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Comparative Inhibition of DNA Polymerases from Varicella Zoster Virus (TK$^+$ and TK$^-$) Strains by (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine 5′-Triphosphate

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SUMMARY

The inhibitory effect of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) 5′-triphosphate on varicella zoster virus (VZV) DNA polymerase was studied using the parent strain (TK$^+$-VZV) and the mutant strain (TK$^-$-VZV). The mutant strain was deficient in thymidine kinase (TK)-inducing activity and resistant to BVDU. In the absence of BVDU, TK$^-$-VZV and TK$^+$-VZV induced an equivalent level of viral DNA polymerase activity in human embryo fibroblasts. In the presence of 5 μM BVDU, TK$^-$-VZV still induced viral DNA polymerase activity, whereas TK$^+$-VZV failed to do so. BVDU 5′-triphosphate (BVDUTP) was considerably more inhibitory to the TK$^+$- and TK$^-$-VZV DNA polymerases than to the cellular DNA polymerases. There were no significant differences in the affinity for dTTP as substrate and the sensitivity to BVDUTP as inhibitor between the TK$^+$- and TK$^-$-VZV DNA polymerases. The K_m value for dTTP and the K_i value for BVDUTP of the VZV DNA polymerases were 1.43 μM and 0.55 μM, respectively. The inhibitory effect of BVDUTP to VZV DNA polymerase was competitive with respect to the natural substrate.

INTRODUCTION

Several nucleoside analogues have been introduced in the chemotherapy of herpesviruses. Among them ranks BVDU[3] as one of the most potent and selective anti-herpes agents (1–10). BVDU inhibits VZV and HSV-1 replication in cell culture at a very low concentration and has minimal toxicity for uninfected cells. However, BVDU is less active against HSV-2 than are other antiherpes compounds such as ACV and 2′-fluoro-5-iodoaracytosine (3, 11, 12). After entry into the virus-infected cell, BVDU is phosphorylated to BVDU 5′-monophosphate and 5′-diphosphate by the virus-coded 2′-deoxy TK (10, 13, 14). BVDU 5′-diphosphate is subsequently processed to BVDUTP by cellular kinases. The selective inhibition of VZV and HSV-1 replication by BVDU appears to reside in a specific interaction of BVDU with the viral TK and of BVDUTP with the viral DNA polymerase.

The inhibitory effects of BVDUTP on the DNA polymerases of human herpesviruses, i.e., HSV-1, HSV-2, and EBV, have been reported previously (15). HSV-1 DNA polymerase proved more sensitive to BVDUTP than did EBV DNA polymerase and the cellular DNA polymerases α, β, and γ (15). However, the sensitivity of VZV DNA polymerase to BVDUTP has not been the subject of previous studies. Recently, we isolated a TK$^-$-VZV strain that was resistant to several antiherpes compounds and did not induce TK activity in infected cells (16, 17). This TK$^-$-VZV strain was also resistant to BVDU. To determine the role of VZV DNA polymerase in the selective inhibition of VZV replication by BVDU, we examined the inhibitory effects of BVDUTP on the VZV DNA polymerase purified from the TK$^+$-VZV- and TK$^-$-VZV-infected cells.

MATERIALS AND METHODS

VZV. The parent VZV strain (Kanno, TK$^+$-VZV), which had been isolated from a patient with herpes zoster, was cloned and passaged in HEF cells. The mutant VZV strain (Kanno-Kohmura, TK$^-$-VZV), which is resistant to BVDU, was isolated by repeated passage of the parent VZV strain in the presence of IDU and BDU. The details of the isolation procedures of the TK$^-$ strain have been described in a previous report (17).

Reagents and chemicals. BVDU and its 5′-triphosphate were synthe-

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[3] The abbreviations used are: BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; VZV, varicella zoster virus; HSV-1 and HSV-2, herpes simplex virus types 1 and 2; ACV, acycloguanosine; TK, thymidine kinase; BVDUTP, BVDU 5′-triphosphate; EBV, Epstein-Barr virus; HEF cells, human embryo fibroblast cells; IDU, 5-iodo-2′-deoxyuridine; BDU, 5-bromo-2′-deoxyuridine; BDUTP, bromodeoxyuridine triphosphate; IDUTP, iododeoxyuridine triphosphate; PAA, phosphonoacetic acid; ddTTP, 2′,3′-dideoxythymidine triphosphate.

