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MODE OF ACTION OF ACYCLOVIR TRIPHOSPHATE ON HERPESVIRAL AND CELLULAR DNA POLYMERASES

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The effect of 5'-triphosphate of acyclovir (ACV) on DNA polymerases of two human herpesviruses, herpes simplex virus type-1 (HSV-1) and Epstein–Barr virus (EBV) as well as human cellular DNA polymerases α and β has been examined. Of the enzymes tested, HSV-1 DNA polymerase was the most sensitive to inhibition by acyclovir triphosphate (ACVTP). The EBV DNA polymerase and DNA polymerase β were less sensitive. ACVTP inhibition was competitive with dGTP with K_i values of 0.03, 0.15, 9.8 and 11.9 μM for HSV-1 DNA polymerase, DNA polymerase α, EBV DNA polymerase and DNA polymerase β, respectively. Substituting a synthetic primer template $(dG)_{\sim 15} \cdot (dC)_n$ for activated DNA template did not alter the pattern of inhibition. In a time course experiment, addition of ACVTP instead of dGTP did not increase DNA synthesis and it appeared to act as a chain terminator in DNA replication catalyzed by either HSV-1 DNA polymerase or DNA polymerase α. Although EBV DNA polymerase was less sensitive to ACVTP inhibition, the nucleoside analog itself was inhibitory to EB virus production by P3HR1 cell line as determined by a reduction in the percentage of cells expressing virus capsid antigen (VCA). On day 4, ACV at 10 and 25 μg/ml reduced the cell growth by 10% and 32%, respectively, while it reduced the VCA-positive cells by 80% and 84%, respectively. These results indicate that inhibition of EBV DNA polymerase activity by ACVTP may not be the primary mechanism responsible for ACV inhibition of EBV replication.

acyclovir DNA polymerases chain termination

INTRODUCTION

Considerable progress has been made in recent years toward developing highly potent and selective antiviral agents [10, 11]. Among these, 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV) has emerged as one of the most promising anti-herpesvirus agents. It selectively inhibits in vitro replication of herpes simplex virus types 1 and 2 (HSV-1, HSV-2), varicella-zoster virus [4] and Epstein–Barr virus (EBV) [7, 9]. It had, however, no effect on cytomegalovirus replication [8]. ACV has been shown to be effective against herpesvirus-induced lesions in experimental animals [18, 23–28]. In clinical trials, topical application of ACV was effective against corneal epithelial lesions caused by herpes simplex infections [17, 22]. Parenteral administration of ACV to cancer patients

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with cutaneous and/or systemic herpes zoster or herpes simplex infections arrested progression of the disease [30].

The selective anti-herpesvirus action of ACV may be due to two factors. Firstly, it is preferentially phosphorylated by the herpesvirus-encoded thymidine kinase [12, 15] and, secondly, the 5′-triphosphate formed in virus-infected cells inhibits the viral DNA polymerase more than the host cellular polymerases; in vitro inhibition by ACVTP of purified herpesviral and cellular DNA polymerases has been reported [9, 14, 32]; moreover, a limited incorporation of the nucleotide analog into replicating DNA may prevent further elongation [12, 14]. ACV selectively inhibits the herpesviral DNA synthesis in HSV-1-infected cells [20] and in EBV-producing cells [7]; genetic studies with HSV mutants resistant to ACV also indicated the possibility of two distinct loci responsible for the resistance [6, 13, 29]. To further investigate the mechanism of selectivity we have examined the effect of ACVTP on the activities of extensively purified cellular and herpesviral DNA polymerases.

MATERIALS AND METHODS

Cells and viruses

Human KB cells infected with HSV-1 strain HF were kindly provided by J.C. Drach, University of Michigan, Ann Arbor. The culture conditions have been described previously [31]. P3HR-1 cells, originally derived from a patient with Burkitt's lymphoma were grown in RPMI 1640 medium supplemented with 10% calf serum, 100 I.U. penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone [33]. This cell line was 8% virus capsid antigen (VCA)-positive.

Cell growth and VCA determination

P3HR-1 cells were seeded in 75 cm^2 flasks (Corning) at a density of 3.5×10^5 cells/ml with or without ACV (10 and 25 μg/ml). The cell density was determined by counting samples in a Coulter cell counter. VCA determination of P3HR-1 cells was kindly performed by Dr. J. Robinson, Department of Pediatrics and Epidemiology, Yale University, using an established procedure [16]. The percentage of VCA-positive cells was determined on days 0, 4, and 6.

