0026-895X/91/040591-06$03.00/0

MOLECULAR PHARMACOLOGY, 40:591-596

Inhibition of Herpes Simplex Virus Type 1 DNA Polymerase by [1*R*(1α,2β,3α)]-9-[2,3-Bis(hydroxymethyl)cyclobutyl]guanine

BRIAN J. TERRY, CHRISTOPHER W. CIANCI, and MOIRA E. HAGEN

Department of Virology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000

Received February 25, 1991; Accepted June 24, 1991

SUMMARY

(±)-(1α,2β,3α) -9- [2,3-Bis(hydroxymethyl)cyclobutyl]guanine [(±)-BHCG] is a nucleoside analog with potent *in vitro* activity against herpesviruses [*Tetrahedron Lett.* 30:6453–6456 (1989)]. The two enantiomers have been synthesized, and their biochemical characterization is reported here. [1*S*(1α,2β,3α)]-9-[2,3-Bis(hydroxymethyl)cyclobutyl]guanine [(*S*)-BHCG] was phosphorylated by herpes simplex virus type 1 (HSV-1) thymidine kinase (V_{max} = 8 nmol/hr/μg of enzyme), whereas [1*R*(1α,2β,3α)]-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine [(*R*)-BHCG] was a poor substrate for the viral thymidine kinase under these conditions. The triphosphate of each enantiomer was enzymatically synthesized, and both enantiomers competitively inhibited HSV-1 DNA polymerase with respect to dGTP. However, the potency of (*R*)-BHCG-TP was 4 orders of magnitude greater than that of (*S*)-BHCG-TP. (*R*)-BHCG-TP inhibited HeLa DNA polymerase α, but the inhibition constant was 30-fold higher than that for the viral DNA polymerase. In comparison, (*S*)-BHCG-TP was a very poor inhibitor of DNA polymerase α. (*R*)-[^{3}H]BHCG-TP could be incorporated into a synthetic DNA template by HSV-1 DNA polymerase at 80% the extent of dGTP under the assay conditions used and, therefore, could act as an alternative substrate. Incorporation of (*R*)-BHCG-TP was similar to that observed for acyclovir triphosphate and ganciclovir triphosphate, based on maximal velocities. In contrast, HSV-1 DNA polymerase did not incorporate (*S*)-BHCG-TP into DNA. Compared with dGTP, only limited extension (10%) of the DNA primer by HSV-1 DNA polymerase occurred after incorporation of (*R*)-BHCG-TP and, therefore, (*R*)-BHCG-TP acts as a nonobligate chain terminator.

The potent and selective inhibitor of herpesvirus replication (±)-BHCG, [(*R,S*)-BHCG] has previously been described (1–4). This compound has an ED_{50} value of 0.08–0.8 μM for different strains of HSV-1 in plaque reduction assays and was efficacious in an HSV-1 intraperitoneal infection in mice (3). In addition, (±)-BHCG was shown to be effective against human immunodeficiency virus *in vitro* (4–6). (±)-BHCG could be phosphorylated by HSV-1 thymidine kinase, and the enzymatically synthesized triphosphate was a potent inhibitor of HSV-1 DNA polymerase (3).

Recently we synthesized (*R*)-BHCG and (*S*)-BHCG (Fig. 1) and reported that (*R*)-BHCG has potent antiherpetic activity and that (*S*)-BHCG is inactive (7). This confirmed concurrent reports (2, 8) that (+)-BHCG[1] has antiviral activity against HSV-1, HSV-2, and human cytomegalovirus. In this report, we have characterized (*R*)-BHCG and (*S*)-BHCG phosphorylation by HSV-1 thymidine kinase and synthesized the triphosphate of each enantiomer. We now report that (*R*)-BHCG-TP is an excellent inhibitor of HSV-1 DNA polymerase, whereas (*S*)-BHCG-TP is a poor inhibitor. In addition, we show that (*R*)-BHCG-TP acts as an alternative substrate for dGTP and is incorporated into DNA by HSV-1 DNA polymerase. Once incorporated into DNA, (*R*)-BHCG-MP is a poor primer for subsequent nucleotide addition. In contrast, (*S*)-BHCG-TP is not incorporated into DNA by HSV-1 DNA polymerase and does not appear to be an alternative substrate. These results offer strong evidence that the mechanism of antiviral activity of (*R*)-BHCG is due to inhibition of viral DNA polymerase.

