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On the mechanism of selective inhibition of herpesvirus replication by (*E*)-5-(2-bromovinyl)-2′-deoxyuridine

(antiviral activity/DNA polymerases)

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ABSTRACT **Bromovinyldeoxyuridine (BVdUrd) is a potent antiherpesvirus compound with low cytotoxicity. To gain an insight into its selectivity and mechanism of inhibition, we chemically synthesized the 5′-triphosphate of BVdUrd, BVdUTP, and tested its effect on the activities of DNA polymerases [DNA nucleotidyltransferase (DNA directed), EC 2.7.7.7] of two herpesviruses—i.e., herpes simplex virus type 1 (HSV-1) and Epstein–Barr virus (EBV)—as well as cellular DNA polymerases α, β, and γ. The effects on the DNA polymerases were determined under assay conditions optimal for the individual polymerases. We found that the BVdUTP was considerably more inhibitory to the utilization of dTTP by the HSV-1 DNA polymerase than by the cellular DNA polymerases. For instance, as little as 1 μM BVdUTP inhibited the utilization of dTTP by HSV-1 DNA polymerase 50%, whereas the same concentration inhibited the DNA polymerase α and the DNA polymerase β activities only 9% and 3%, respectively. The BVdUTP inhibited DNA synthesis by competing with the natural substrate, dTTP. The K_m for dTTP and the K_i for the BVdUTP of the HSV-1 DNA polymerase were 0.66 and 0.25 μM, respectively. Kinetic analyses with the DNA polymerases α and β and the EBV DNA polymerase also reflected a similar difference in sensitivity between the HSV-1 enzyme and other enzymes. Increasing the concentration of either the DNA template or the enzyme in the reaction mixture did not bring about a significant change in the extent of inhibition. Preincubation of the inhibitor with the enzyme was not necessary for inhibition. Studies on time course of inhibition revealed that the compound is inhibitory even after the initiation of DNA synthesis. These studies indicate that the ability of BVdUTP to preferentially inhibit the HSV-1 DNA polymerase may contribute towards its selective inhibition of the viral DNA replication in infected cells.**

In the past few years several compounds have been developed with antiviral activity; however, they have not been clinically useful because they are also toxic to uninfected cells. However, recent studies on the cellular events that occur after virus infection have led to an understanding of many steps specific for the infecting virus. There are several viral functions that can be clearly distinguished from the cellular functions, thus allowing the development of newer antiviral agents based on the difference in viral-host metabolism.

Mammalian cells contain three classes of DNA polymerases [DNA nucleotidyltransferase (DNA directed), EC 2.7.7.7], designated as α, β, and γ. After infection, herpes simplex virus (HSV) induces synthesis of many enzymes involved in DNA replication; the most notable examples are a unique thymidine kinase (1, 2) and a DNA polymerase (3, 4); in addition, a DNase (5) and a nucleoside phosphotransferase (6) are induced in cells upon infection with HSV. These virus-induced enzymes are significantly different from their cellular counterparts in many of their properties (7–9). For example, the HSV-induced DNA polymerase differs from the host cellular DNA polymerases α, β, and γ in elution profile on ion-exchange columns, molecular weight, primer template preference, effect of monovalent and divalent cations, and other requirements for maximal activity. From correlative studies to delineate specific roles that the cellular DNA polymerases play in DNA replication, it appears that the DNA polymerase α may be involved in chromosomal replication, DNA polymerase β in DNA repair, and DNA polymerase γ in mitochondrial DNA synthesis (7–10). Genetic studies with HSV type 1 (HSV-1) have demonstrated that the virus-induced DNA polymerase is responsible for the replication of the viral DNA (11).

