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Inhibition of Cellular α DNA Polymerase and Herpes Simplex Virus-Induced DNA Polymerases by the Triphosphate of BW759U

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The triphosphate form of the acyclovir analog BW759U (9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl] guanine) inhibited the DNA polymerases (EC 2.7.7.7) from several strains of herpes simplex virus type 1. Two acyclovir triphosphate-resistant DNA polymerases were as sensitive to BW759U-triphosphate as were the DNA polymerases induced by wild-type viruses (K_i = 0.05 to 0.1 μM). The K_i value for cellular α DNA polymerase was 35- to 50-fold greater than those for the DNA polymerases induced by the various herpes simplex virus strains investigated. Incubation of Vero cells infected by the KOS strain of herpes simplex virus type 1 with the acyclovir analog resulted in the formation of substantial quantities of (9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine) triphosphate.

An analog of acyclovir, BW759U (9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine) (Fig. 1), also known as BIOLF-62 (14), 9-[(1,3-dihydroxy-2-propoxy)methyl]-guanine (2), DHPG (2), and 2′-nor-2′-deoxyguanosine (6), has been shown to be a potent inhibitor of herpes simplex virus (HSV) (2, 6, 14). As is the case with acyclovir (5), BW759U is phosphorylated to the triphosphate form in HSV-infected cells (J. Germershausen, R. Bostedor, R. Liou, H. Perry, A. K. Field, and J. D. Karkas, Abstr. Annu. Meet. Fed. Am. Soc. Exp. Biol. 1983, 42, p. 2111). Again, in a similar fashion with acyclovir, BW759U-triphosphate is an inhibitor of HSV-induced DNA polymerases (6). However, several HSV-1 strains found to be resistant to inhibition by acyclovir have been shown to retain sensitivity to BW759U (2, 14). To further understand the mechanism of action of BW759U, it was important to investigate the amount of BW759U-triphosphate made in HSV-infected cells and the ability of BW759U-triphosphate to inhibit the activity of HSV-induced DNA polymerases.

MATERIALS AND METHODS

Cells and viruses. HeLa S-3 and Vero cells (both obtained from American Type Culture Collection, Rockville, Md.) were maintained in Joklik-modified minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% newborn calf serum (MA Bioproducts, Walkersville, Md.) and Eagle minimal essential medium (GIBCO Laboratories) supplemented with 5% fetal bovine serum (Sterile Systems, Inc., Logan, Utah), respectively. The KOS strain of HSV-1 and its mutant PAA^{r}5 were obtained from Priscilla A. Schaffer (Sidney Farber Research Center, Boston, Mass.), and the Patton strain of HSV-1 and its mutant BW^r were gifts of Kendall O. Smith (Department of Microbiology, The University of Texas, San Antonio, Tex.). PAA^{r}5 was prepared in KOS-infected Vero cells exposed to phosphonoacetic acid, and BW^r was isolated from Patton-infected human fetal lung cells treated with acyclovir.

Plaque reduction assays were performed by the method of Collins and Bauer (4). Cytoxicity assays were performed as described previously (8). Briefly, actively growing Vero cells were treated for 76 h with acyclovir or BW759U at concentrations varying from 1 to 1,000 μM. The cells were detached by incubation with 1.5 ml of trypsin (0.25%, GIBCO) and suspended in growth medium. After dilution, the cell numbers were determined by using a model Z_b Coulter Counter.

Purification of DNA polymerase. Herpesvirus-specified DNA polymerase and HeLa S-3 α DNA polymerase were purified from cell extracts by DEAE-cellulose chromatography and phosphocellulose column chromatography as described by Huang (11) and Weissbach et al. (17) and modified by Elion et al. (5) and Furman et al. (9). Further purification of the HSV-induced enzymes was accomplished with the use of DNA cellulose chromatography as described by Potuzak and Wintersberger (13). HeLa S-3 α DNA polymerase was further purified by using a heparin Sepharose column and eluting with a gradient of 0.05 to 1.0 M NaCl. The purified DNA polymerases were stored at −20°C in 60% glycerol containing 1 mg of bovine serum albumin per ml.