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sized at the Rega Institute (Katholieke Universiteit Leuven) by R. Bueson, L. Colla, and H. Vanderhaeghe. Tritiated deoxyribonucleoside triphosphates ([^{3}H]dTTP and [^{3}H]dGTP) were obtained from New England Nuclear Corporation (Boston, Mass.). Synthetic polynucleotide-oligodeoxynucleotides were purchased from P-L Biochemicals (Milwaukee, Wis.). Calf thymus DNA was converted to the activated form by treatment with DNase I, according to the procedure of Schlabach *et al.* (18). BDUTP and IDUTP were purchased from Sigma biochemicals. PAA was obtained from Bodmen Chemicals (Richmond, Va.), and aphidicolin was purchased from Wako Chemicals.

DNA polymerases. The cellular DNA polymerases were isolated from HeLa cells as described previously (15, 19). The cells were collected by centrifugation; suspended in 3 volumes of a solution consisting of 20 mM phenylmethylsulfonyl fluoride, 800 mM KCl, and 20% (v/v) glycerol; and disrupted by using a Brinkmann Polytron homogenizer (six 30-sec pulses). Triton X-100 was added to a final concentration of 0.5%, and the assay mixtures were stirred for 30 min and centrifuged at 90,000 × *g* for 30 min. The supernatant was dialyzed against Buffer A [20 mM potassium phosphate buffer (pH 7.0), 1 mM dithiothreitol, and 20% (v/v) glycerol]. The nucleic acids were removed from the extract by chromatography on a DEAE-cellulose column equilibrated in Buffer A, and DNA polymerases were eluted with Buffer A containing 0.3 M KCl. The DNA polymerases were separated by chromatography on a phosphocellulose column equilibrated with Buffer B [50 mM Tris-HCl buffer (pH 8.0), 1 mM dithiothreitol, and 20% (v/v) glycerol] and eluted with a linear gradient of 0–600 mM KCl in Buffer B. DNA polymerase α was eluted at a concentration of 180 mM KCl. DNA polymerase γ, which was eluted at a concentration of 200 mM KCl, had to be further separated from the contaminating DNA polymerase α by subsequent chromatography on a DEAE-cellulose column. VZV DNA polymerase was isolated from HEF cells infected with VZV. HSV-1 DNA polymerase was isolated from HeLa cells infected with HSV-1 (Miyama strain). The purification scheme for these DNA polymerases was similar to that described for the cellular DNA polymerases. VZV DNA polymerase was eluted from the phosphocellulose column at a concentration of 350 mM KCl (20) and HSV-1 DNA polymerase was eluted at a concentration of 100 mM KCl.

DNA polymerase assays. The DNA polymerase assays (20, 21) were based upon the incorporation of tritium-labeled nucleotide triphosphate into an acid-precipitable product. In System A, with activated calf thymus DNA as template, the stock reaction mixture (2.5 times concentration) contained the following: Tris-HCl (pH 7.8), 0.1 M; $MgCl_2$, 0.025 M; 2-mercaptoethanol, 0.01 M; bovine serum albmin, 1.25 mg/ml; dATP, dCTP, and dGTP, 0.25 mM each; and dTTP, 0.025 mM. For the assay, 0.1 ml of the stock reaction mixture, 0.1 ml of activated calf thymus DNA (200 mg/ml), 0.05 ml (unless stated otherwise) of enzyme, and 1 μCi of [^{3}H]dTTP (specific activity 48 Ci/mM) were incubated at 37° for 1 hr. The reaction was terminated by adding 2 ml of chilled 10% trichloroacetic acid and 1% sodium pyrophosphate. The precipitate was collected on glass-fiber paper (GF/C, Whatman) and washed once each with 1% trichloroacetic acid, 0.1% sodium pyrophosphate, and 50% ethanol. Then, the filters were washed with 50% alcohol and dried at 37°, and their radioactivity was estimated in a liquid scintillation counter. The activity of the cellular DNA polymerases α and γ was determined under assay conditions optimal for the individual polymerase (22).