These virus-induced enzymes lend themselves as exploitable targets for selective antiviral chemotherapy. Recent findings that some of the nucleoside analogs were preferentially phosphorylated by the herpesvirus-induced thymidine kinase marked a significant step forward in the development of antiviral chemotherapy (12). Examples of such analogs include 1-β-D-arabinofuranosylthymine (Ara-T) (13), 5-alkyl deoxyuridines (14), 9-(2-hydroxyethoxymethyl)guanine (acyclovir) (15), 2-fluro-5-iodo-1-β-D-arabinofuranosylcytosine (FIAC) (16), 5-substituted 2′-deoxycytidines and deoxyuridines, and 5-iodo-5′-amino-2′,5′-dideoxyuridine (AIdUrd) (17). Acyclovir, one of the most promising antiherpesviral agents developed in recent years, possesses antiviral activity against both types of HSV (type 1 and type 2) in cell culture as well as in experimental animal infections (18).

A more recently synthesized nucleoside analog, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdUrd), was found to be a very potent and selective antiherpesvirus agent (19). It inhibited HSV-1 replication in cell culture at concentrations as low as 0.007–0.01 μg/ml, levels 1/10,000th of the concentration at which normal cell metabolism was altered. In animal model systems—i.e., cutaneous herpesvirus infection in athymic *nude* mice—either topical or systemic administration of BVdUrd suppressed the development of herpetic skin lesions and mortality.

BVdUrd belongs to the group of nucleoside analogs that are preferentially phosphorylated by the herpesvirus-induced thymidine kinase, because it is ineffective in suppressing herpesvirus replication when cells are infected with thymidine kinase mutants of HSV-1 (20). However, the mechanism of the antiviral effect of the analog is not known. Presumably, BVdUrd is converted to an active triphosphate form only in the infected cells and inhibits herpesvirus DNA replication by inhibiting the viral DNA polymerase. However, it is not known whether the analog, after its conversion to the triphosphate, can inhibit the

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Abbreviations: HSV, herpes simplex virus, HSV-1, HSV type 1; EBV, Epstein–Barr virus; BVdUrd, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; BVdUMP and BVdUTP, 5′-monophosphate and 5′ triphosphate of BVdUrd.

cellular DNA polymerases as well. In order for an analog to be more selective, it should be preferentially phosphorylated by the viral thymidine kinase and be a selective inhibitor of viral DNA polymerase. To examine further the selectivity of BVdUrd, we chemically synthesized the 5′-triphosphate of BVdUrd (BVdUTP), purified the herpesvirus DNA polymerases as well as cellular DNA polymerases α, β, and γ, and tested the effect of BVdUTP on the activities of these polymerases. Our results show that, among the polymerases tested, the viral polymerase is the most sensitive to BVdUTP inhibition.

MATERIALS AND METHODS

Materials. Tritiated deoxyribonucleoside triphosphates were obtained from New England Nuclear and from ICN Chemical and Radioisotope (Irvine, CA). Nucleoside triphosphates and synthetic primer templates were purchased from P-L Biochemicals. The oligonucleotides contained 12–18 nucleotides. Calf thymus DNA was converted to the activated form by treatment with DNase I, according to the procedure of Schlabach *et al.* (21). The synthesis of BVdUrd was described by Jones *et al.* (22).

BVdUrd was converted to the corresponding 5′-monophosphate (BVdUMP) in high yield by $POCl_2/PO(OEt)_3$ (23) and purified by column chromatography on DEAE-Sephadex using a linear gradient of triethylammonium bicarbonate between 0 and 400 mM at 4°C. The monophosphate eluted at 200 mM salt. Activation of the monophosphate with 1,1′-carbonyldiimidazole afforded the corresponding phosphorimidazolidate, which was condensed with di(tri-*n*-butylammonium) pyrophosphate (24). Purification on DEAE-Sephadex with a linear gradient of triethylammonium bicarbonate between 0 and 800 mM afforded the desired product (elution at 400 mM). Phosphate assay by the method of Fischer (25) demonstrated the presence of three phosphate residues per nucleoside. After treatment of the triphosphate with apyrase and alkaline phosphatase, analysis by reverse-phase high-pressure liquid chromatography [Partisil ODS-2 (Whatman); 15% vol/vol $MeOH/H_2O$] resulted in a single nucleoside peak that was identical in chromatographic mobility to the authentic BVdUrd.

