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Inhibition of Herpes Simplex Virus-Induced DNA Polymerases and Cellular DNA Polymerase α by Triphosphates of Acyclic Guanosine Analogs

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SUMMARY

The triphosphates of the antiherpes acyclic guanosine analogs (*R*)- and (*S*)-enantiomers of 9-(3,4-dihydroxybutyl)guanine [BCVTP and (*S*)-DHBGTP], 9-(4-hydroxybutyl)guanine (HBGTP), and 9-(2-hydroxyethoxymethyl)guanine (ACVTP) were investigated for their effects on partially purified DNA polymerases of herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) as well as cellular DNA polymerase α of calf thymus and Vero cells. The triphosphates of the four analogs were all competitive inhibitors when dGTP was the variable substrate with both the viral and the cellular DNA polymerases with activated calf thymus DNA or poly(dC)oligo(dG)$_{12-18}$ as template. No inhibition was observed with deoxythymidine 5′-triphosphate as substrate and poly(dA)oligo(dT)$_{12-18}$ as template. All analogs were preferential inhibitors of the viral DNA polymerases. Ordering the compounds according to their decreasing binding affinities, as reflected by their increasing inhibition constants for the viral DNA polymerases, gave ACVTP>HBGTP>BCVTP>(*S*)-DHBGTP. The DNA polymerase from the HSV-1 mutant, Cl(101)P_2C_5, resistant to ACV, showed a stronger decrease in sensitivity for ACVTP and HBGTP than for BCVTP compared to the effects on DNA polymerase from the wild-type strain Cl(101). The analogs were not able to support DNA synthesis in the absence of the competing substrate dGTP. A decrease in the ability of calf thymus DNA to serve as primer template for HSV-2 DNA polymerase was observed after preincubation with the triphosphates of the acyclic guanosine analogs. The analogs showed a progressive inhibition of the HSV-2 DNA polymerase activity with incubation time, and the inhibition could be reversed by high concentrations of dGTP both with and without addition of fresh enzyme or fresh template. However, no reversion was obtained when fresh enzyme or template was added if dGTP was omitted. The data indicate that these analogs inhibited the DNA polymerases by a similar mechanism and that the inhibition was reversible.

The antiherpes compounds ACV, HBG, and the (*R*)- and (*S*)-enantiomers of DHBG, BCV and (*S*)-DHBG, respectively, have been shown to require the HSV-1- or HSV-2-induced thymidine kinase (TK) for their antiviral activities (1–4). In contrast to ACV, which was shown to have low affinity to the viral TKs as well as a low phosphorylation rate (2, 4), the other analogs were characterized by having high affinities for the viral TKs (2–4). Especially the (*R*)-enantiomer of DHBG (BCV) was shown to have both a high affinity and a high phosphorylation rate to the viral TKs (3, 4). The monophosphates of these analogs were further shown to be substrates for cellular GMP kinase (5, 6)[1] and the triphosphates could then be formed by cellular kinases (5–7). Thus, once the monophosphates of these analogs are formed, cellular metabolism will dictate the formation of the triphosphates. In principle, two steps in the anti-HSV mechanism are involved for this type of antiherpes inhibitor, which play a role in their selective modes of action: first, the "activation step," e.g., the ability of the analogs to act as substrates for the virus-induced TK compared to the cellular TKs, and second, the "inhibition step," e.g., the ability of these analogs in their triphosphorylated forms to preferentially interact with the viral DNA polymerases as compared to cellular DNA polymerases. The antivirally active form of ACV has previously been shown to be ACVTP which is a highly potent and selective inhibitor of HSV-induced DNA polymerases (8, 9), and recently BCVTP was also shown to be an inhibitor of HSV-1 and HSV-2 DNA polymerases (6). In cell culture, ACV inhibited HSV multiplication about 10 times better than did either BCV or HBG and about 40 times better than did (*S*)-DHBG (4, 10). All of these analogs were earlier shown to inhibit viral DNA synthesis in HSV-infected cells at concentrations where no effect was observed on the cellular DNA synthesis in infected or uninfected cells (2, 3, 11).

[1] A. Larsson, unpublished observations.

ABBREVIATIONS: ACV, 9-(2-hydroxyethoxymethyl)guanine; BCV, (R)-DHBG, (*R*)-9-(3,4-dihydroxybutyl)guanine; (*S*)-DHBG, (*S*)-9-(3,4-dihydroxybutyl)guanine; DHBG, 9-3,4-dihydroxybutyl)guanine; HBG, 9-(4-hydroxybutyl)guanine; DHPG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; BCVTP, BCV 5′-triphosphate; (*S*)-DHBGTP, (*S*)-DHBG 5′-triphosphate; HBGTP, HBG 5′-triphosphate; ACVTP, ACV 5′-triphosphate; foscarnet, trisodium phosphonoformate; dNTP, deoxynucleoside 5′-triphosphates; HSV-1 and HSV-2, herpes simplex virus types 1 and 2; TK, thymidine kinase; GMP kinase, guanosine monophosphate kinase.

The aim of this study was to investigate whether the triphosphates of HBG and (*S*)-DHBG really also are the active forms of these analogs and, if so, to investigate the relative potency of these triphosphates and of BCVTPs as well as their mechanism of actions as DNA polymerase inhibitors relative to ACVTP. We also wanted to explain why ACV is a more potent inhibitor of HSV multiplication *in vitro* than the other analogs.

Materials and Methods

Reagents and chemicals. ACV and HBG were synthesized at Astra Läkemedel AB according to published methods (2). The synthesis of the (*R*)- and (*S*)-enantiomers of DHBG was made at Astra Läkemedel AB and will be described separately.[2]

The deoxynucleoside triphosphates were from Sigma Chemical Co., St. Louis, MO. [*Methyl*-^{3}H]dTTP (specific activity 79.4 Ci/mmol) and [8,5-^{3}H]dGTP (specific activity 27.1 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA. Activated calf thymus DNA was prepared as described by Schlabach *et al.* (12). Poly(dC)oligo(dG)$_{12-18}$ and poly(dA)oligo(dT)$_{12-18}$ were from Pharmacia P-L Chemicals, Uppsala, Sweden.