Isolation of cellular and viral polymerases

Cellular DNA polymerases α and β were purified from leukocytes of a patient with acute myelogenous leukemia. HSV-1 DNA polymerase was isolated from human KB cells infected with HSV-1 strain HF [31]. EBV DNA polymerase was purified from the P3HR-1 cell line. The isolation procedures of the polymerases were published previously [1].

Chemicals

All nucleoside triphosphates were purchased from Sigma Chemical Company (St.

Louis, MO). $(dG)_{\sim 15} \cdot (dC)_n$ was obtained from P.-L. Biochemicals, Inc. (Milwaukee, WI). Tritiated deoxyribonucleoside triphosphates were obtained from the New England Nuclear Corp. (Boston, MA) and from the ICN Chemical and Radioisotope Division (Irvine, CA). Calf thymus DNA was converted to the activated form by treatment with DNase-I. Acyclovir was a gift from Dr. G.B. Elion (Burroughs Wellcome, Chapel Hill, NC). ACV was converted to its 5′-triphosphate (ACVTP) using methods described previously [2]. Analysis of ACVTP by [^{31}P] NMR demonstrated the presence of triphosphate and its [^{1}H] NMR obtained on a Bruker HX-270 spectrometer was consistent with its structure. Analysis by high-pressure liquid chromatography on an Altex Model 332 gradient liquid chromatograph (Partisil SAX column, 0.4 M NaH_2PO_4 buffer, pH 3.3; flow rate, 1.5 ml/min) showed a single peak eluting at 48 min.

DNA polymerase assays

DNA polymerase α activity was assayed in a 50 μl reaction mixture containing 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol (DTT), 8 mM $MgCl_2$, 100 μM each of dATP, dCTP and dTTP, and 3–5 μM [^{3}H]dGTP (1060 c.p.m./pmol), 10 μg activated calf thymus DNA, 10–20 μg bovine serum albumin (BSA), 5–10% glycerol and enzyme. Incubation, was at 37°C for 30 min. Acid-insoluble radioactivity was collected on nitrocellulose filters (Gelman or Millipore, 0.45 μm) washed several times using a Millipore filtration manifold with 5% trichloroacetic acid containing 2 mM sodium pyrophosphate, once with 70% ethanol, dried, and measured in a liquid scintillation counter.

DNA polymerase β activity was assayed under similar conditions except that a pH 9.0 Tris-HCl buffer, 50 μM of non-radioactive triphosphates, 20 μM [^{3}H] dGTP, and 40 mM KCl were used.

The reaction mixture for assaying HSV-1 DNA polymerase activity contained 50 mM Tris-HCl, pH 8.3, 2 mM DTT, 4 mM $MgCl_2$, 10 μM each of dATP, dCTP and dTTP, and 0.5–1.0 μM [^{3}H]dGTP (4300 c.p.m./pmol), 5 μg activated calf thymus DNA, 50 mM ammonium sulfate, 10 μg BSA, 5–10% glycerol and enzyme. Other conditions were similar to those described for measuring DNA polymerase α activity.

EBV DNA polymerase activity was assayed under similar conditions for DNA polymerase α except that 4 mM $MgCl_2$, 10 μM [^{3}H] dGTP and 100 mM KCl were used.

RESULTS

Relative sensitivities of the different polymerases to ACVTP inhibition

The effect of ACVTP on the utilization of dGTP by viral and cellular DNA polymerases is illustrated in Fig. 1. The polymerases used in these experiments were purified by DNA-cellulose column chromatography and were free of cross-contamination, i.e. HSV-1 DNA polymerase was free of polymerase α and vice versa. All enzyme assays were performed under conditions optimal for the individual enzymes. The concentrations of [^{3}H]dGTP were maintained at 2–3 times the K_m value for the corresponding enzyme.

Fig. 1 shows that the HSV-1 DNA polymerase was the most sensitive among the enzymes tested. DNA polymerase β and EBV DNA polymerase were less sensitive. The concentrations of ACVTP required to reduce [^{3}H]dGMP incorporation by 50% of HSV-1 DNA polymerase, EBV DNA polymerase, DNA polymerases α and β were 0.18, 33, 0.45 and 40 μM, respectively. We have also examined the effect of ACVTP in assays wherein the activated DNA template was replaced with a synthetic primer template, $(dG)_{\sim 15} \cdot (dC)_n$ and the results are summarized in Table 1. The inhibitory pattern did not change with use of the synthetic primer template.