Cells and Viruses. Monolayer cultures of human KB cells were grown in minimal essential medium supplemented with 10% calf serum. The HF strain of HSV-1 was used to infect KB cells. Other growth conditions and isolation procedures of both uninfected and HSV-1-infected KB cells were as described by Shipman *et al.* (26).

P3HR-1K, an Epstein–Barr virus (EBV)-producing human cell line (27) was propagated as exponential suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum.

Isolation of Cellular and Viral DNA Polymerases. The cellular DNA polymerases α, β, and γ were isolated from KB cells. The isolation of enzymes was performed at 2–4°C unless otherwise specified. The cells were suspended in 4 vol of a solution consisting of 20 mM potassium phosphate buffer at pH 7.0, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 800 mM KCl, and 20% (vol/vol) glycerol and were disrupted by using a Brinkmann Polytron homogenizer (six 30-s pulses). Triton X-100 was added to a final concentration of 0.5% and the contents were stirred for 30 min and centrifuged at 90,000 × g for 30 min. The supernatant was dialyzed in buffer A, which consists of 20 mM potassium phosphate buffer at pH 7.0, 1 mM dithiothreitol, and 20% (vol/vol) glycerol. The nucleic acids in the extract were removed by passing it through a DEAE-cellulose column equilibrated in buffer A and eluting with buffer A containing 0.3 M KCl. The DNA polymerases were separated on a phosphocellulose column equilibrated in buffer B, which consists of 50 mM Tris·HCl buffer at pH 8.0, 1 mM dithiothreitol, and 20% (vol/vol) glycerol with a linear gradient of 0–600 mM KCl in buffer B. The DNA polymerase α eluted at 180 mM KCl, and the DNA polymerase β eluted at 400 mM KCl. The DNA polymerase γ, which eluted at 200 mM KCl, had to be further separated from the contaminating DNA polymerase α by a subsequent chromatography on a DEAE-cellulose column (28).

The HSV-1 DNA polymerase was purified from KB cells infected with HSV-1 strain HF. The EBV-specific DNA polymerase was purified from an exponentially growing P3HR1-K cell line. The initial steps of purification of the herpesvirus-specific DNA polymerases were similar to those described for the cellular DNA polymerases. The HSV-1 DNA polymerase eluted on a phosphocellulose column at 100 mM KCl and the EBV DNA polymerase eluted at 50 mM KCl. These polymerases were further purified by column chromatography on single-stranded DNA-cellulose with a linear KCl gradient between 0 and 400 mM. The details of purification of the herpesvirus-specific DNA polymerases will be described elsewhere.

Enzyme Assays. DNA polymerase α activity was assayed in a 50-μl reaction mixture that contained 50 mM Tris·HCl at pH 8.0, 2 mM dithiothreitol, 8 mM $MgCl_2$, 100 μM each of dATP, dCTP, and dGTP, 20 μM [^{3}H]dTTP (530 cpm/pmol), 10 μg of activated calf thymus DNA, 10–20 μg of bovine serum albumin, 5–10% glycerol, and enzyme. Incubation was at 37°C for 30 min. Acid-insoluble radioactive material was collected on a nitrocellulose filter (Gelman or Millipore, 0.45 μm pore diameter), washed several times with 5% trichloroacetic acid containing 2 mM sodium pyrophosphate and once with 70% (vol/vol) ethanol, dried, and measured in a liquid scintillation counter (29).

DNA polymerase β activity was assayed under similar conditions except that a pH 9.0 Tris·HCl buffer, 50 μM nonradioactive triphosphates, 40 μM [^{3}H]dTTP, and 40 mM KCl were used.

The reaction mixture for assaying DNA polymerase γ activity contained 50 mM Tris·HCl at pH 8.5, 2 mM dithiothreitol, 0.8 mM $MnCl_2$, 10 μM [^{3}H]dTTP (4300 cpm/pmol), 1 μg of $(dT)_{\approx 15} \cdot (A)_n$, 100 mM KCl, 20 μg of bovine serum albumin, and enzyme. Other conditions were similar to those described above for measuring DNA polymerase α activity.