Calf thymus DNA polymerase α was from Worthington Biochemical Corp., Freehold, NJ. Hog brain GMP kinase, creatine kinase, and phosphocreatine were purchased from Boehringer-Mannheim Biochemicals, Indianapolis, IN. Tris-tri-*n*-butylamonium pyrophosphate was synthesized according to the method of Ruth and Cheng (13) by S. Kovacs, Astra Läkemedel AB. The monophosphates of BCV and (*S*)-DHBG (BCVMP and (*S*)-DHBGMP) were synthesized enzymatically by K. Eklind, Astra Läkemedel AB, with wheat shoot extracts as has been described earlier (6) in a yield of 36%. This method was preferred since a chemical phosphorylation of BCV yielded a mixture of monophosphates including a phosphate group on the secondary hydroxyl group as well as a 3,4-cyclic monophosphate (data not shown). BCVMP made by wheat shoot extracts was shown by ^{13}C NMR and ^{31}P NMR analysis to be phosphorylated at the primary hydroxyl group.

The BCVTP and (*S*)-DHBGTP were made enzymatically as follows. About 13 mg of BCVMP or (*S*)-DHBGMP were incubated in a mixture containing 50 mM Tris-acetate, pH 7.6, 5 mM $MgCl_2$, 10 mM ATP, 2 mM dithiothreitol, 130 mM phosphocreatine, 250 units/ml of creatine kinase, 2.5 mM NaF, 800 μg of GMP kinase, and 10 mg/ml of bovine serum albumin in a total volume of 5 ml. The mixture was incubated for 24 hr at 30°. The triphosphates formed were purified by HPLC on a preparative anion-exchange column (Whatman, Partisil 10 SAX 1.0 × 25 cm) isocratically with 0.45 M KH_2PO_4, pH 3.5, and desalted on a DEAE-Sephadex A 25 column (1.5 × 10 cm) using a linear gradient of 0.2–1.0 M triethylammonium formate, pH 7.3. The buffer was removed by repeated lyophilizations. The synthesis of the triphosphates of ACV and HBG, starting with 68 mg of ACV and 25 mg of HBG, were made as described by Ruth and Cheng (13). The triphosphates formed were purified on a DEAE-Sephadex A-25 column as described above. The yields were about 20% for BCVTP and (*S*)-DHBGTP, 7% for ACVTP, and about 11% for HBGTP. The purity determined by analytical high pressure liquid chromatography was > 98% for the synthesized triphosphates.

Isolation of viral and cellular DNA polymerases. HSV-1 strain C42 and HSV-2 strain 91075 have been described earlier (11) and HSV-1 Cl(101) and HSV-1 Cl(101)P_2C_5 were kindly provided by Dr. H. Field. The purification of HSV-1 and HSV-2 DNA polymerases from infected baby hamster kidney cells as well as DNA polymerase α from Vero cells was done as described previously (14).

Enzyme assay. DNA polymerase assays were performed according to published methods (14). Unless otherwise specified, standard DNA polymerase reactions (50 μl) contained: buffer A [100 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 100 mM KCl (not included in the DNA polymerase α reaction), 5 mM dithiothreitol, 45 μg of bovine serum albumin, 250 μg/ml of activated calf thymus DNA], 100 μM dATP, dCTP, dGTP, and 5 μM [^{3}H]dTTP (3500 cpm/pmol), and DNA polymerase. The synthetic template poly(dC)oligo(dG)$_{12-18}$ or poly(dA)oligo(dT)$_{12-18}$ was used, at concentrations of 2 μg/ml and 20 μg/ml, respectively. One unit of DNA polymerase activity was defined as the incorporation of 1 pmol of [^{3}H]dTMP/min. The reaction mixtures were incubated for 30 min at 37° and trichloroacetic acid-insoluble radioactivity was determined from 20-μl samples. The enzyme-kinetic experiments were done in buffer A with dNTP concentrations of 100 μM each of dATP, dCTP, dTTP and by varying the concentration of [^{3}H]dGTP using 2.0 unit of HSV-1 and HSV-2 DNA polymerases, respectively, and 0.02 unit of cellular DNA polymerase α from calf thymus and from Vero cells, respectively. The kinetic constants, K_M and K_I, were determined after linear regression analysis of the data in double reciprocal plots and using the relationship described by Lineweaver and Burk for competitive inhibitors that:

$$\text{slope } I = \text{slope } O\left(1 + \frac{[I]}{K_I}\right) \quad \text{(Slope } I = \text{slope for inhibited reaction)}$$

slope O = slope for uninhibited reaction, and $[I]$ = inhibitor concentration, and K_I = inhibition constant). The rate of DNA polymerization was linear at the dNTP concentration used during the incubation period, 30 min, and the nucleoside analogs were stabile as analyzed by HPLC during the assay conditions (not shown).

Ability to support DNA synthesis in the absence of dGTP. The ability of the analogs to support HSV or cellular DNA synthesis in the absence of dGTP was evaluated in buffer A with 100 μM dATP and dCTP each, 10 μM [^{3}H]dTTP (1780 cpm/pmol), 0.2 unit of HSV-1 and HSV-2 DNA polymerase, 0.02 unit of calf thymus DNA polymerase α, and a 100 μM concentration of the acyclic guanosine analogs, respectively. The activity was compared with the activity obtained with 100 μM dGTP added instead of the nucleoside analog.

Effect on DNA primer efficacy. The analogs were incubated for different times (0–60 min) in a 50-μl reaction mixture containing buffer A except that 5 μg of activated calf thymus DNA and 0.2 or 1 unit of HSV-2 DNA polymerase were used. After the indicated times of incubation, the reaction mixture was heated to 65° (in 65° water bath) for 15 min to inactivate the enzyme and then it was slowly cooled in room temperature for 1 hr. Incubation with fresh enzyme (0.2 unit) after addition of 1 mM dGTP and 2 μM [^{3}H]dTTP was then continued for 30 min at 37°.

Time-dependent inhibition of HSV-2 DNA polymerase activity. Enzyme assays were performed in buffer A with dNTP concentrations of 100 μM dATP and dCTP, respectively, 0.2 μM dGTP (for BCVTP) or 2 μM dGTP (for ACVTP and HBGTP), and 10 μM [^{3}H]dTTP. At the indicated times 20 μl of the mixtures were added on Whatman cellulose paper and counted as described earlier.

Results

Inhibition of DNA polymerases. Kinetic experiments showed that the triphosphates of ACV, HBG, BCV (Fig. 1, A–C), and (*S*)-DHBG (not shown) were competitive inhibitors of the HSV-2 DNA polymerization reaction as well as with the cellular DNA polymerase α (Fig. 1, D–F) with respect to the varied substrate dGTP. Similar results were observed when HSV-1 DNA polymerase was used (not shown).