DNA polymerase assay. DNA polymerase assays were carried out as described by Elion et al. (5) and Furman et al. (9). Briefly, the reaction mixture (150 μl total volume) contained 50 μM Tris-hydrochloride (pH 8.5), 12 mM $MgCl_2$, 0.1 mM each dCTP, dATP, and [^{3}H]dTTP (specific activity, 70 to 210 mCi/mmol), 0.05 to 10 μM dGTP, 1.2 mM dithiothreitol, 0.25 mg of activated calf thymus DNA per ml, and 0.5 to 10 units of enzyme (one unit is the amount of enzyme catalyzing the incorporation of 1 nmol of [^{3}H]dTTP per h at 37°C). When assaying HSV-1 DNA polymerases, we included 50 mM ammonium sulfate in the reaction mixture. Reaction mixtures were incubated at 37°C for 15 min. Samples (20 μl) of the reaction mixture were transferred at 3-min intervals to Whatman DE-81 paper and processed by the procedure of Altman and Lerman (1). Reaction rates were calculated from the initial linear portion of [^{3}H]dTTP incorporation. Kinetic constants were determined by directly fitting the data to a hyperbola by the method of Wilkinson (18) by the use of the computer program of Cleland (3). Enzyme inhibition was analyzed by the method of Spector and Hajian (15).

Synthesis of BW759U and BW759U-triphosphate. BW759U was synthesized at Wellcome Research Laboratories by unpublished procedures. The BW759U-triphosphate was enzymatically synthesized from BW759U, using HSV-1-induced thymidine kinase (10), GMP kinase, and phospho-

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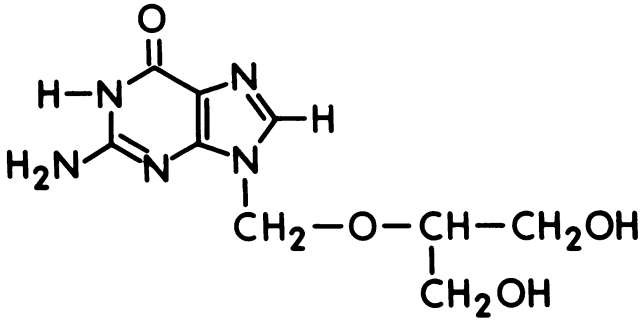

FIG. 1. Structure of BW759U.

glycerate kinase (12). The purified BW759U-triphosphate had a UV spectrum identical to that of GTP. It had a base/inorganic phosphate ratio of 1:3.1 and was cleaved to BW759U by alkaline phosphatase.

High-pressure liquid chromatography. Vero cells infected at a multiplicity of infection of 10 PFU per cell were exposed to the indicated concentrations of BW759U or acyclovir at 1 h postinfection and harvested after 7 h of treatment. The cells were extracted with 3.5% perchloric acid, and the nucleotide content of the extracts was analyzed by high-pressure liquid chromatography. BW759U-triphosphate in the extracts was identified by comparison of retention times with enzymatically synthesized BW759U-triphosphate. Using an isocratic elution of 0.55 M KH_2PO_2 (pH 3.5) and an elution rate of 30 ml per h, BW759U-triphosphate eluted at 2,600 s, between GTP and dGTP.

RESULTS

Inhibition of viral and cellular growth by B759U. In plaque-reduction assays in Vero cells the KOS and Patton strains of HSV-1 had 50% inhibitory dose (ID_{50}) values for BW759U of 0.2 and 0.7 μM, respectively (Table 1). PAA^{r}5, the DNA polymerase mutant of the KOS strain, and BW^r, the DNA polymerase mutant of the Patton strain, although resistant to acyclovir, were as sensitive to inhibition by BW759U as were their wild-type counterparts. As was observed for acyclovir (5), BW759U was relatively nontoxic to uninfected Vero cells, exhibiting an ID_{50} value of 460 μM.