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

EBV DNA polymerase activity was assayed under conditions similar to those for DNA polymerase α except 4 mM $MgCl_2$, 40 μM [^{3}H]dTTP, and 100 mM KCl were used.

RESULTS

Relative Sensitivities of the Polymerases to BVdUTP Inhibition. The polymerases purified by DNA-cellulose column chromatography were used in all experiments, except the one to determine the relative sensitivities to BVdUTP inhibition. These enzymes were free of cross-contamination; for example, the HSV-1 DNA polymerase was free of DNA polymerase α and vice versa. The HSV-1 DNA polymerase and DNA polymerase α were clearly separated on a phosphocellulose column; they were further purified by DNA-cellulose column chromatography. Furthermore, the assay conditions optimal for HSV-1 DNA polymerase were inhibitory to the DNA polymerase α activity. For example, at conditions optimal for HSV-1 DNA

polymerase activity only 10% of the DNA polymerase α activity was expressed. The ability of BVdUTP to inhibit the utilization of dTTP by viral and cellular DNA polymerases is depicted in Fig. 1. The enzyme assays were performed under conditions optimal for the individual enzymes, with the concentration of [^{3}H]dTTP maintained at 2–3 times the K_m value for the corresponding enzyme. The HSV-1 DNA polymerase was the most sensitive of the enzymes tested. The EBV DNA polymerase, however, was relatively insensitive. The DNA polymerase α activity was inhibited at a concentration lower than that required to inhibit the activity of either DNA polymerase β or DNA polymerase γ. The difference in sensitivity between HSV-1 DNA polymerase and other DNA polymerases was significant, particularly at lower concentrations of BVdUTP. For example, at 1 μM BVdUTP, the HSV-1 DNA polymerase was inhibited by 50% whereas the DNA polymerase α and β activities were inhibited by only 6% and 3%, respectively. The amounts required to effect a 50% inhibition of [^{3}H]dTMP incorporation into DNA by the individual polymerases are listed in Table 1. However, BVdUTP did not have any effect on the incorporation of [^{3}H]dGMP in reactions in which [^{3}H]dTTP was replaced with nonradioactive dTTP and [^{3}H]dGTP.

There was no significant variation in the extent of BVdUTP inhibition of the HSV-1 DNA polymerase at different states of purity. For example, 1 μM BVdUTP inhibited 59.5% and 58% of the polymerase purified by phosphocellulose column chromatography and a subsequent DNA-cellulose column chromatography, respectively. Addition of either BVdUrd or BVdUMP to the reaction mixture did not inhibit the enzyme activity.

Kinetics of BVdUTP Inhibition. To characterize further the nature of the BVdUTP inhibition, we determined the extent of inhibition with increasing concentrations of the substrate. In assays with activated DNA template, [^{3}H]dTTP was used as the rate-limiting substrate and the other three triphosphates were in excess. A Lineweaver and Burk (30) plot shows that straight lines could be drawn intersecting on the ordinate, indicating that the inhibition was competitive with dTTP in the case of HSV-1 DNA polymerase (Fig. 2).