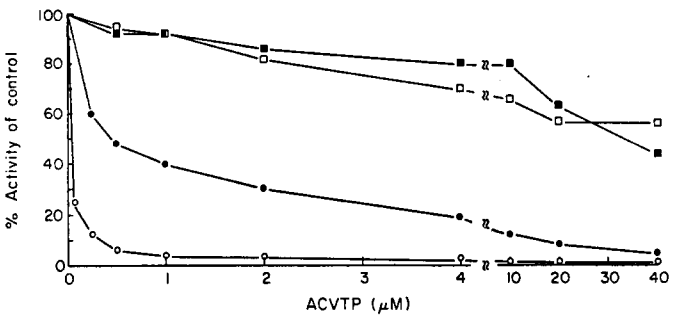

Fig. 1. ACVTP inhibition of DNA polymerases. The polymerase activities were measured by [^{3}H]-dGMP incorporation to activated DNA template under assay conditions optimal to the individual enzymes. The concentrations of [^{3}H]dGTP used were 2–3 times the K_m values of the corresponding enzymes; the other three triphosphates were in excess. 100% activity of HSV-1 DNA polymerase, DNA polymerase α, DNA polymerase β, and EBV DNA polymerase represents: 10.5 (○), 15.5 (●), 13.0 (■), and 15.0 (□) pmol [^{3}H]dGMP incorporation, respectively.

TABLE 1

ACVTP inhibition HSV-1 DNA polymerase activity with activated DNA and $(dG)_{\sim 15} \cdot (dC)_n$

Concentration ACVTP (μM)	Template	
	Activated DNA	$(dG)_{\sim 15} \cdot (dC)_n$
0	100	100
0.25	44	44
0.5	34	30
1	32	28
2	21	22
4	19	12
10	4	9

The reaction mixture with the synthetic primer template contained in a total volume of 50 μl, 50 mM Tris-HCl, pH 8.0, 20 mM sodium fluoride, 2 mM DTT, 50 μg BSA, 4 mM $MgCl_2$, 2 μg $(dG)_{\sim 15} \cdot (dC)_n$, 3 μM [^{3}H]dGTP and enzyme. Other conditions were similar to those described for assays with activated DNA template. A 100% activity represents 9–12 pmol [^{3}H] dGMP incorporation.

Kinetics of ACVTP inhibition

To investigate further the mode of inhibition of polymerase activity by ACVTP, the extent of inhibition with increasing concentrations of the substrate was determined. In these assays, [^{3}H] dGTP was the rate-limiting substrate, while the other three triphosphates were in excess. Lineweaver–Burk plots [21] show that the inhibition was competitive with dGTP. Figs. 2, 3 and 4 depict the inhibitory pattern of activities of HSV-1 DNA polymerase, DNA polymerase α and EBV DNA polymerase, respectively. The K_m values for dGTP and the K_i values for ACVTP are summarized in Table 2. The K_i value

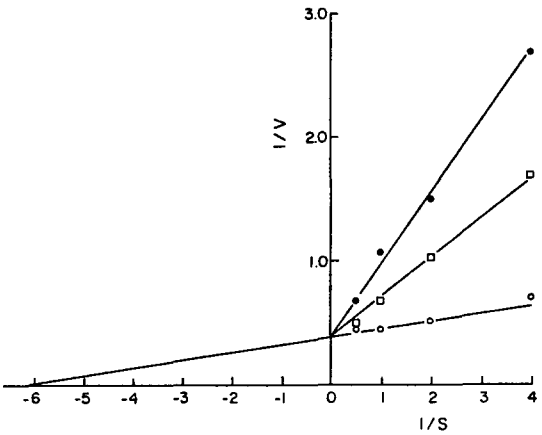

Fig. 2. Effect of ACVTP on HSV-1 DNA polymerase reaction in the presence of different concentrations of [^{3}H] dGTP. No inhibitor (○); 0.05 μM ACVTP (□); and 0.25 μM ACVTP (●).

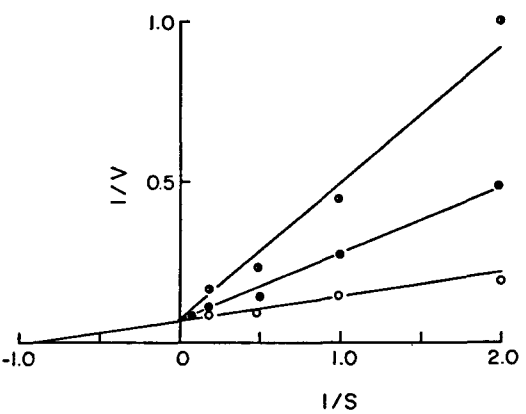

Fig. 3. Effect of ACVTP on DNA polymerase α activity in the presence of different concentrations of [^{3}H] dGTP. No inhibitor (○); 0.25 μM ACVTP (●); and 1 μM ACVTP (◑).