[1] The absolute configurations of (*R*)-BHCG and (*S*)-BHCG have been determined (7). Nomenclature used by others indicates that (*R*)-BHCG corresponds to (+)-carbocyclic OXT/G-8 (2) and (+)-cyclobut-G (8).

Materials and Methods

Compounds. The cyclobutane guanosine analog (*R,S*)-BHCG was synthesized as described by Slusarchyk *et al.* (1). The two enantiomers of BHCG, (*R*)-BHCG and (*S*)-BHCG, were synthesized as described by Bisacchi *et al.* (7). The enantiomeric purity of both (*R*)-BHCG and (*S*)-BHCG was >99.9% (7). The syntheses of ACV and DHPG have been described previously in Netherlands Patent 7709458 (1978) and by Martin *et al.* (9), respectively.

[8-^{3}H]ACV, [8-^{3}H]DHPG, (*R*)-[8-^{3}H]BHCG, and (*S*)-[8-^{3}H]BHCG were synthesized by Amersham Corporation, by catalytic reduction of the corresponding 8-bromo analog with tritium gas. The tritiated

ABBREVIATIONS: (*R*)-BHCG, [1*R*(1α,2β,3α)]-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine; (*S*)-BHCG, [1*S*(1α,2β,3α)]-9-bis(hydroxymethyl)cyclobutyl]guanine; HSV-1, herpes simplex virus type 1; ACV, acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine; DHPG, ganciclovir, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine; DTT, dithiothreitol; dNTP, deoxynucleotide triphosphate; BHCG-TP, (1α,2β,3α)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine triphosphate; BHCG-MP, (1α,2β,3α)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine monophosphate; HPLC, high performance liquid chromatography; DHPG-TP, ganciclovir triphosphate; ACV-TP, acyclovir triphosphate; BSA, bovine serum albumin.

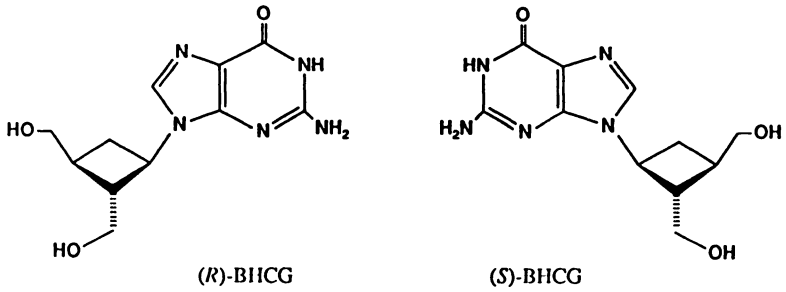

Fig. 1. Structures of (*R*)-BHCG and (*S*)-BHCG.

nucleosides were purified by reverse phase HPLC and had the following specific activities: [8-^{3}H]ACV, 6 Ci/mmol; [8-^{3}H]DHPG, 12 Ci/mmol; (*R*)-[8-^{3}H]BHCG, 19.9 Ci/mmol; and (*S*)-[8-^{3}H]BHCG, 20.7 Ci/mmol. 5′-TCGCAGCTC and 5′-AAACCCTTAGCGAGCTGCGA oligonucleotides (10) were synthesized using an Applied Biosystems 380B DNA synthesizer programmed to retain the trityl group. Crude oligonucleotides were deprotected and purified by OPC cartridges (11). Oligonucleotide concentrations were determined by UV spectroscopy. Each oligonucleotide was 5′-end labeled with polynucleotide kinase and [γ-^{32}P]ATP and run on a DNA-sequencing gel. The 9-mer oligonucleotide was >95% pure, and the purity of the 20-mer was >90%.