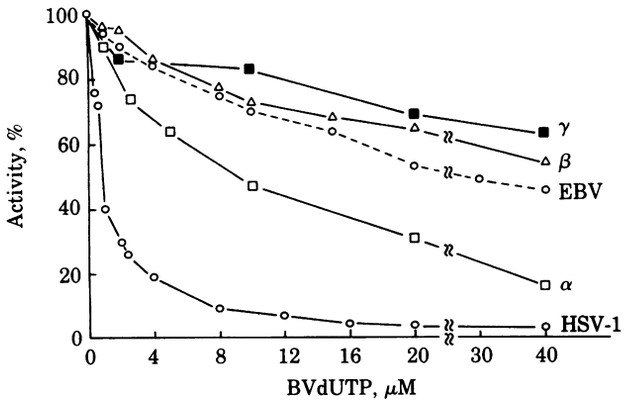

FIG. 1. BVdUTP inhibition of DNA polymerases. The enzyme activities were measured by [^{3}H]dTMP incorporation; activated DNA template was used for all the polymerases except for DNA polymerase γ; the DNA polymerase γ activity was assayed with $(dT)_{\sim 15}\cdot(A)_n$ primer-template. The concentrations of [^{3}H]dTTP used in these experiments were 2–3 times the K_m values of the corresponding enzymes; the other three triphosphates were in excess. One hundred percent activity of DNA polymerase α represents 18.8 pmol of [^{3}H]dTMP incorporation; that of DNA polymerase β is 22.0; DNA polymerase γ is 4.6; HSV-1 DNA polymerase is 4.8; and EBV-DNA polymerase is 17.5.

Table 1. Relative sensitivity of viral and cellular DNA polymerases to BVdUTP inhibition

Enzyme	IC_{50}, μM	Relative sensitivity
HSV-1 DNA polymerase	0.75	1
EBV DNA polymerase	41	55
Human DNA polymerase α	9.2	12
Human DNA polymerase β	47.0	63
Human DNA polymerase γ	70	93

The DNA polymerase activity was assayed by measuring [^{3}H]dTMP incorporation. The concentrations of [^{3}H]dTTP used were 2–3 times the K_m values of the corresponding enzymes. The template and other conditions are same as described for Fig. 1. IC_{50}, 50% inhibitory concentration.

Similar experiments with other DNA polymerases showed that the mode of inhibition for all the polymerases is the same, although concentrations of the inhibitor necessary to bring about a 50% inhibition were different. EBV DNA polymerase results are shown in Fig. 3; DNA polymerase α, in Fig. 4; and DNA polymerase β, in Fig. 5. The K_m values for dTTP and the K_i values for BVdUTP are shown in Table 2.

Time Course. To determine whether BVdUTP could inhibit DNA synthesis even after the reaction was initiated, it was added to the ongoing reaction at different times and the activity was monitored. Fig. 6 illustrates that the compound caused an instantaneous inhibition whether it was added at the beginning or after the initiation of the reaction.

Preincubation. To determine whether preincubation of the inhibitor with either the enzyme or the template was necessary, BVdUTP was mixed with either the HSV-1 DNA polymerase or the activated DNA template and maintained at 0°C or 37°C for 30 min; other ingredients were added after the preincubation period and the polymerase reaction was performed as usual. We found that there was no difference in the extent of BVdUTP inhibition, whether or not the analog was preincubated with

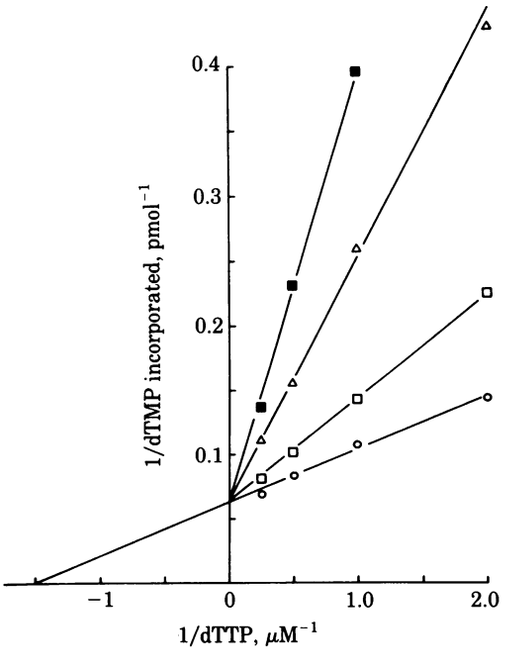

FIG. 2. Effect of BVdUTP on HSV-1 DNA polymerase reaction in the presence of different concentrations of [^{3}H]dTTP with activated DNA template. ○, No inhibitor; □, 0.26 μM BVdUTP; △, 0.9 μM BVdUTP; and ■, 1.8 μM BVdUTP.