The inhibition constants (Table 1) revealed that ACVTP was the most potent inhibitor with K_I values for HSV-1 (C42) and HSV-2 (91075) DNA polymerases of 0.0014 μM and 0.0016 μM, respectively. HBGTP, which was much less inhibitory than ACVTP, with K_I values of 0.12 μM and 0.11 μM for HSV-1 (C42) and HSV-2 (91075) DNA polymerase, respectively, was more inhibitory than BCVTP and (*S*)-DHBGTP.

ACVTP showed about the same affinity for the cellular DNA

[2] Manuscript in preparation.

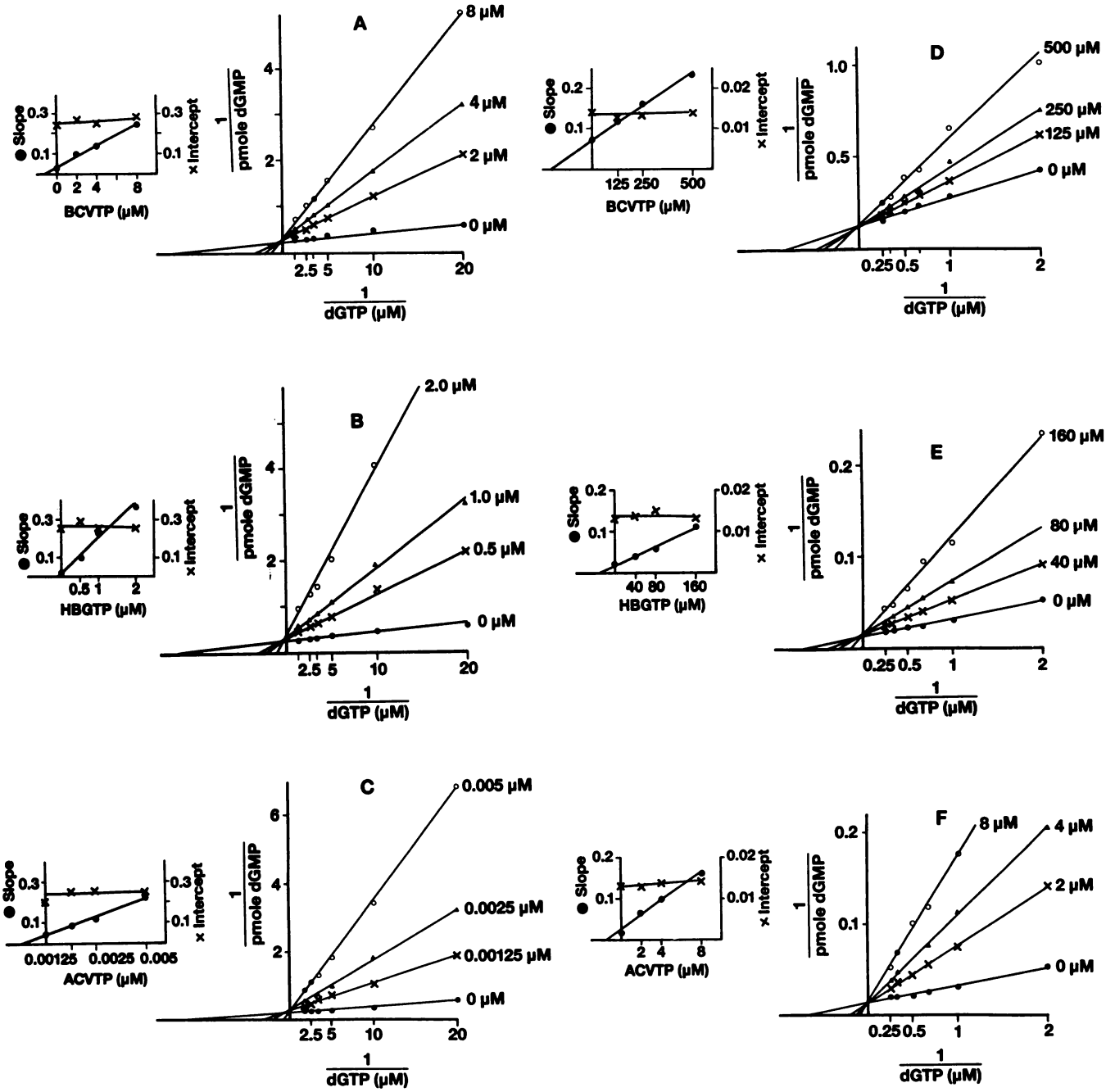

Fig. 1. Competitive inhibition of HSV-2 DNA polymerase (strain 91075) and calf thymus α DNA polymerase by triphosphates of acyclic guanosine analogs. The data are presented as Lineweaver Burk plots for HSV-2 (A–C) and as cellular DNA polymerase α (D–F) for BCVTP (A, D) HBGTP (B, E) and ACVTP (C, F) as inhibitors, with dGTP as the variable substrate and activated calf thymus DNA as template. The assay is described in Materials and Methods.

polymerase α as the natural substrate dGTP, whereas about 100 times higher affinity was observed for ACVTP than for dGTP when using the viral DNA polymerases. In contrast, HBGTP showed similar affinity for the viral DNA polymerases as dGTP, whereas lower affinity was observed for the cellular DNA polymerase α. BCVTP showed, in comparison with dGTP, lower affinity for both the viral and, especially, the cellular DNA polymerase α. Although BCVTP had about 10 times higher affinity for the viral DNA polymerases than (*S*)-DHBGTP, binding affinities of about the same magnitude were observed when the cellular DNA polymerase α was studied for these two enantiomers.

The DNA polymerase purified from the HSV-1 strain Cl(101)P_2C_5 showed a markedly decreased sensitivity to ACVTP (about 100 times) compared to the enzyme of the wild-type strain Cl(101) (Table 1). The same order of decreased sensitivity was also observed for the triphosphate of HBG, whereas a lower decrease was observed for BCVTP when the affinities to the purified DNA polymerases from the HSV-1 strains Cl(101) and Cl(101)P_2C_5 were compared. These differences also corresponded to the antiviral activities in cell culture (Vero cells) for these HSV-1 strains where the ID_{50} values for Cl(101) compared to Cl(101)P_2C_5 for ACV were 1.4 μM and 33 μM, for HBG 10 μM and 200 μM, and for BCV 7.2 μM and 24 μM.

TABLE 1

Inhibition constants (K_i) of triphosphates of acyclic guanosine analogs for DNA polymerases

The inhibition constants were determined as described in Materials and Methods; results shown are mean (± standard deviation) values from five different determinations.