Phosphorylation of nucleosides. HSV-1 thymidine kinase was affinity purified from HSV-1 (KOS)-infected cells (3, 12). Phosphorylation of nucleosides by thymidine kinase was determined under the following conditions: 50 mM Tris·HCl pH 7.5, 5 mM $MgCl_2$, 30 mM KCl, 2 mM DTT, 0.5 mM nucleoside, 5 mM ATP, 0.075 mg/ml BSA, and 0.25 unit/ml thymidine kinase, at 37°. Samples were removed at 0–8 hr for rate determinations, quenched at 80° for 3 min, and frozen at −80° until analysis by HPLC. Reactions were analyzed by HPLC on a C_{18} column with 10 mM potassium phosphate, 2 mM tetrabutylammonium phosphate, and a 0–35% acetonitrile gradient (13). Nucleoside monophosphates were identified by UV spectra, utilizing a LKB rapid spectral detector. Rates of nucleoside phosphorylation were calculated from the linear portion of the reaction curve. Determinations of K_m and maximal velocity (V_{max}) were performed with varied (*R*)-[^{3}H]BHCG and (*S*)-[^{3}H]BHCG concentrations in the buffer described above and were assayed by a DE81 filter method (14).

Synthesis of nucleoside triphosphates. (*R*)-BHCG-TP, (*S*)-BHCG-TP, ACV-TP, and DHPG-TP were prepared by incubating 2 mg/ml nucleoside with HSV-1 thymidine kinase in 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 30 mM KCl, 2 mM DTT, 5 mM ATP, 30 mM creatine phosphate, 0.075 mg/ml BSA, 8 units/ml creatine kinase, at 37°. After 24 and 48 hr, thymidine kinase, 1 unit/ml guanylate kinase, 5 mM phospho(enol)pyruvate, 4 units/ml pyruvate kinase, and 8 units/ml creatine kinase were added, and incubation was continued at 37°. Reactions were allowed to proceed for an additional 48 hr for ACV, DHPG, and (*S*)-BHCG. Additional thymidine kinase was added to (*R*)-BHCG every 24 hr for 72 hr; phospho(enol)pyruvate and pyruvate kinase were added when ADP began to accumulate. The reaction products were separated by HPLC, using a Synchropak AX100 column with a linear gradient of 0.04–1 M KH_2PO_4, pH 5.6. Nucleoside triphosphates were then desalted on individual DEAE-Sephadex A-25 columns using a gradient of 0.05–0.6 M ammonium bicarbonate (pH 8) or 0.1–2 M triethylammonium acetate (pH 7.5), lyophilized several times, and quantitated by UV spectroscopy. The overall yields were as follows: ACV-TP, 63%; DHPG-TP, 69%; (*R*)-BHCG-TP, 2%; and (*S*)-BHCG-TP, 20%. (*R,S*)-BHCG-TP was enzymatically synthesized as previously described (3). Triphosphates were stored in 20 mM Tris·HCl pH 7.6, 0.2 mM EDTA, at −80°.

Radioactive triphosphates were synthesized and isolated as described above, starting with 300 μCi (15–50 nmol) of each nucleoside. Overall yields for [8-^{3}H]ACV-TP, [8-^{3}H]DHPG-TP, (*R*)-[8-^{3}H]BHCG-TP, and (*S*)-[8-^{3}H]BHCG-TP were >80%. The triphosphates were stored in 10 mM Tris·HCl, pH 7.6, 0.1 mM EDTA, 50% ethanol, at −80°.

DNA polymerase inhibition studies. HSV-1 DNA polymerase was purified from HSV-1 (KOS)-infected HeLa S3 cells by DEAE-cellulose, denatured DNA-cellulose, Mono S, and denatured DNA-cellulose chromatography, as previously described (15). Immunoaffinity-purified HeLa DNA polymerase α was purchased from Molecular Biology Resources (Milwaukee, WI) and stored at −80°. One unit of this DNA polymerase α was completely neutralized by 5 μg of a monoclonal antibody to DNA polymerase α, SJK-132-20 (purified from hybridoma cell line obtained from the American Type Culture Collection) (16). In addition, 2.5 μM N^2-(*p*-*n*-butylphenyl)dGTP (17) and 500 μM aphidicolin inhibited this polymerase by 99% and 90%, respectively.