In System B, with synthetic poly(dA)·oligo(dT)$_{12-18}$ as the template primer, the reaction mixture contained the same ingredients as in System A, except that dATP, dCTP, and dGTP were omitted. The synthetic template primer (2 μg/0.1 ml) was used instead of the activated calf thymus DNA. In System C, with synthetic poly(dC)·oligo(dG)$_{12-18}$ as the template primer, only one substrate, dGTP (0.025 mM for 2.5 times concentration), together with 1 μCi of [^{3}H]dGTP (specific activity 45 Ci/mM), was used; the template primer concentration was again 2 μg/0.1 ml.

RESULTS

Induction of VZV DNA polymerase activity after infection of HEF cells with VZV. As previously reported by Mar (20), infection of HEF cells with VZV results in the induction of a novel DNA polymerase activity that can be distinguished from host cell DNA polymerase by its high-salt requirement for maximal activity. Such high-salt-dependent DNA polymerase activity was also detected in HEF cells following VZV infection. The HEF cells were infected with either TK$^+$-VZV or TK$^-$-VZV at a ratio of 10^3 VZV-infected cells/2 × 10^6 HEF cells. At every 24-hr period after infection, 2 × 10^6 virus-infected or mock-infected cells were harvested and sonicated for 1 min in Buffer A. The suspensions were centrifuged at 20,000 rpm for 30 min, and the resulting supernatant fluids were assayed for DNA polymerase activity in the presence of high salt concentration [60 mM $(NH_4)_2SO_4$], as described under Materials and Methods. When DNA polymerase activity was assayed in the presence of 60 mM $(NH_4)_2SO_4$, only VZV DNA polymerase could be detected. When BVDU was absent from the cell culture medium, the VZV DNA polymerase activity increased proportionally with virus focus formation, as noted for cells infected with either TK$^+$-VZV or TK$^-$-VZV (Fig. 1). The TK$^-$-VZV strain induced levels of viral DNA polymerase similar to those induced by the TK$^+$-VZV strain. When the TK$^-$-VZV- and TK$^+$-VZV-infected cell cultures were incubated in the presence of 5 μM BVDU, the TK$^-$-VZV strain proved capable of inducing the DNA polymerase, whereas the TK$^+$-VZV strain failed to do so.

Relative sensitivities of DNA polymerases from TK$^+$-VZV- or TK$^-$-VZV-infected cells to inhibition by BVDUTP. The purified VZV DNA polymerases were assayed for their sensitivities to BVDUTP at optimal conditions for the individual enzymes, using activated calf thymus DNA as template. As shown in Fig. 2, the VZV DNA polymerases and the HSV-1 DNA polymerase were the most sensitive to BVDUTP. The TK$^+$-VZV and TK$^-$-VZV DNA polymerases were equally sensitive to the inhibitory effects of BVDUTP; i.e., the amount of BVDUTP required to effect 50% inhibition of [^{3}H]dTMP incorporation into DNA was 1.7 μM for both TK$^+$-VZV DNA polymerase and TK$^-$-VZV DNA polymerase. DNA polymerase α was inhibited by BVDUTP to a greater extent than was DNA polymerase γ, although both were significantly less sensitive to BVDUTP than were the VZV DNA polymerases. The differences in sensitivity between the VZV DNA polymerases and the DNA polymerases α and γ were particularly marked at low concentrations of BVDUTP.

Inhibition of VZV DNA polymerase by BDUTP and IDUTP. The ability of BDUTP and IDUTP to inhibit VZV DNA polymerase activity is shown in Fig. 3. IDUTP and BDUTP inhibited VZV DNA polymerase activity to the same extent as BVDUTP, although IDU and BDU were less inhibitory than BVDU to VZV replication in cell culture (16). VZV DNA polymerase proved also sensitive to ddTTP, a well-known inhibitor of DNA polymerase (data not shown). No significant differences were noted between the TK$^+$-VZV and TK$^-$-VZV DNA