Inhibition of polymerase activity with B759U-triphosphate. The effects of BW759U-triphosphate and acyclo-GTP on the purified DNA polymerases of the two wild-type viruses, their mutants, and HeLa S-3 α DNA polymerase were studied. Representative Lineweaver-Burk plots obtained with three of these polymerases are shown in Fig. 2. In each case, the plots were indicative of competitive inhibition between BW759U-triphosphate and dGTP. The K_i values of BW759U-triphosphate for the DNA polymerases induced by the wild-type virus strains KOS and Patton were approximately 10-fold greater than the corresponding K_i values obtained for the triphosphate of acyclovir (acyclo-GTP). The DNA polymerases induced by the mutants PAA^{r}5 and BW^r were similar in sensitivity to inhibition by BW759U-triphosphate to the corresponding wild-type viruses (Table 1). However, the DNA polymerases of the mutant viruses were significantly less susceptible to inhibition by acyclo-GTP than the DNA polymerases induced by the wild-type strains. BW759U-triphosphate exhibited competitive inhibition of the α DNA polymerase of HeLa S-3 cells (Fig. 2). The K_i for this enzyme (Table 1) was 30- to 50-fold greater than that for the viral DNA polymerases. Similarly, the K_i for acyclo-GTP was 60-fold greater than that determined for the DNA polymerases induced by the KOS and Patton strains of HSV-1 (Table 1).

Formation of B759U-triphosphate. High-pressure liquid chromatographic analysis of perchloric acid extracts of uninfected Vero cells incubated with 100 μM BW759U showed no detectable BW759U-triphosphate formation (Table 2). This is in contrast to the KOS-infected Vero cells which formed 2,700 pmol of BW759U-triphosphate per 10^6 cells under the same conditions. Acyclo-GTP formation, under these conditions, was approximately 10-fold less than BW759U-triphosphate (Table 2).

DISCUSSION

In vitro, BW759U appeared to be a potent and selective antiviral compound showing little toxicity toward uninfected Vero cells (Table 1). The drug is phosphorylated in vitro by the HSV-1 thymidine kinase. (6; J. A. Fyfe, personal communication). In addition, large quantities of BW759U-triphosphate have been detected in HSV-1-infected cells (Table 2). When one compared the levels of acyclo-GTP and BW759U-triphosphate formed in HSV-infected cells treated with identical concentrations of the parent compounds, it was found that nearly 10-fold more BW759U-triphosphate than acyclo-GTP was produced. No BW759U-triphosphate was detected in uninfected cells.

ID_{50} values for BW759U with the KOS and Patton strains of HSV-1 were comparable to values obtained for acyclovir. The acyclovir-resistant HSV-1 mutants, PAA^{r}5 and BW^r,

TABLE 1. Apparent kinetic constants and inhibition of plaque formation

DNA polymerase source	K_m of dGTP (μM)[a]	K_i of acyclo-GTP (μM)[a]	K_i of BW759U-triP (μM)[a]	Viral sensitivity (ID_{50} [μM])[b]	
				ACV[c]	BW759U
HSV-1 (KOS)	0.17 ± 0.01	0.006 ± 0.001	0.05 ± 0.01	0.7	0.2
HSV-1 (Patton)	0.18 ± 0.01	0.006 ± 0.002	0.08 ± 0.01	1.4	0.7
HSV-1 (PAA^{r}5)	0.68 ± 0.006	0.13 ± 0.001	0.05 ± 0.01	14	0.2
HSV-1 (BW^r)	0.61 ± 0.19	0.53 ± 0.11	0.05 ± 0.01	220	0.8
HeLa S-3	1.2 ± 0.22	0.37 ± 0.07	2.5 ± 0.7	ND[d]	ND
Vero	ND	ND	ND	300[e]	460

[a] All values are means from a minimum of two separate determinations, ± standard error.
[b] ID_{50} values were determined in Vero cells.
[c] ACV, acyclovir, as reported by Furman et al. (7).
[d] ND, Not determined.
[e] As reported by Elion et al. (5).