Inhibition of purified HSV-1 DNA polymerase by (*R*)-BHCG-TP and (*S*)-BHCG-TP were determined in 50 mM Tris·HCl, pH 8, 5 mM $MgCl_2$, 1 mM DTT, 0.1 M ammonium sulfate, 5 μM (each) dATP, dCTP, and [^{3}H]dTTP (300 cpm/pmol), 30 μg/ml activated calf thymus DNA, 0.1 mg/ml BSA, HSV-1 DNA polymerase, with varying dGTP. Incubation was for 20 min at 37°, and incorporation of [^{3}H]dTTP into DNA was quantitated by trichloroacetic acid precipitation onto glass fiber discs and scintillation counting.

Inhibition of HeLa DNA polymerase α was studied under the following conditions: 50 mM Tris·HCl, pH 8, 5 mM magnesium acetate, 1 mM DTT, 200 μg/ml activated calf thymus DNA, 0.1 mg/ml BSA, 50 μM (each) dATP, dCTP, and [^{3}H]dTTP (100 cpm/pmol), varying dGTP, and 0.5 unit of DNA polymerase α. Incubation was for 30 min at 37°, and incorporation of radiolabel into DNA was quantitated by trichloroacetic acid precipitation on fiber discs and scintillation counting.

Incorporation of nucleoside analogs into primer/template and extension with dCTP. Incorporation of 5 μM [^{3}H]BHCG-TP, [^{3}H]ACV-TP, [^{3}H]DHPG-TP, and [^{3}H]dGTP (each adjusted to 2.67 Ci/mmol) into primer/template (Fig. 2) was performed in 50 mM Tris·HCl, pH 8, 12 mM $MgCl_2$, 1 mM DTT, 50 mM ammonium sulfate, 0.5 mg/ml BSA, 10 μM annealed primer/template, with HSV-1 DNA polymerase. Incubation was at 25°, as described by Reardon and Spector (10). Aliquots were assayed by HPLC, and the reaction was frozen on dry ice when >80% of the nucleoside triphosphate [except (*S*)-BHCG-TP] was incorporated into the primer/template; the reaction with (*S*)-BHCG-TP was terminated after 90 min. Unreacted radioactive nucleoside triphosphate was removed by HPLC on a C_8 column with triethylammonium acetate (pH 7) and a 0–100% acetonitrile

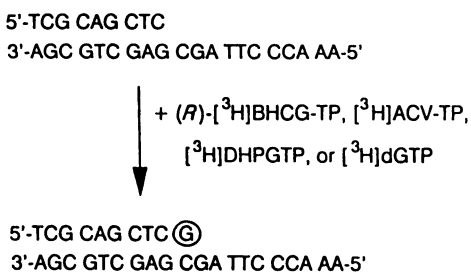

Fig. 2. Incorporation of deoxynucleoside triphosphate analogs into a defined primer/template. The sequence of this 9:20-mer is from Reardon and Spector (10).

gradient. Radioactive primer/template was isolated, lyophilized twice, dissolved in 20 mM Tris·HCl (pH 7.6) plus 0.2 mM EDTA, and stored at −20°.