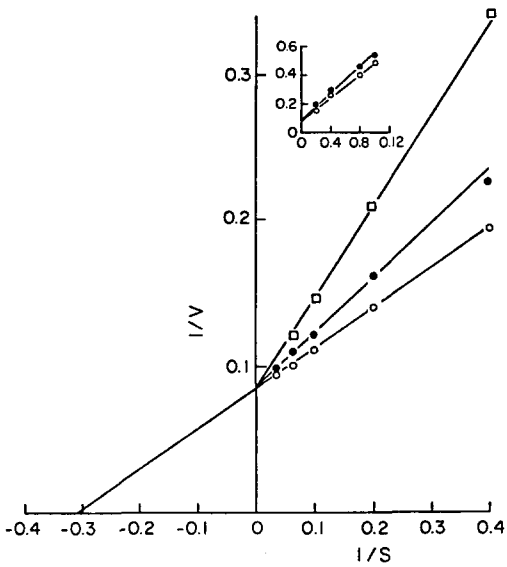

Fig. 4. Effect of ACVTP on the reaction catalyzed by EBV DNA polymerase. No inhibitor (○); 4 μM ACVTP (●); and 20 μM ACVTP (□). Inset: effect on lower concentrations of [^{3}H] dGTP and ACVTP. No inhibitor (○); and 1 μM ACVTP (●).

TABLE 2

Kinetic analysis of ACVTP inhibition of cellular and viral DNA polymerases

Enzyme	K_m (dGTP) (μM)	K_i (ACVTP) (μM)	K_m/K_i
HSV-1 DNA polymerase	0.15	0.03	5
EBV DNA polymerase	3.15	9.8	0.32
DNA polymerase α	1.11	0.15	7.21
DNA polymerase β	7.8	11.9	0.66

of ACVTP for the HSV-1 DNA polymerase with $(dG)_{\sim 15} \cdot (dC)_n$ did not change significantly (0.03 μM); however, the K_m value of dGTP was about 10 times more (1.38 μM) than that with activated DNA template. Our kinetic values for the inhibitor and the competing substrate of EBV DNA polymerase are different from those reported by Datta et al. [9]. To further verify our results we have also determined the polymerase activity at lower concentrations of dGTP and ACVTP and obtained similar results (Fig. 4, inset). Furthermore, we have examined the relative affinities to dGTP and ACVTP of EBV DNA polymerases isolated from three different virus-producing cell lines and the values are indistinguishable (data not shown).

Effect on in vitro DNA synthesis

To determine the effect of ACVTP on DNA synthesis, we performed time course experiments with HSV-1 DNA polymerase (Fig. 5). DNA replication was linear up to 150 min in reaction with all four nucleoside triphosphate substrates. ([^{3}H]dATP was the labelled substrate.) When dGTP was omitted from the reaction mixture, DNA synthesis was reduced to as low as 10% after 120 min. When dGTP was added at the end of 120 min, the DNA synthesis increased as expected. However, when ACVTP was added instead of dGTP, there was no increase in DNA synthesis. Addition of ACVTP instead of dGTP even in the beginning of the reaction did not enhance DNA synthesis. These results are consistent with the notion that ACVTP prevents chain elongation and can be a chain terminator. We obtained identical results with DNA polymerase α (data not presented).

Effect of ACV on cell growth and VCA expression by P3HR1 cell line

Addition of ACV inhibited cell growth only slightly, whereas it inhibited the percent of VCA-positive cells considerably (Table 3). For instance, on day 4, ACV, at 10 and 25 μg/ml, reduced the cell growth by only 10% and 32%, respectively; however, it inhibited VCA expression by 80% and 84%, respectively.