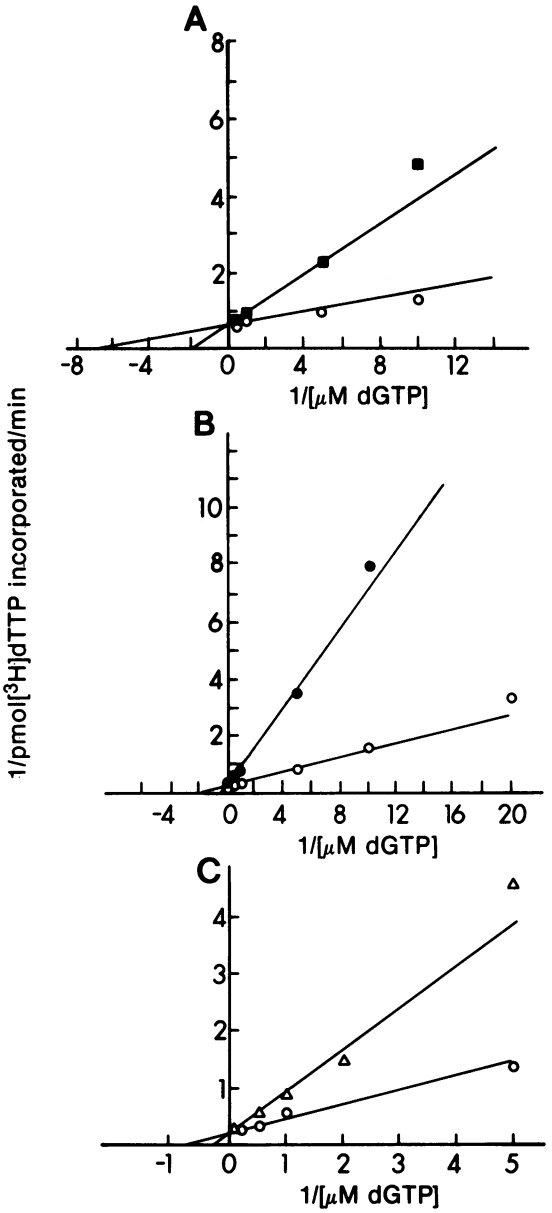

FIG. 2. Lineweaver-Burk plots showing inhibition by BW759U-triphosphate of (A) HSV-1 (strain KOS) DNA polymerase, (B) HSV-1 (strain PAA^{r}5) DNA polymerase, and (C) HeLa S-3 α DNA polymerase. Symbols: ■, 0.30 μM BW759U-triphosphate; ●, 0.20 μM BW759U-triphosphate; △, 4.7 μM BW759U-triphosphate; ○, no BW759U-triphosphate. DNA polymerase activity was measured by following the incorporation of [^{3}H]dTTP.

were as sensitive to BW759U inhibition as were their wild-type counterparts (Table 1).

BW759U-triphosphate, like acyclo-GTP, functions as a competitive inhibitor with respect to dGTP of both viral and cellular α DNA polymerases. Interestingly, the wild-type DNA polymerases were about 10-fold more sensitive to inhibition by acyclo-GTP than to that by BW759U-triphosphate. Yet the ID_{50} values for acyclovir for the KOS and Patton strains of HSV-1 were within twofold of the ID_{50} values for BW759U. The 10-fold greater concentration of BW759U-triphosphate formed in infected cells may offset the 10-fold-greater K_i values observed for BW759U-triphosphate with wild-type DNA polymerases, resulting in the similar ID_{50} values for BW759U and acyclovir for the wild-type viruses.

TABLE 2. Formation of acyclo-GTP or BW759U-triphosphate in HSV-1-infected and uninfected Vero cells[a]

Vero cells infected with:	BW759U in media (μM)	Acyclovir in media (μM)	BW759U-triP (pmol/10^6 cells)	acyclo-GTP (pmol/10^6 cells)
Uninfected	100		<5	
HSV-1 (KOS)	10		480	
HSV-1 (KOS)	100		2,700	
Uninfected		100		5[b]
HSV-1 (KOS)		10		51
HSV-1 (KOS)		100		342

[a] Cells were treated for 7 h beginning at 1 h postinfection.
[b] As reported by Elion et al. (5).

BW759U appears to have a mechanism of action similar to that of acyclovir. However, differences do exist. The decay in the rate of [^{3}H]dTTP incorporation which has been reported for HSV-induced DNA polymerases when incubated in the presence of acyclo-GTP (16) was not observed for BW759U-triphosphate. Acyclovir cannot structurally accommodate chain elongation and therefore appears to terminate the DNA molecule (8), whereas BW759U, with its two available hydroxyl groups, has the potential of being incorporated internally into the growing DNA chain. Finally, a mutation in the DNA polymerase gene rendering a virus resistant to inhibition by acyclovir does not necessarily render the virus resistant to BW759U. On the other hand, a thymidine kinase-negative virus which is obviously resistant to acyclovir would probably also be resistant to BW759U. Studies are in progress to expand our understanding of the mechanism of action of BW759U.