Polymerase	dGTP	K_i			
		BCVTP	(S)-DHBGTP	HBGTP	ACVTP
	K_M		μM		
HSV-1 C42	0.14 ± 0.03	0.76 ± 0.17	8.63 ± 1.21	0.12 ± 0.01	0.0014 ± 0.0004
HSV-1 C(101)	0.064 ± 0.0023	0.64 ± 0.05	ND[a]	0.12 ± 0.002	0.0043 ± 0.0002
HSV-1 C(101)P_2C_5	0.13 ± 0.018	8.8 ± 0.2	ND	21.0 ± 1.8	0.44 ± 0.02
HSV-2 91075	0.075 ± 0.010	0.60 ± 0.08	8.86 ± 1.87	0.11 ± 0.02	0.0016 ± 0.0002
Calf thymus	1.4 ± 0.19	204 ± 17	199 ± 12	34 ± 5	1.1 ± 0.2
Vero cell[b]	0.8	130	93	29	1.8

[a] ND, not done.
[b] Mean values from two different determinations.

As mentioned above, these analogs were competitive inhibitors to dGTP with activated calf thymus DNA as template. No competition was observed between triphosphates of the nucleoside analogs and the other dNTPs in their inhibition of the DNA polymerases when activated calf thymus DNA was used as template (not shown).

This corresponds to the results shown in Table 2, where the effects of the triphosphates for these nucleoside analogs were investigated with two different synthetic templates, poly(dC)-oligo(dG)$_{12-18}$ and poly(dA)oligo(dT)$_{12-18}$. All analogs were competitive inhibitors with dGTP when poly(dC)oligo(dG)$_{12-18}$ was used as template, whereas no inhibition (ID_{50} > 150 μM) was observed when the effect of these analogs was studied against the incorporation of dTTP with poly(dA)oligo(dT)$_{12-18}$ as template.

Ability to support DNA synthesis in the absence of competing substrate. The ability of the acyclic guanosine analogs to support HSV or cellular DNA polymerase synthesis is shown in Table 3. In a reaction mixture in which the competing substrate dGTP was omitted, a low DNA polymerase activity was observed. Addition of dGTP increased the activity, whereas addition of either ACVTP, BCVTP, or HBGTP instead of dGTP decreased the DNA polymerase activity, even more than was observed when dGTP was omitted. This shows that none of these analogs can substitute for dGTP in the DNA polymerase reaction. These data also indicate that the inhibition of DNA polymerase caused by the triphosphates of these analogs may involve more mechanisms of action than only a competitive inhibition of dGMP incorporation.

Effect on primer template efficacy. The triphosphates of ACV, BCV, and HBG were incubated at concentrations 60 times their corresponding K_i values with HSV-2 DNA polymerase and a limiting amount of calf thymus DNA. The primer template efficiency was then evaluated in the presence of a saturating concentration of normal substrates and with fresh enzyme and compared with unmodified control. As shown in Fig. 2, neither ACVTP, BCVTP, nor HBGTP (not shown) had any effect on the ability of the activated DNA to serve as primer template with the same amount of enzyme used as in the enzyme-kinetic experiments where the inhibition constants were determined. However, by increasing the amount of HSV-2 DNA polymerase 5 times to 1 unit during the preincubation, a decrease in the ability of the activated DNA to serve as primer-template was observed for these analogs (Fig. 2; for HBGTP, not shown). In contrast, the inhibition of the HSV-2 DNA polymerase activity in a standard assay (or in enzyme-kinetic experiments) was not dependent on the amount of enzyme used (not shown).

TABLE 2

Effects of triphosphates of acyclic guanosine analogs for HSV-2 91075 DNA polymerase using synthetic templates

The inhibition constants with poly(dC)·oligo(dG)$_{12-18}$ with different concentrations of [^{3}H]dGTP as well as the ID_{50} values with poly(dA)·oligo(dT)$_{12-18}$ as templates using 1 μM [^{3}H]dTTP were performed as described in Materials and Methods.

Triphosphate	K_i with poly(dC)·oligo(dG)$_{12-18}$	ID_{50} with poly(dA)·oligo(dT)$_{12-18}$
	μM	
dGTP	0.57	
BCVTP	2.40	>150
(S)-DHBGTP	1.46	>150
HBGTP	0.30	>150
ACVTP	0.016	>150

TABLE 3

The ability of triphosphates of acyclic guanosine analogs to support DNA synthesis in the absence of dGTP

The DNA polymerase assays were performed as described in Materials and Methods.

Compound added	Origin of DNA polymerase		
	HSV-1 (C42)	HSV-2 (91075)	Cell α (Vero)
	relative % DNA synthesis		
dGTP	100	100	100
BCVTP	2	2	28
HBGTP	2	2	10
ACVTP	3	3	6
No addition	15	10	41

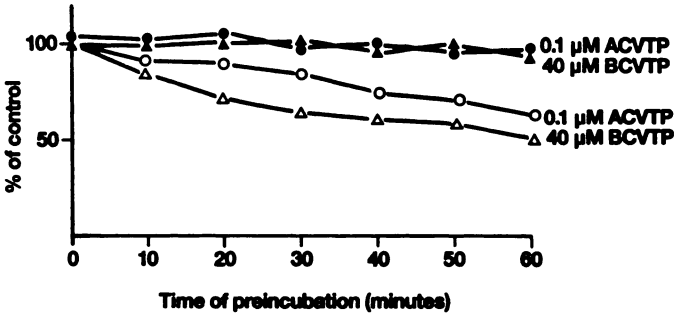

Fig. 2. Effect of triphosphates of acyclic guanosine analogs on DNA primer efficiency. ACVTP and BCVTP incubated at their 60 × K_i values, respectively, with buffer A, 100 μM dATP, dCTP, 5 μg of calf thymus DNA, and two different concentrations of HSV-2 DNA polymerase as described in Materials and Methods. ●, 0.1 μM ACVTP, or ▲, 40 μM BCVTP + 0.2 unit of HSV-2 DNA polymerase; ○, 0.1 μM ACVTP, or △, 40 μM BCVTP + 1.0 unit of HSV-2 DNA polymerase.

Time-dependent inhibition of HSV-2 DNA polymerase. In an HSV-2 DNA polymerase reaction, where the incorporation of [^{3}H]dTMP into activated calf thymus DNA was followed, a deceleration in the rate of incorporation of [^{3}H]dTMP was observed for ACVTP, BCVTP, and HBGTP (Fig. 3) after about 15 min of incubation. The rate of deceleration increased with increasing concentration of the analogs. This deceleration was independent of the amount of primer template used (not shown).