The primer/template (10 pmol) described above was heated to 75° with a 5-fold molar excess of the 20-mer (template) and allowed to cool slowly to room temperature, to minimize single-stranded radiolabeled primer. Concentrated buffer was added to give final concentrations of 50 mM Tris·HCl, pH 8, 12 mM $MgCl_2$, 1 mM DTT, 50 mM ammonium sulfate, 10 μM (each) [α-^{32}P]dCTP (74 Ci/mmol), dATP, and dTTP, 0.5 mg/ml BSA, and 1.7 μM annealed primer/template. HSV-1 DNA polymerase was added and allowed to react for 1 hr at 25° (final volume, 6 μl). The reaction was quenched with an equal volume of 95% formamide/0.05% bromophenol blue and heated to 100° for 2 min, and an aliquot (6 μl) was separated on an 8% polyacrylamide DNA-sequencing gel. The gel was dried under vacuum and radioactive bands were localized by autoradiography, excised, and quantitated in a scintillation counter. All bands larger than 10 nucleosides in length were pooled. Oligonucleotides (9-mer and 20-mer) labeled at the 5′ end with polynucleotide kinase and [γ-^{32}P]ATP were used as size markers. These markers were also used to determine the counting efficiency of dried gel slices.

Results

Phosphorylation of (*R,S*)-BHCG, (*R*)-BHCG, and (*S*)-BHCG. Initial studies of the phosphorylation of (*R*)-BHCG by HSV-1 thymidine kinase indicated that this enantiomer was a surprisingly poor substrate, compared with (*R,S*)-BHCG (Fig. 3). The initial rate of phosphorylation of (*R*)-BHCG was approximately 1/25th that of phosphorylation of (*S*)-BHCG, even though (*R*)-BHCG is the enantiomer with antiviral activity (7). This result confirms the recent report by Kohlbrenner *et al.* (8), in which they observed that (+)-cyclobut G[1] [(*R*)-BHCG] was phosphorylated at 1/20th the rate of (−)-cyclobut G [(*S*)-BHCG]. Further evaluation of HSV-1 thymidine kinase phosphorylation indicated that the maximal velocities for thymidine, (*R*)-BHCG, and (*S*)-BHCG phosphorylation were 9.8, 4.2, and 8.0 nmol/hr/μg of thymidine kinase, respectively. The corresponding K_m values under our experimental conditions were determined to be 1 μM for thymidine, 500 μM for (*R*)-BHCG, and 60 μM for (*S*)-BHCG. The reason these K_m values differ from those reported for thymidine kinase partially purified from HSV-1 (R3820)-infected Vero cells [K_m = 350 μM and 150 μM for (*R*)-BHCG and (*S*)-BHCG, respectively] (8) is not obvious. Our results suggest that (*R*)-BHCG is a poorer thymidine kinase substrate than (*S*)-BHCG primarily due to the low affinity of thymidine kinase for (*R*)-BHCG, rather than the phosphorylation step.

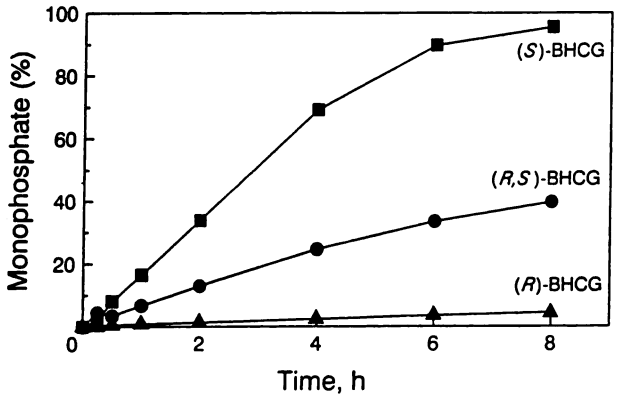

Fig. 3. Phosphorylation of (*R,S*)-BHCG, (*R*)-BHCG, and (*S*)-BHCG by HSV-1 thymidine kinase. Nucleoside (0.5 mM; 500 nmol) was incubated with HSV-1 thymidine kinase, and samples were analyzed by HPLC (for details see Materials and Methods). ●, (*R,S*)-BHCG; ▲, (*R*)-BHCG; ■, (*S*)-BHCG.