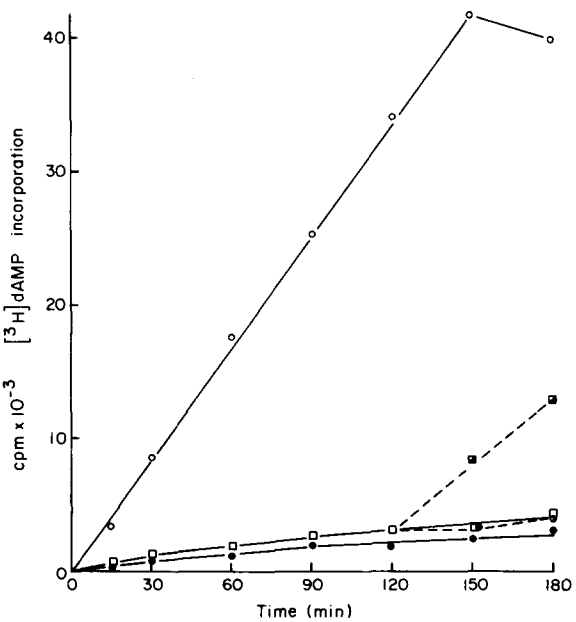

Fig. 5. HSV-1 DNA polymerase reaction in the presence of ACVTP. [^{3}H]dATP (10 μM) was the labelled substrate; other conditions were as described in the text. All four natural triphosphate substrates (○); three nucleotides only, without dGTP (□); 1 μM dGTP was added at the end of 120 min (◪); three nucleotides and 1 μM ACVTP (●); and three nucleotides, 1 μM ACVTP (◑) added at the end of 120 min.

TABLE 3

Effect of ACV on cell growth and VCA expression

Day	Cell No. ($\times 10^{-5}$)			% VCA-positive cells		
	Control	ACV 10 μg/ml	ACV 25 μg/ml	Control	ACV 10 μg/ml	ACV 25 μg/ml
0	3.5	3.5	3.5	8.0	8.0	8.0
1	4.5	3.9	4.1			
4	9.4	8.5	6.4	7.5	1.50	1.25
5	13.2	11.0	9.1			
6	22.5	15.5	12.8	7.0	2.0	1.0

DISCUSSION

A comparison of relative sensitivities of herpesviral and cellular DNA polymerases to ACVTP inhibition is essential in delineating its mechanism of selectivity. Our results show that of the polymerases tested HSV-1 DNA polymerase is the most sensitive to ACVTP inhibition. Our kinetic data with HSV-1 DNA polymerase are in agreement with values reported by Furman et al. [14] and St. Clair et al. [32]. However, we noticed that DNA polymerase α purified from human leukemic leukocytes was also sensitive to ACVTP inhibition. In DNA polymerization reaction catalyzed by either HSV-1 DNA polymerase or DNA polymerase α, addition of ACVTP did not enhance DNA synthesis and perhaps prevented further chain elongation reaction. The inability of ACVTP to promote chain elongation is consistent with its structure. However, the 5′-triphosphate of another potent anti-herpesviral nucleoside analog, *E*-5-(2-bromovinyl)-2′-deoxyuridine, was found to be an alternative substrate for DNA polymerases and the analog was incorporated into replicating DNA in virus-infected cells [3].

We noticed that EBV DNA polymerase was not sensitive to ACVTP inhibition. The K_m values for dGTP and K_i values for ACVTP of EBV DNA polymerase are different from those obtained by Datta et al. [9], who reported that EBV DNA polymerase was more sensitive to ACVTP inhibition than the cellular DNA polymerases. To further verify our results we have examined ACVTP inhibition also at lower concentrations of the substrate and inhibitor (Fig. 4, inset), and these results support our original values. Furthermore, we have tested the inhibitory effect of ACVTP on EBV DNA polymerase isolated from three different virus-producing human cell lines and obtained similar values. It is also worth noting that the K_m values of DNA polymerase α we have isolated from P3HR1 cell line as well as from human leukemic leukocytes are similar to the values reported by Furman et al. [14] and others for DNA polymerase α isolated from various cell sources (1.5–2.5 μM); however, the K_m value reported by Datta et al. [9] for dGTP of DNA polymerase α from a human cell line is unusually low (0.18 μM). Although ACVTP was not inhibitory to the EBV DNA polymerase activity, we noticed that addition

of ACV to EBV-producing cells in culture inhibited the virus production; this is in agreement with the results of Datta et al. [9]. Therefore, it appears that EBV DNA polymerase is not a primary target for ACV inhibition of EBV production. It should also be pointed out that Colby et al. [7] have noticed a significant quantity of covalently circular EBV DNA remaining in P3HR-1 cells after ACV treatment. The identification of enzymes responsible for the phosphorylation of ACV to its 5′-triphosphate in EBV-producing cell lines will be of interest because of several conflicting reports concerning EBV thymidine kinase(s) in the cell lines. It will also be useful to determine the relative levels of ACVTP in different EBV cell lines in delineating the mechanism of ACV inhibition of EBV-production. Thus, further information on the enzyme(s) responsible for the conversion of the nucleoside analog to its active form, the relative levels of ACVTP in different EBV cell lines and specific roles of various DNA polymerases in replication of the viral and cellular DNAs will be essential in understanding the mode of action of ACV in inhibiting EBV production.

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