Reversal of inhibition of HSV-2 DNA polymerase. To determine whether the inhibition of the viral DNA polymerase could be restored, e.g., if the inhibition was a reversible or an irreversible process, fresh template or fresh enzyme with or without an excess of dGTP was added to the reaction mixture. It can be seen in Fig. 4 that the HSV-2 DNA polymerase activity, which was strongly inhibited by 60 times the K_I values of BCVTP or ACVTP (or HBGTP, not shown), respectively, could be restored nearly to the rate of the untreated control if, after 15 min preincubation, an excess of dGTP was added but a short lag period was observed. However, if the reaction mixtures contained a limiting concentration of either primer-template or low amounts of HSV-2 DNA polymerase, the inhibition with BCVTP or ACVTP (or HBGTP, not shown) could not be reversed if either fresh template (Fig. 5) or fresh enzyme, respectively (not shown), was added without an excess of dGTP. However, if an excess of dGTP was added to prevent further inhibition with the analogs, together with either fresh template or fresh enzyme, the rate of HSV-2 DNA polymerization was comparable to or even higher than the untreated control.

Discussion

The acyclic guanosine analogs represent a class of nucleoside analogs with an unmodified base and a modification in the sugar moiety. They differ from the other class of nucleoside analogs investigated as antiherpes agents, 5-substituted 2′-deoxyuridines or 2′-deoxycytidines (15–17), which usually are modified in the base both with and without an additional modification in the sugar moiety. The triphosphates of analogs of these two classes of nucleoside analogs have earlier been shown to be the active forms which can interact with both viral and, to a lesser extent, cellular DNA polymerases (8, 13, 18). The results summarized in Table 1 show that the acyclic guanosine analogs investigated in this study were inhibitors of viral DNA polymerases as well as of cellular DNA polymerase α. The performed enzyme-kinetic experiments show that the analogs were competitive with dGTP for viral as well as for cellular DNA polymerase α. However, there were great differences in their affinities with ACVTP being the most potent inhibitor of viral DNA polymerases and cellular DNA polymerase α. ACVTP also showed the largest difference between inhibiting the viral DNA polymerases and the cellular DNA polymerase α. About 800 times lower K_I values were obtained for the viral DNA polymerases compared to the cellular DNA polymerase α, whereas a ratio of 300 was observed for BCV and HBG. This corresponds to the previous observations that ACV was a more selective inhibitor of viral DNA synthesis in HSV-1-infected cells than were BCV and HBG (2, 3, 11). Compared to HBGTP, ACVTP binds about 100 times more tightly to the viral DNA polymerases showing that the introduction of a methylene group instead of an oxygen in the 2-position of the carbon chain, which replaces the sugar moiety in these acyclic guanosine analogs, markedly reduced the affinity to the DNA polymerases. The introduction of an additional hydroxyl group at the 3-carbon in the four carbon chain, as in BCV and (*S*)-DHBG, even further lowered the affinity for the viral and cellular DNA polymerases compared to ACVTP and HBGTP (Table 1). A similar decrease in affinity was earlier observed when the triphosphate of the acyclovir analog DHPG (BW 759U, Biolf-62, 2′-NDG) was compared with ACVTP in its effect on different DNA polymerases revealing that addition of a hydroxymethyl group in the sugar moiety decreased the affinity of DHPGTP for both viral and cellular DNA polymerases as compared to ACVTP (19). Furthermore, the (*R*)-configuration of DHBG (BCVTP), in which the secondary hydroxyl group might better simulate the 3′-hydroxyl group in

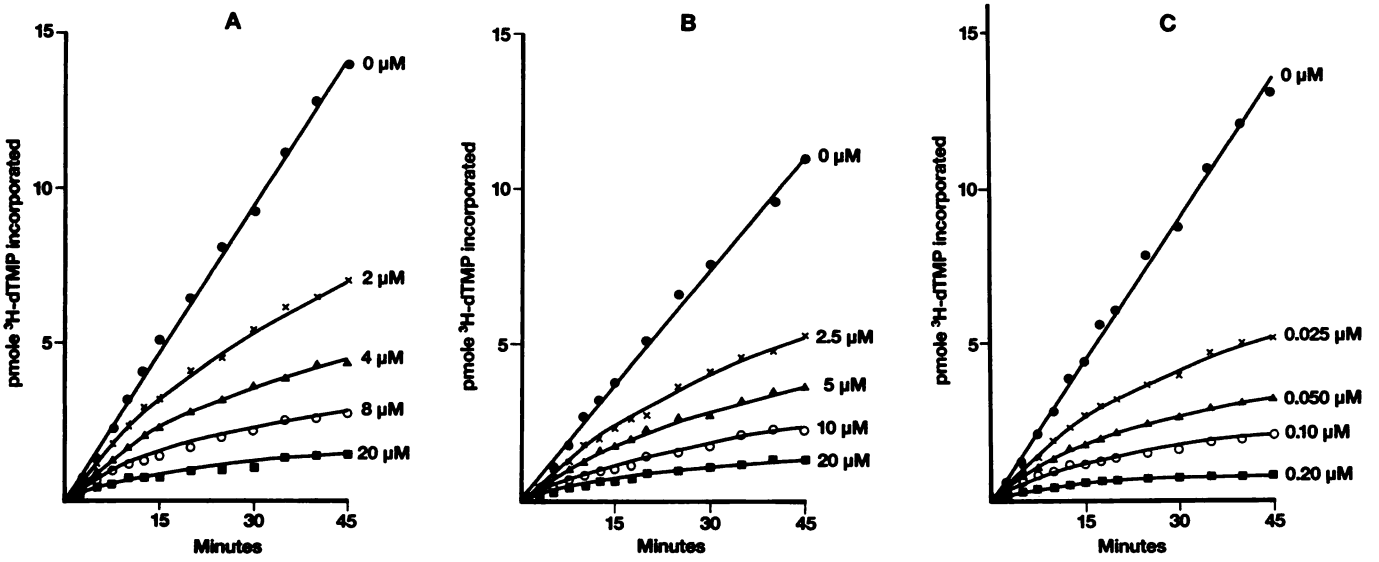

Fig. 3. Time-dependent inhibition of HSV-2 DNA polymerase by triphosphates of acyclic guanosine analogs. The assays were performed as described in Materials and Methods by following the incorporation of [^{3}H]dTTP (3200 cpm/pmol) in the presence of the indicated concentrations of BCVTP (A), HBGTP (B), ACVTP (C), and 0.5 unit of enzyme.