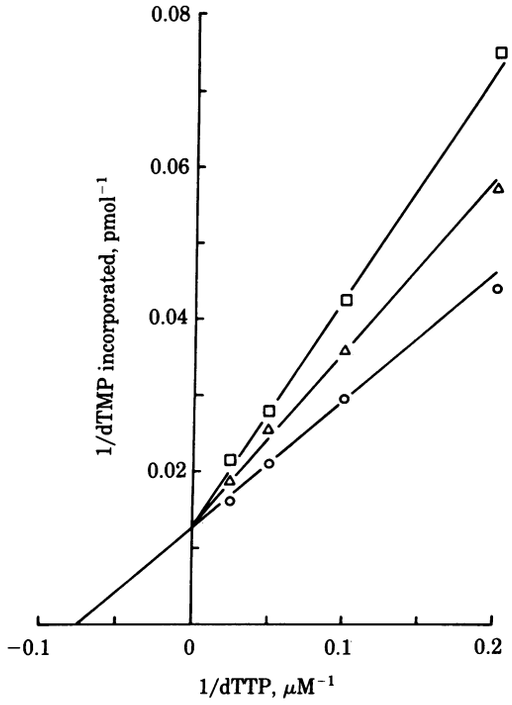

FIG. 3. Effect of BVdUTP on EBV DNA polymerase reaction in the presence of different concentrations of [^{3}H]dTTP with activated DNA template. Other assay conditions used were optimal for EBV DNA polymerase activity. ○, No inhibitor; △, 6.0 μM BVdUTP; and □, 12.0 μM BVdUTP.

either the enzyme or the template. This indicated that preincubation was not required for inhibition.

DISCUSSION

The results presented here provide an explanation for the known antiviral activity of the recently developed nucleoside analog BVdUrd. For a comparison of BVdUTP inhibition of the herpesviral and cellular DNA polymerases, assay conditions optimal for the individual enzymes were used; more importantly, the concentrations of dTTP used were 2–3 times the K_m values for the respective enzymes; this is essential because BVdUTP is competitive with dTTP. It is clear from our results

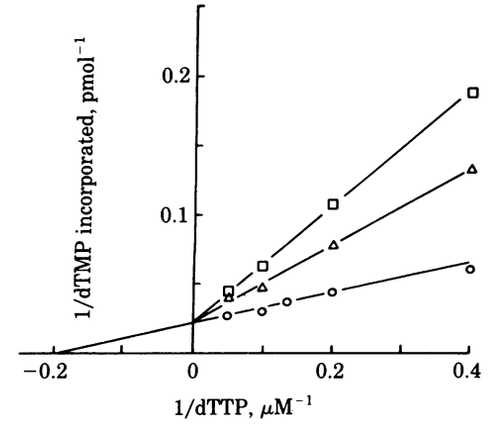

FIG. 4. Effect of BVdUTP on the reaction catalyzed by DNA polymerase α in the presence of different concentrations of [^{3}H]dTTP with activated DNA template. Other assay conditions used were optimal for the DNA polymerase α activity. ○, No inhibitor, △, 5 μM BVdTUP; and □, 10 μM BVdUTP.

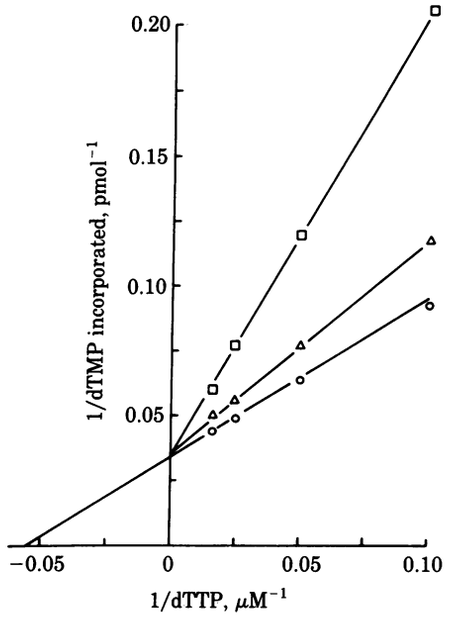

FIG. 5. Effect of BVdUTP on the reaction catalyzed by DNA polymerase β in the presence of different concentrations of [^{3}H]dTTP. Other assay conditions used were optimal for the DNA polymerase β reaction. ○, No inhibitor; △, 5.0 μM BVdUTP; and □, 20 μM BVdUTP.