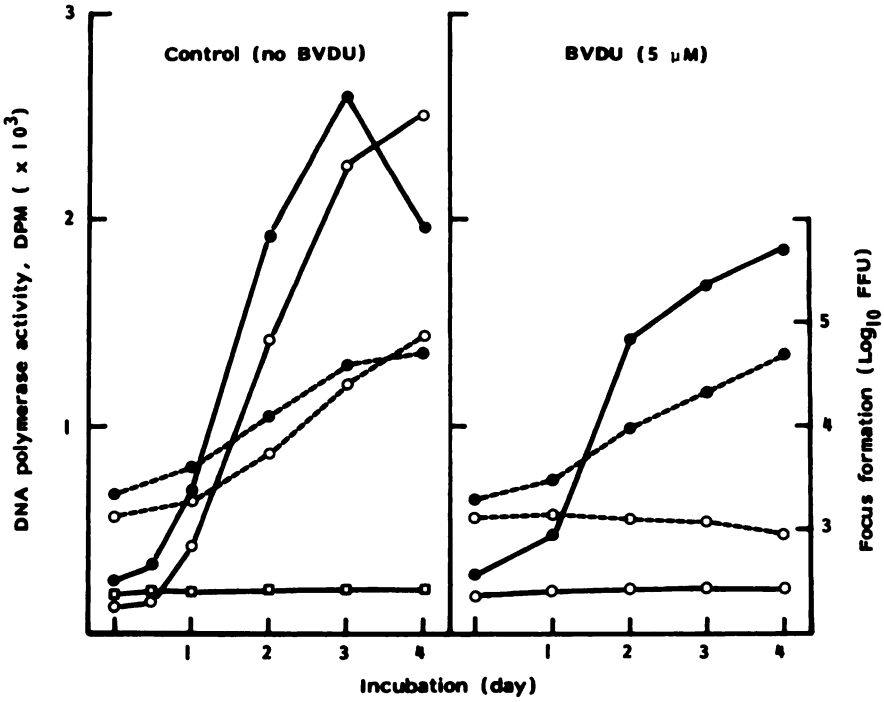

FIG. 1. *Induction of virus-specific DNA polymerase in VZV-infected cells*

HEF cells were infected with the TK⁺- and the TK⁻-VZV strains (10^3 infected cells/2 × 10^6 cells per bottle) and further incubated in the absence or presence (5 μM) of BVDU. At the indicated times after infection, 2 × 10^6 virus-infected or mock-infected HEF cells were harvested and sonicated for 1 min in Buffer A. The suspensions were centrifuged at 20,000 rpm for 30 min, and the resulting supernatants were assayed for DNA polymerase activity in the presence of 60 mM $(NH_4)_2SO_4$, as described under Materials and Methods. *Solid lines* indicate DNA polymerase activity. *Broken lines* indicate focus formation in focus-forming units (*FFU*). ○, TK⁺-VZV infected cells; ●, TK-VZV infected cells; □, mock-infected cells.

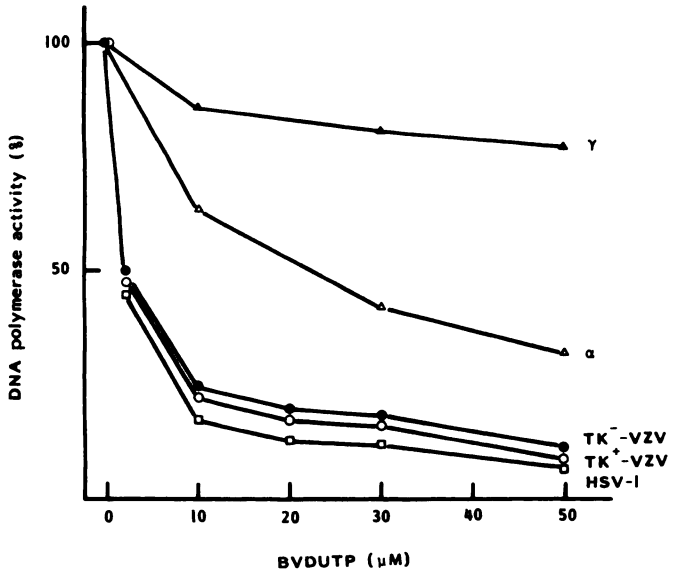

FIG. 2. *Inhibition of DNA polymerases of various origin by BVDUTP*

The polymerase activities were monitored by [³H]dTMP incorporation; activated calf thymus DNA was used as template. ○, TK⁺-VZV DNA polymerase; ●, TK⁻-VZV DNA polymerase; □, HSV-1 DNA polymerase; △, DNA polymerase α; ▲, DNA polymerase γ.