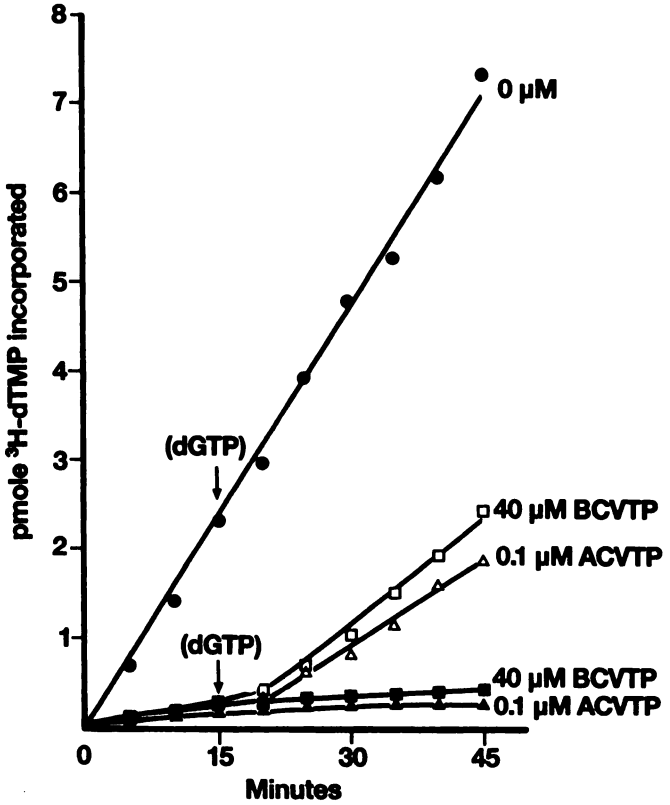

Fig. 4. Reversal of HSV-2 DNA polymerase inhibition of triphosphates of acyclic guanosine analogs by dGTP. The incorporation of [^{3}H]dTTP was followed with 60 × K_i values of BCVTP and ACVTP. The assay mixtures (250 µl) contained: buffer A, 100 µM each of dATP and dCTP, 0.2 µM dGTP, 10 µM [^{3}H]dTTP (1780 cpm/pmol), 250 µg/ml of activated calf thymus DNA and 0.5 unit of enzyme. After 15 min incubation, 20 mM dGTP was added to give a final concentration of 1 mM dGTP and the incubation was continued. Then, 20-µl samples were counted at the indicated times. ●, control; □, 40 µM BCVTP + 1 mM dGTP at 15 min; △, 0.1 µM ACVTP + 1 mM dGTP at 15 min; ■, 40 µM BCVTP + 0 mM dGTP at 15 min; ▲, 0.1 µM ACVTP + 0 mM dGTP at 15 min.

a deoxysugar than does the secondary hydroxyl group in the (*S*)-configuration, has a higher affinity for the viral DNA polymerases, whereas similar affinities for the cellular DNA polymerase α were observed for both of these enantiomers. The data clearly show that all of these analogs have a high and preferential affinity for the viral DNA polymerases and also that neither of these analogs have significantly different affinity for HSV-1 DNA polymerase compared to HSV-2 DNA polymerase.

The partially purified DNA polymerase from HSV-1 strain Cl(101)P_2C_5 has a more reduced sensitivity for ACVTP and HBGTP than for BCVTP compared to the DNA polymerase from the wild-type strain Cl(101), whereas the affinity for dGTP was only marginally changed (Table 1). This shows that the DNA polymerase of this mutant has changed its binding site characters more for ACV and HBG than for BCV. Similar results were earlier observed with other DNA polymerases of other mutants where a DNA polymerase with decreased sensitivity for ACVTP still was sensitive for inhibition by the triphosphate of DHPG (20, 21) which, together with BCV, has a more "bulky" side chain substituent with two hydroxyl groups in contrast to ACV and HBG.

From the enzyme-kinetic experiments with calf thymus DNA as template as well as with poly(dC)oligo(dG)$_{12-18}$ and poly(dA)oligo(dT)$_{12-18}$ (Table 2), it is clear that these analogs interfere with the dGTP-binding site of the DNA polymerases. As shown in Table 3, dGTP cannot be replaced by any of these analogs either with viral or cellular DNA polymerases. These analogs were therefore not alternative substrates for DNA elongation in the absence of dGTP and an inhibition of the DNA polymerization reaction occurs with the involvement of "something more" than only being a competitive inhibitor to dGTP. To further investigate the inhibition pattern of these analogs which lack the ability to support DNA synthesis, the DNA polymerase and analog were preincubated with a limiting amount of activated calf thymus DNA. As shown in Fig. 2, neither BCVTP nor ACVTP (nor HBGTP, not shown) showed any effect on the ability of the primer-template to be fully functional after preincubation of the analog triphosphates with HSV-2 DNA polymerase at assay conditions where the normal inhibition values of the analogs were determined. In contrast, by increasing the amount of HSV-2 DNA polymerase during the preincubation with the analogs, a decrease in the ability of HSV-2 DNA polymerase to utilize the primer-template was observed (Fig. 2). These data indicate the BCV (and HBG) also might have the possibility of being incorporated into the DNA-template as earlier has been shown to be the case for ACVTP (8, 9). However, the observation that the primer-template was fully functional after preincubation with the same amount of enzyme used as in the enzyme-kinetic experiments indicate that the inhibition observed cannot be explained only by the eventual incorporation of the analogs into the template.