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LITERATURE CITED

1. **Altman, S., and L. S. Lerman.** 1970. Kinetics and intermediates in the intracellular synthesis of bacteriophage T_1 deoxyribonucleic acid. J. Mol. Biol. **50**:235–261.
2. **Cheng, Y.-C., E.-S. Huang, J.-C. Lin, E.-C. Mar, J. S. Pagano, G. E. Dutschman, and S. P. Grill.** 1983. Unique spectrum of activity of 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine against herpesviruses *in vitro* and its mode of action against herpes simplex virus type 1. Proc. Natl. Acad. Sci. U.S.A. **80**:2767–2770.
3. **Cleland, W. W.** 1963. Computer programmes for processing enzyme kinetic data. Nature (London) **198**:463–465.
4. **Collins, P., and D. J. Bauer.** 1977. Relative potencies of anti-herpes compounds. Ann. N.Y. Acad. Sci. **284**:49–50.
5. **Elion, G. B., P. A. Furman, J. A. Fyfe, P. de Miranda, L. Beauchamp, and H. J. Schaeffer.** 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. U.S.A. **74**:5716–5720.
6. **Field, A. K., M. E. Davies, C. DeWitt, H. C. Perry, R. Liou, J. Germershausen, J. D. Karkas, W. T. Ashton, D. B. R. Johnston, and R. L. Tolman.** 1983. 9-[[2-Hydroxy-1-(hydroxymethyl) ethoxy]methyl]guanine: a selective inhibitor of herpes group virus replication. Proc. Natl. Acad. Sci. U.S.A. **80**:4139–4143.
7. **Furman, P. A., D. M. Coen, M. H. St. Clair, and P. A. Schaffer.** 1981. Acyclovir-resistant mutants of herpes simplex virus type 1 express altered DNA polymerase or reduced acyclovir phosphorylating activities. J. Virol. **40**:936–941.
8. **Furman, P. A., P. V. McGuirt, P. M. Keller, J. A. Fyfe, and G. B. Elion.** 1980. Inhibition by acyclovir of cell growth and

DNA synthesis of cells biochemically transformed with herpesvirus genetic information. Virology **102**:420–430.

9. **Furman, P. A., M. H. St. Clair, J. A. Fyfe, J. L. Rideout, P. M. Keller, and G. B. Elion.** Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl)guanine and its triphosphate. J. Virol. **32**:72–77.
10. **Fyfe, J. A., P. M. Keller, P. A. Furman, R. L. Miller, and G. B. Elion.** 1978. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. J. Biol. Chem. **253**:8721–8727.
11. **Huang, E.-S.** 1975. Human cytomegalovirus. III. Virus-induced DNA polymerase. J. Virol. **16**:298–310.
12. **Miller, W. H., and R. L. Miller.** 1982. Phosphorylation of acyclovir diphosphate by cellular enzymes. Biochem. Pharmacol. **31**:3879–3884.
13. **Potuzak, H., and U. Wintersberger.** 1976. DNA covalently linked to carboxymethyl-cellulose and its application in affinity chromatography. Febs Lett. **63**:167–170.
14. **Smith, K. O., K. S. Galloway, W. L. Kennell, K. K. Ogilvie, and B. K. Radatus.** 1982. A new nucleoside analog, 9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine, highly active in vitro against herpes simplex virus types 1 and 2. Antimicrob. Agents Chemother. **22**:55–61.
15. **Spector, T., and G. Hajian.** 1981. Statistical methods to distinguish competitive, noncompetitive, and uncompetitive enzyme inhibitors. Anal. Biochem. **115**:403–409.
16. **St. Clair, M. H., P. A. Furman, C. M. Lubbers, and G. B. Elion.** 1980. Inhibition of cellular α and virally induced deoxyribonucleic acid polymerases by the triphosphate of acyclovir. Antimicrob. Agents Chemother. **18**:741–745.
17. **Weissbach, A., S. Hong, J. Aucker, and R. Miller.** 1973. Characterization of herpes simplex virus-induced deoxyribonucleic acid polymerase. J. Biol. Chem. **218**:6270–6277.
18. **Wilkinson, G. N.** 1961. Statistical estimations in enzyme kinetics. Biochem. J. **80**:324–332.