primer end on the phosphonate of CDVpp and departure of pyrophosphate were at rates similar to the rate of addition of dCMP at the primer end. This is partially supported by the finding that nucleoside phosphonates analogous to CDV are isoelectronic to nucleoside phosphates and have similar pK_as [17]. Therefore, the V_{max} of incorporating CDVpp is not affected to any significant extent by the lack of a rigid deoxyribose ring and the presence of a phosphonate in CDVpp.

The difference in the overall slower rate of incorporation of CDVpp (V_{max}/K_m) was due mainly to the higher K_m value for the interaction of CDVpp with the enzyme–primer–template complex when compared with dCTP. CDV is a chiral molecule with a defined stereochemistry at the C-2 position of the side chain, and, due to its flexible acyclic side chain, possesses considerable conformational mobility. Therefore, without much energetic constraint, CDV can achieve remarkably high spacial superimposition

TABLE 1. Kinetic constants of dCTP and CDVpp toward HCMV DNA polymerase*

	dCTP			CDVpp		
Primer-template	**K_m(μM)**	**V_{max} (pmol/min/unit)**	**V_{max}/K_m pmol/min/unit/μM)**	**K_m(μM)**	**V_{max} (pmol/min/unit)**	**V_{max}/K_m (pmol/min/unit/μM)**
Synthetic DNA	0.67 ± 0.07	0.39 ± 0.08	0.582	18.7 ± 1.4	0.27 ± 0.09	0.014
Synthetic DNA with 3′-CDV terminus	3.8 ± 1.4	0.19 ± 0.02	0.050	35.3 ± 6.7	0.11 ± 0.02	0.003

* All kinetic constants are the averages of at least 3 separate experiments. For K_m and V_{max}, values are means ± SD.