In a previous report (6) it was not possible to detect [^{3}H]BCVMP into primer-template in an HSV-2 DNA polymerase reaction, perhaps due to a low detection limit. If BCVMP is incorporated into the primer-template, it was less than 1% compared to dGTP (6). The inhibition of HSV-2 DNA polymerase activity was shown to be nonlinear during the incubation time for these analogs (Fig. 3), and this time-dependent progressive inhibition was independent of the amount of primer-template (not shown). This differs from what was observed with the reversible noncompetitive DNA polymerase inhibitor, foscarnet, which caused a linear inhibition profile in similar experiments[3] but is in agreement with a previous report for ACVTP for HSV-1 DNA polymerase (22). These data indicate that some kind of "inactivating process" might be involved in the mode of inhibition of the HSV-2 DNA polymerase for these analogs as observed previously (22). However, the time-dependent progressive inhibition of HSV-2 DNA polymerase by these nucleoside analogs was a reversible process. As shown in Figs. 4 and 5, the inhibition caused by BCVTP and ACVTP (and HBGTP, not shown) could be reversed with an excess of competing substrate dGTP, but not by fresh template (or fresh enzyme, not shown) only. These results differ from previous observations (22), showing that the inhibition of HSV-1 DNA polymerase by ACVTP could be reversed by an excess of dGTP and fresh enzyme but not by an excess of dGTP and fresh template. The reason for this difference is unclear. Nevertheless, the inhibition of HSV-2 DNA polymerase by BCVTP, ACVTP, and HBGTP was reversible with an excess of dGTP in our experiments. This indicates that the binding of the analog to the enzyme is a reversible process and that a dissociation of the analogs from the enzyme occurs by

[3] A. Larsson, unpublished results.

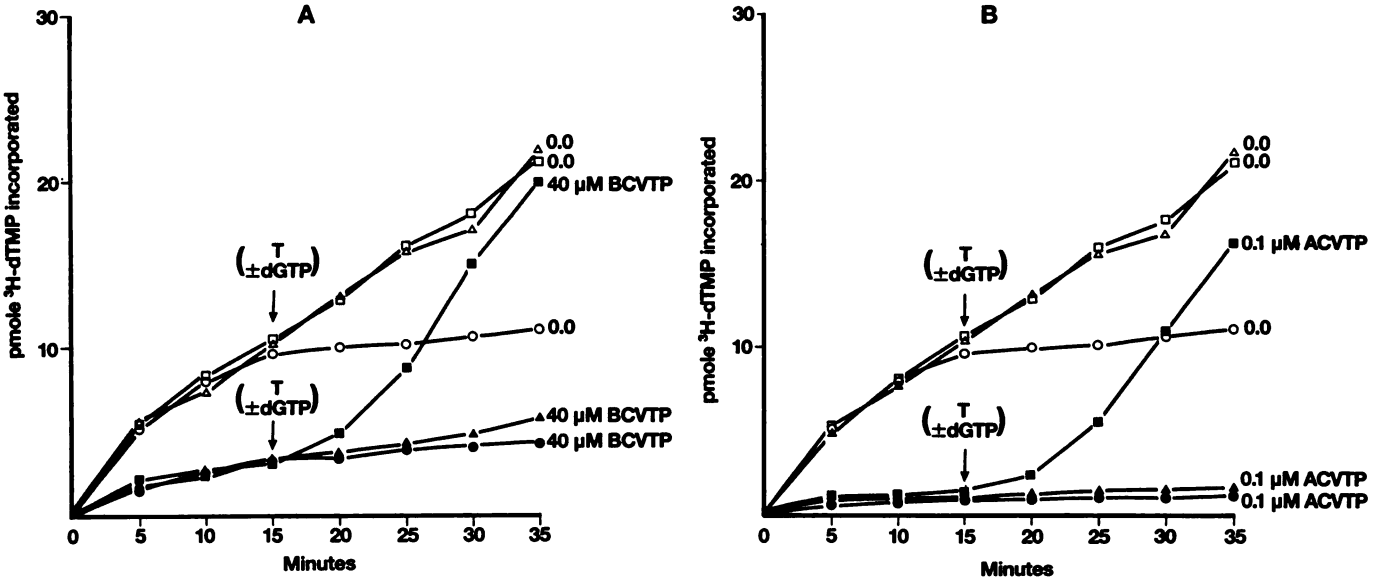

Fig. 5. Determination of functional HSV-2 DNA polymerase activity following inhibition by triphosphates of acyclic guanosine analogs. The incorporation of [^{3}H]dTMP was followed with 60 × K_i values of BCVTP (A) and ACVTP (B). The assay mixture (250 μl) contained: buffer A, 100 μM each of dATP and dCTP, 0.2 μM dGTP, 10 μM [^{3}H]dTTP (1780 cpm/pmol), 1 μg/ml of activated calf thymus DNA, and 0.5 unit of enzyme. After 15 min incubation, 20 mM dGTP and 10 mg/ml of activated calf thymus DNA were added to give final concentrations of 1 mM and 250 μg/ml, respectively. Then, 20-μl samples were counted at the indicated times. A. ○, control; △, control + template at 15 min; □, control + dGTP + template at 15 min; ●, 40 μM BCVTP; ▲, 40 μM BCVTP + template at 15 min; ■, 40 μM BCVTP + dGTP + template at 15 min. B. ○, control; △, control + template at 15 min; □, control + dGTP + template at 15 min; ●, 0.1 μM ACVTP; ▲, 0.1 μM ACVTP + template at 15 min; ■, 0.1 μM ACVTP + dGTP + template at 15 min.

addition of an excess of dGTP but not by high amounts of template or enzyme. Two possible steps might be involved in the DNA polymerase inhibition caused by these analogs, one step being binding to the dGTP-binding site of the DNA polymerase, thereby preventing further elongation possibly by causing "conformation" changes or inactivating the polymerase in some way. The other step could be incorporation of the analog into DNA and thereby, due to the lack of 3′-hydroxyl group, as for ACV, acting as a chain terminator (8, 9), which also seems possible for HBG and for BCV. A combination of these two interacting steps for these analogs could, of course, also be possible. However, the lack of detection of [^{3}H]BCVMP incorporation into primer-template (6) and the low incorporation of ACVMP (8) (and also for DHPGMP) into primer-template, in combination with the present results showing that the inhibition was reversible, might favor the first inhibition mechanism as mentioned above. That the formation of an enzyme-analog complex or enzyme-analog-template complex was reversible is not surprising since covalent linkage of inhibitor or "template-inhibitor" is not expected to be formed between enzyme and these nucleoside analogs. The explanation of why ACVTP is the most potent inhibitor of the DNA polymerases could be that ACV more easily causes a "conformation change" than the other analogs but it can be restored in the same manner as for the other analogs investigated in this study. Another explanation might be that ACVMP is more easily incorporated into the DNA than the other analogs, thereby preventing further DNA synthesis. Further comparative studies with radioactive-labeled triphosphates of these analogs may eventually explain more clearly the underlying mechanisms of action.