that the HSV-1 DNA polymerase is the most sensitive to BVdUTP inhibition among the enzymes tested. Furthermore, BVdUrd is preferentially phosphorylated by the herpesvirus thymidine kinase (Y. C. Cheng, personal communication). These studies indicate that the compound exerts its preferential antiviral effect in two ways—i.e., at the viral thymidine kinase as well as at the DNA polymerase levels. Therefore, it appears that two different herpesvirus-coded enzymes are involved in eliciting its selective antiviral activity; at least one more of the recently developed nucleoside analogs with antiherpesvirus activity, acyclovir, seems to have this feature (15, 18, 31).

The sensivity of HSV-1 DNA polymerase to BVdUTP inhibition was further confirmed by differences in K_i values for BVdUTP of the viral and cellular polymerases. For instance, there was a 14-fold difference in K_i values between the HSV-1 DNA polymerase and DNA polymerase α, although the K_m/K_i ratio for the viral polymerase was only 2 times more than that for the cellular polymerase. Both DNA polymerase β and EBV DNA polymerase have similar K_m and K_i values. The K_i values for the triphosphate of acyclovir (acyclo-GTP) reported by Furman *et al.* (15) were between 0.08 and 1.42 μM for the different strains of HSV-1 used. However, it is difficult to draw a direct comparison of the K_i values for the triphosphate of acyclovir and BVdUrd because the differences in values between the two analogs may also be due to variations in the (*i*) strain of HSV-1, (*ii*) assay conditions, and (*iii*) state of purity of the polymerases used in these studies.

A comparison of the relative sensitivities to BVdUTP inhibition of the polymerases used shows that the DNA polymerase

Table 2. Kinetic analysis of BVdUTP inhibition

Enzyme	K_m for dTTP, μM	K_i for BVdUTP μM	K_m/K_i
HSV-1 DNA polymerase	0.66	0.25	2.64
EBV DNA polymerase	13.0	16.1	0.81
Human DNA polymerase α	5.3	3.6	1.47
Human DNA polymerase β	17.8	16.4	1.08

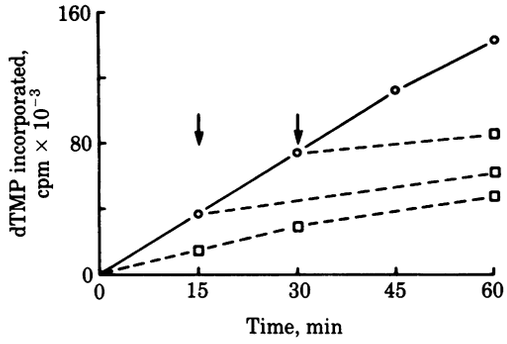

FIG. 6. Time course of BVdUTP inhibition of the HSV-1 DNA polymerase reaction. BVdUTP at a final concentration of 1 μM was added at the time intervals shown after the initiation of the enzyme reaction with [^{3}H]dTTP as the rate-limiting substrate and activated DNA template; other conditions used were optimal for the HSV-1 DNA polymerase activity.

γ is the least sensitive enzyme. No further experiment was performed with this polymerase because it utilizes the activated DNA template poorly; furthermore, the low level of the enzyme present in dividing cells makes difficult its further purification in sufficient quantity.

Further studies to determine whether the analog is incorporated into a growing chain of DNA indicate that this indeed is the case—i.e., BVdUTP can act as an alternative substrate (unpublished data). Although BVdUrd is a potent inhibitor of HSV-1 replication, it is not as effective against HSV type 2 replication. The difference in sensitivity between these two viruses to BVdUrd could be due to differences either at the phosphorylation level or at the polymerase level. To determine whether there is any difference in BVdUTP inhibition between DNA polymerases specific to HSV types 1 and 2, it is necessary to purify DNA polymerase of HSV type 2 and determine its sensitivity to the triphosphate.

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