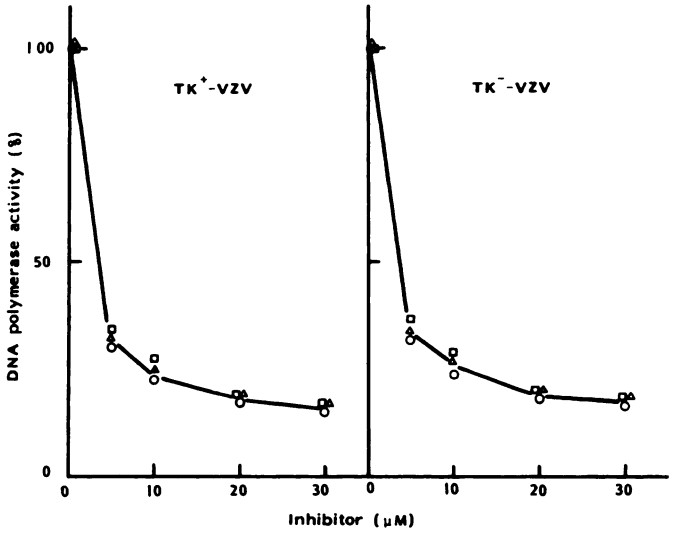

FIG. 3. *Inhibition of TK⁺- and TK⁻-VZV DNA polymerases by BVDUTP, BDUTP, and IDUTP*

The polymerase activities were monitored by [³H]dTMP incorporation; activated calf thymus DNA was used as template. ○, BVDUTP; □, BDUTP; △, IDUTP.

polymerases in their sensitivity to the deoxyribonucleoside triphosphates (Fig. 3).

VZV DNA polymerase activity measured with several template primers. It has been reported previously that VZV DNA polymerase efficiently uses poly(dC)·oligo(dG)$_{12-18}$ as well as poly(dA)·oligo(dT)$_{12-18}$ as template primers (20). We also found poly(dA)·oligo(dT)$_{12-18}$ and poly(dC)·oligo(dG)$_{12-18}$ to be better templates for VZV DNA polymerase than activated calf thymus DNA

(data not shown). The cellular DNA polymerase α and γ used both synthetic templates less efficiently than activated calf thymus DNA. In System B, using poly(dA)·oligo(dT)$_{12-18}$ as template primer (Fig. 4A), incorporation of [^{3}H]dTMP was inhibited by BVDUTP, IDUTP, BDUTP, aphidicolin, and PAA. However, in System C, using poly(dC)·oligo(dG)$_{12-18}$ as template primer (Fig. 4B), the incorporation of [^{3}H]dGMP was not inhibited by BVDUTP, IDUTP, and BDUTP. These results suggest that the inhibition achieved by BVDUTP, BDUTP, IDUTP, and BVDUTP was competitive with respect to dTTP. On the other hand, aphidicolin and PAA inhibited VZV DNA polymerase activity, irrespective of the synthetic template primer used (Fig. 4A and B). These results suggest that the inhibition of VZV DNA polymerase by aphidicolin and PAA is not competitive with respect to dTTP.

Kinetics of the inhibition of VZV DNA polymerase activity by BVDUTP. To characterize further the mechanism of VZV DNA polymerase inhibition by BVDUTP, we followed the extent of this inhibition at increasing concentrations of substrate. In this assay, activated calf thymus DNA served as template and [^{3}H]dTTP was used as the rate-limiting substrate, the other three triphosphates being added in excess. From the Lineweaver-Burk plots of the results, it is clear that BVDUTP inhibited VZV DNA polymerase in a manner that was competitive with the natural substrate, dTTP (Fig. 5). The K_m value of the TK$^+$-VZV DNA polymerase for dTTP was 1.43 μM, and the K_i value for BVDUTP was 0.55 μM. A similar competitive type of inhibition was observed with the TK$^-$-VZV DNA polymerase (Fig. 6).