In summary, the data show that the explanation of why ACV is the most potent inhibitor of HSV-1 and HSV-2 multiplication in cell culture compared to the other analogs investigated in this study (4, 10) is most likely that ACVTP is a much more potent inhibitor of the viral DNA polymerases than the triphosphates of the other analogs. The inhibition mechanisms of the DNA polymerases caused by the triphosphates of the guanosine analogs studied seem to be similar, despite the observed differences in their affinities to the studied DNA polymerases. The more or less tight binding to the dGTP-binding site of the DNA polymerase of the analogs may result in the formation of an enzyme-analog (or even enzyme-analog-template) complex which prevents further elongation. This complex could be dissociated by addition of high amounts of the natural competing substrate dGTP, and the observed inhibitions are reversible.

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References

1. Fyfe, J. A., P. M. Keller, P. A. Furman, R. L. Miller, and G. B. Elion. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. *J. Biol. Chem.* **253**:8721–8727 (1978).
2. Larsson, A., S. Alenius, N.-G. Johansson, and B. Öberg. Antiherpetic activity and mechanism of action of 9-(4-hydroxybutyl)guanine. *Antiviral Res.* **3**:77–86 (1983).
3. Larsson, A., B. Öberg, S. Alenius, C.-E. Hagberg, N.-G. Johansson, B. Lindborg, and G. Stening. 9-(3,4-Dihydroxybutyl)guanine, a new inhibitor of herpesvirus multiplication. *Antimicrob. Agents Chemother.* **23**:664–670 (1983).
4. Larsson, A., and P.-Z. Tao. Phosphorylation of four acyclic guanosine analogs by herpes simplex virus type 2 thymidine kinase. *Antimicrob. Agents Chemother.* **25**:524–526 (1984).
5. Miller, W. H., and R. L. Miller. Phosphorylation of acyclovir (acycloguanosine) monosphosphate by GMP kinase. *J. Biol. Chem.* **255**:7204–7207 (1980).
6. Stenberg, K., A. Larsson, and R. Datema. Metabolism and mode of action of buciclovir ((*R*)-9-(3,4-dihydroxybutyl)guanine), in herpes simplex virus infected Vero cells. *J. Biol. Chem.* **261**:2134–2139 (1986).
7. Miller, W. H., and R. L. Miller. Phosphorylation of acyclovir diphosphate by cellular enzymes. *Biochem. Pharmacol.* **31**:3879–3884 (1982).
8. 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 (1979).
9. Derse, D., Y.-C. Cheng, P. A. Furman, M. H. St. Clair, and G. B. Elion. Inhibition of purified human and herpes simplex virus-induced DNA polymerases by 9-(2-hydroxyethoxymethyl)-guanine triphosphate. *J. Biol. Chem.* **256**:11447–11451 (1981).
10. Ericson, A.-C, A. Larsson, F. Y. Aoki, W.-A. Yisak, N.-G. Johansson, B. Öberg, and R. Datema. Antiherpes effects and pharmacokinetic properties of 9-(4-hydroxybutyl)guanine and the (*R*) and (*S*) enantiomers of 9-(3,4-dihydroxybutyl)guanine. *Antimicrob. Agents Chemother.* **27**:753–759 (1985).
11. Larsson, A., and B. Öberg. Selective inhibition of herpesvirus deoxyribonucleic acid synthesis by acycloguanosine, 2′-fluoro-5-iodo-aracytosine, and (E)-5-(2-bromovinyl)-2′-deoxyuridine. *Antimicrob. Agents Chemother.* **19**:927–929 (1981).
12. Schlabach A., B. Fridlender, A. Bolden, and A. Weissbach. DNA-dependent DNA polymerases from HeLa cell nuclei. II. Template and substrate utilization. *Biochem. Biophys. Res. Commun.* **44**:879–885 (1971).
13. Ruth, J. L., and Y.-C. Cheng. Nucleoside analogues with clinical potential in antivirus chemotherapy: the effect of several thymidine and 2′-deoxycytidine analogue 5′-triphosphates on purified human (α, β) and herpes simplex virus (types 1, 2) DNA polymerases. *Mol. Pharmacol.* **20**:415–422 (1981).
14. Eriksson, B., A. Larsson, E. Helgstrand, N.-G. Johansson, and B. Öberg. Pyrophosphate analogues as inhibitors of herpes simplex virus type 1 DNA polymerase. *Biochim. Biophys. Acta* **607**:53–64 (1980).
15. De Clercq, E. Antiviral and antitumor activities of 5-substituted 2′-deoxyuridines. *Methods Find. Exp. Clin. Pharmacol.* **2**:253–267 (1980).
16. De Clercq, E., J. Balazarini, J. Descamps, G.-F. Huang, P. F. Torrence, D. E. Bergstrom, A. S. Jones, P. Serafinowski, G. Verhelst, and R. T. Walker. Antiviral, antimetabolic, and cytotoxic activities of 5-substituted 2′-deoxycytidines. *Mol. Pharmacol.* **21**:217–223 (1981).
17. De Clercq, E., and R. T. Walker. Synthesis and antiviral properties of 5-vinylpyrimidine nucleoside analogs. *Pharmacol. Ther.* **26**:1–44 (1984).
18. Frank, K. B., J.-F. Chiou, and Y.-C. Cheng. Interaction of herpes simplex virus-induced DNA polymerase with 9-(1,3-dihydroxy-2-propoxymethyl)guanine triphosphate. *J. Biol. Chem.* **259**:1566–1569 (1984).
19. St. Clair, M. H., W. H. Miller, R. L. Miller, C. U. Lambe, and P. A. Furman. Inhibition of cellular α DNA polymerase and herpes simplex virus-induced DNA polymerases by the triphosphate of BW759U. *Antimicrob. Agents Chemother.* **25**:191–194 (1984).
20. Cheng, Y.-C., E.-S. Huang, J.-C. Lin, E.-C. Mar, J. S. Pagano, G. E. Dutschman, and S. P. Grill. Unique spectrum of activity of 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine against herpes-viruses *in vitro* and its mode of action against herpes simplex virus type 1. *Proc. Natl. Acad. Sci. USA* **80**:2767–2770 (1983).
21. Crumpacker, C. S., P. N. Kowalsky, S. A. Oliver, L. E. Schnipper, and A. K. Field. Resistance of herpes simplex virus to 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]guanine: physical mapping of drug synergism within the viral DNA polymerase locus. *Proc. Natl. Acad. Sci. USA* **81**:1556–1560 (1984).
22. Furman, P. A., M. H. St. Clair, and T. Spector. Acyclovir triphosphate is a suicide inactivator of the herpes simplex virus DNA polymerase. *J. Biol. Chem.* **259**:9575–9579 (1984).

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