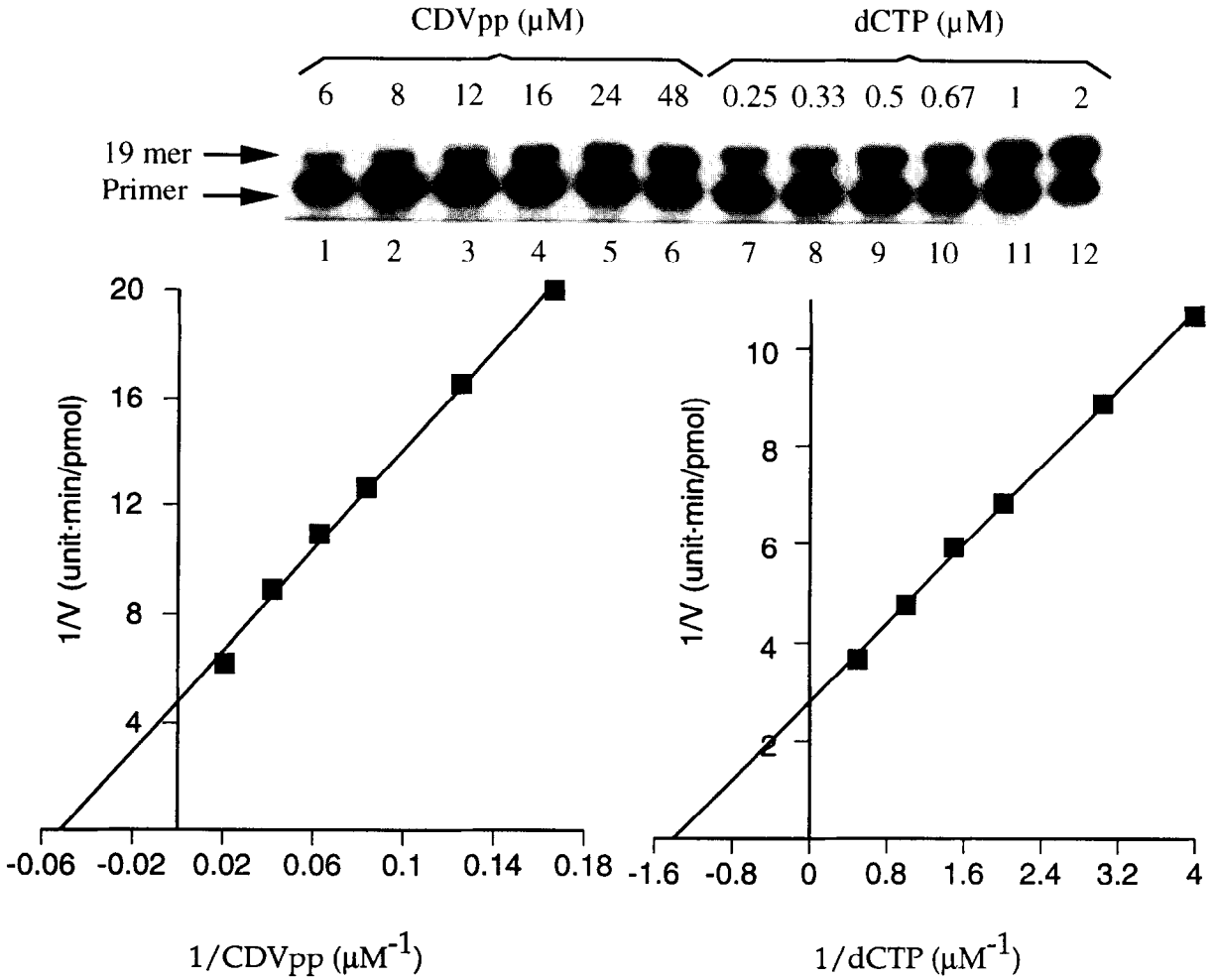

FIG. 1. Top panel: Incorporation of CDVpp and dCTP by HCMV DNA polymerase in a primer extension assay. Two picomoles of ^{32}P-end-labeled primer SX1 annealed at a 1:2 molar ratio to template SX2 was used in each 20-μL reaction.

SX1 (5′-TGA-CCA-TGT-AAC-AGA-GAG-3′)
SX2 (3′-ACT-GGT-ACA-TTG-TCT-CTC-GTT-CTC-TCT-CTC-TTC-TCT-5′)

HCMV DNA polymerase (0.096 unit) was mixed with increasing concentrations of CDVpp (lanes 1–6) or dCTP (lanes 7–12) in HCMV DNA polymerase buffer. The primer extension reactions were carried out for 40 min at 37°. Primer extension products were separated on 15% polyacrylamide/8 M urea gel electrophoresis. An Ambis Image Acquisition and Analysis scanner was used for quantification of the primer extension products (the 19 mers). Bottom left panel: Double-reciprocal plot of CDVpp incorporation. Bottom right panel: Double-reciprocal plot of dCTP incorporation.

on 2′-deoxycytidylate, as shown in Fig. 2. In the conformation shown in Fig. 2, the cytosine ring, and the hydroxyl and the phosphonate functionalities of CDV overlay very well with those of 2′-deoxycytidylate.

HCMV DNA polymerase could also use a synthetic DNA primer containing a single molecule of CDV at the 3′-terminus with a K_m value of 165 ± 42 nM. This K_m value was only slightly higher than the K_m value of 90 ± 8 nM with the 3′-unsubstituted synthetic DNA and indicates that both the synthetic DNA and synthetic DNA containing one CDV molecule at the 3′-terminus have similar affinities toward the HCMV DNA polymerase. In addition, this suggests that the incorporation of a single molecule of CDV at the 3′-terminus does not cause dissociation of the primer–template containing CDV at the 3′-terminus from the enzyme active site and that incorporation of one single molecule of CDV does not cause DNA chain termination. The K_m values for CDVpp and dCTP using synthetic DNA containing one molecule of CDV at the 3′-terminus were 35.3 ± 6.7 and 3.8 ± 1.4 μM, respectively. Table 1 also shows that the V_{max} values for the incorporation of dCTP and CDVpp, using the primer containing one CDV at the 3′-terminus, were 49 and 41%, respectively, of the rates compared with natural substrate. The V_{max}/K_m ratio of dCTP to CDVpp was 17 with synthetic DNA containing one molecule of CDV at the 3-terminus as the primer–template. Therefore, the lower catalytic efficiency in the incorporation of a second consecutive CDV is due to the higher K_m value of CDVpp to the enzyme–primer–template complex compared with dCTP.

The reported K_i values of CDVpp toward human DNA polymerases α, β, and γ are 51, 520, and 299 μM, respec-

FIG. 2. Super-imposition of a conformation of CDV (blue) with 2′-deoxycytidylate (yellow). All atoms were omitted for clarity.

tively [10, 18]. The low affinity of CDVpp toward these three human DNA polymerases suggests that CDV would be expected to have minimal inhibitory effects on lagging DNA strand synthesis, DNA repair, and mitochondrial DNA synthesis. The low affinity of CDVpp toward these three human DNA polymerases and CMV DNA polymerase may be a common characteristic of this compound toward DNA polymerases.

CDV is an acyclic nucleoside phosphonate analog [6], and selected analogs of this class exhibit broad spectrum antiviral activities partially due to metabolic activation which is independent of virally encoded kinases. Selected antiviral acyclic nucleoside phosphonate analogs exhibit prolonged antiviral activities after dosing *in vitro* and *in vivo*. Our results demonstrate that the lower catalytic efficiency in the incorporation of either the first or second consecutive CDV molecule into viral DNA is due mainly to the higher K_m value of CDVpp toward the enzyme–primer–template complexes. Our data also indicate that incorporation of a single molecule of CDV into DNA by HCMV DNA polymerase does not lead to DNA chain termination. Although CDVpp (V_{max}/K_m) is incorporated into viral DNA at a lower rate than the natural substrate dCTP, CDV exhibits more potent and longer lasting anti-CMV activity than ganciclovir, both *in vitro* and *in vivo* [9]. The effect of incorporation of CDV into DNA on viral DNA synthesis is under investigation.

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