DISCUSSION

Recently we isolated a mutant VZV strain which was resistant to several antiherpes compounds, including BVDU and ACV (17). It was relatively resistant to BDU and IDU, but sensitive to ara-A, ara-C, and PAA (16). This mutant VZV strain appeared to be deficient in its ability to express viral TK activity (17).

Mar (20) showed that infections of cells with VZV resulted in the induction of a new DNA polymerase activity which could be differentiated from the host cell DNA polymerase by its high-salt requirement for maximal activity. As shown here, the VZV DNA polymerases

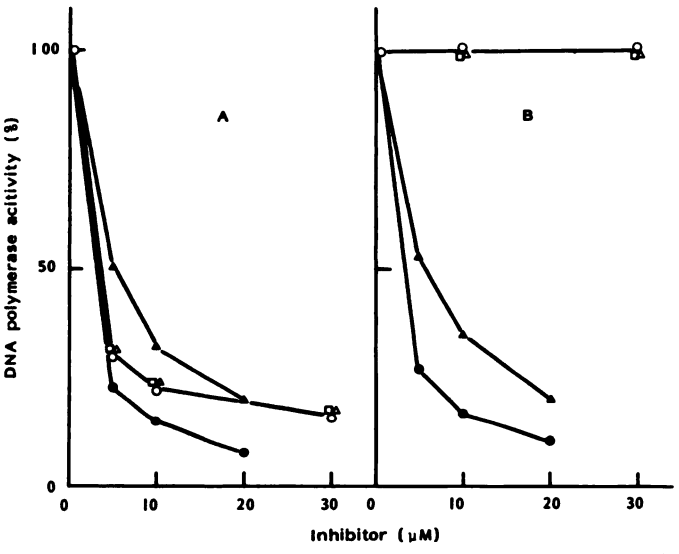

FIG. 4. *Effect of different template primers on inhibition of TK$^+$-VZV DNA polymerase by various inhibitors*

In A, poly(dA)·oligo(dT)$_{12-18}$ served as template primer, and [^{3}H]dTMP incorporation was measured. In B, poly(dC)·oligo(dG)$_{12-18}$ served as template primer, and [^{3}H]dGMP incorporation was measured. ○, BVDUTP; □, BDUTP; △, IDUTP; ●, aphidicolin; ▲, PAA.

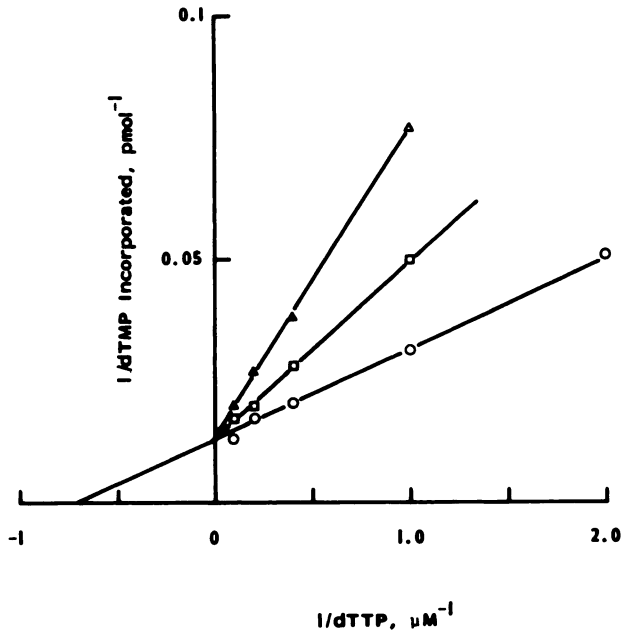

FIG. 5. *Effect of BVDUTP on TK$^+$-VZV DNA polymerase activity with activated DNA as template in the presence of different concentrations of [^{3}H]dTTP*

○, No inhibitor; □, 0.5 μM BVDUTP; △, 1.0 μM BVDUTP.

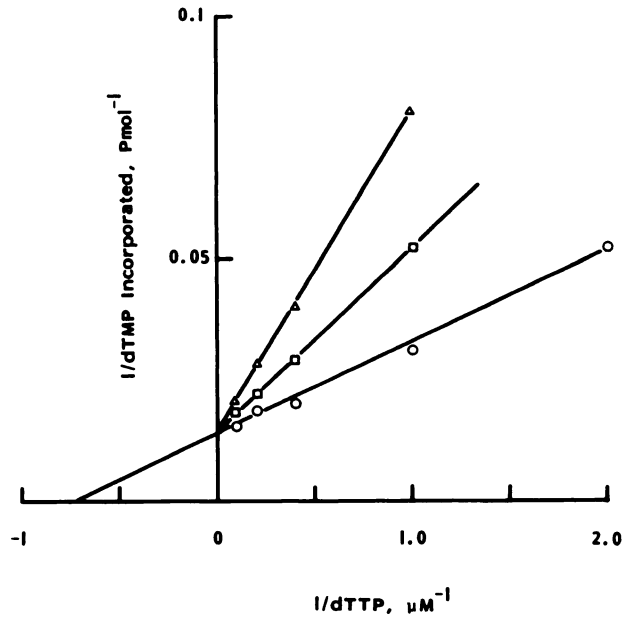

FIG. 6. *Effect of BVDUTP on TK$^-$-VZV DNA polymerase activity (with activated DNA as template) in the presence of different concentration of [^{3}H]dTTP*

○, No inhibitor; □, 0.5 μM BVDUTP; △, 1.0 μM BVDUTP.

also differed from the host DNA polymerases by its elution pattern on ion exchange columns, template primer preference, and sensitivity to aphidicolin and ddTTP. The amount of high-salt dependent DNA polymerase activity induced by the TK$^-$-VZV was similar to that induced by TK$^+$-VZV. In the presence of BVDU, the replication of TK$^+$-VZV was shut off and no high-salt dependent DNA polymerase was induced. On the other hand, BVDU did not suppress the replication of TK$^-$-VZV, and, consequently, cells infected with this mutant strain continued to express the virus-specific DNA polymerase.

The purified VZV DNA polymerases were more sensitive to the inhibitory effects of BVDUTP than the cellular enzymes. The amount required to effect a 50% inhibition of [^{3}H]dTMP incorporation into DNA was 1.7 μM. The K_i value of the enzymes for BVDUTP was 0.55 μM. The sensitivity of VZV DNA polymerases to BVDUTP was similar to that of HSV-1 DNA polymerase. BVDUTP inhibited the DNA polymerases of TK$^+$-VZV and TK$^-$-VZV to a similar extent. Allaudeen *et al.* (1) and Sim *et al.* (23) reported that HSV-1 DNA polymerase is more sensitive to BVDUTP than are the cellular DNA polymerases α, β, and γ. BVDUTP appears to interact equally well with HSV-1 and HSV-2 DNA polymerases (23), although BVDU is less active against HSV-2 than against HSV-1. This implies that the selectivity of BVDU as an anti-HSV-1 agent primarily resides at the level of its interaction with the viral TK (23). From our results, it appears that the DNA polymerases derived from BVDU-sensitive (TK$^+$) and BVDU-resistant (TK$^-$) VZV strains are equally sensitive to the inhibitory effects of BVDUTP. Furthermore, BVDUTP did not appear to be more inhibitory to VZV DNA polymerases than the 5′-triphosphates of IDU and BDU, two nonselective inhibitors of VZV replication. This, again, reinforces the concept that the selective inhibitory action of BVDU on VZV replication primarily depends on a specific interaction with the viral TK.

However, VZV DNA polymerases are more sensitive to inhibition by BVDUTP than are the cellular DNA polymerases. Likewise, HSV-1 DNA polymerase is more readily inhibited by BVDUTP than are the cellular DNA polymerase (15). These differential inhibitory effects of BVDUTP at the DNA polymerase level may contribute further to the selective inhibition of HSV-1 and VZV replication by BVDU.

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