Inhibition of HSV-1 DNA polymerase by (*R*)-BHCG-TP and (*S*)-BHCG-TP. Titration of (*R*)-BHCG-TP and (*S*)-BHCG-TP in the presence of 5 μM dNTPs was performed to evaluate these triphosphates as inhibitors of HSV-1 DNA polymerase (Fig. 4). Clearly, inhibition of viral DNA polymerase by (*R*)-BHCG-TP was orders of magnitude better than inhibition by (*S*)-BHCG-TP. The inhibition of HSV-1 DNA polymerase by (*R*)-BHCG-TP was investigated further (Fig. 5). The double-reciprocal plot indicates that (*R*)-BHCG-TP inhibition was competitive with respect to dGTP; this inhibition was quite impressive (K_i = 0.017 μM). Additional experiments indicated that inhibition of HSV-1 DNA polymerase by (*R*)-BHCG-TP was uncompetitive with respect to dATP (data not shown).

Inhibition constants were also determined for (*R,S*)-BHCG-TP, (*S*)-BHCG-TP, and ACV-TP with purified HSV-1 DNA polymerase (Table 1). All three guanine analog triphosphates were competitive inhibitors with respect to dGTP. (*S*)-BHCG-TP was a poor inhibitor of HSV-1 (KOS) DNA polymerase, with an inhibition constant of approximately 400 μM. Surprisingly, the inhibition constant determined for (*R,S*)-BHCG-TP was similar to that observed for (*R*)-BHCG-TP, rather than twice the value. (*R,S*)-BHCG-MP was enzymatically synthesized previously by using thymidine kinase cloned and expressed in *Escherichia coli*, which has a reduced substrate specificity for cyclobutyl nucleoside analogs; the triphosphate

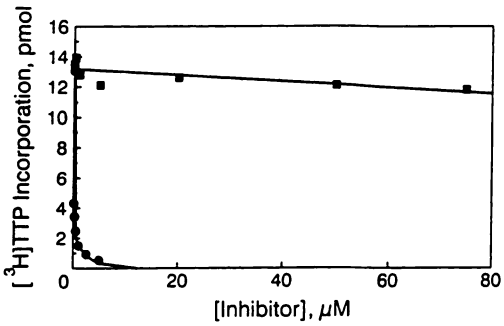

Fig. 4. Inhibition of HSV-1 DNA polymerase by (*R*)-BHCG-TP and (*S*)-BHCG-TP. Inhibition by (*R*)-BHCG-TP and (*S*)-BHCG-TP was determined in 50 mM Tris·HCl, pH 8, 5 mM $MgCl_2$, 1 mM DTT, 0.1 M ammonium sulfate, 5 μM (each) dATP, dGTP, dCTP, and [^{3}H]dTTP, 30 μg/ml activated calf thymus DNA, 0.1 mg/ml BSA, HSV-1 DNA polymerase, at 37°. ●, (*R*)-BHCG-TP; ■, (*S*)-BHCG-TP.

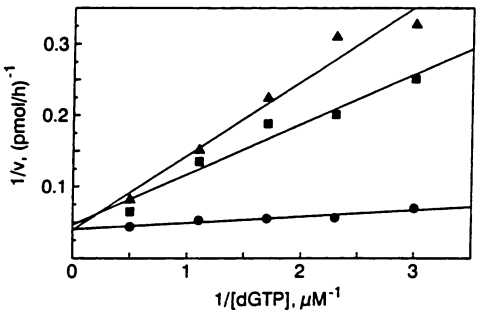

Fig. 5. Inhibition of HSV-1 DNA polymerase by (*R*)-BHCG-TP. Incorporation of [^{3}H]dTTP into DNA was quantitated by trichloroacetic acid precipitation, as described in Materials and Methods. ●, 0 μM; ■, 0.10 μM; ▲, 0.20 μM (*R*)-BHCG-TP.

TABLE 1

Inhibition of DNA polymerases by (*R*)-BHCG-TP and (*S*)-BHCG-TP

HSV-1 DNA polymerase was purified from KOS-infected HeLa cells (14), and DNA polymerase α was purchased from Molecular Biology Resources. Inhibition constants were determined from slope replots (1/slope versus [*I*]) of Lineweaver-Burk plots. Results are the average ± standard deviation of four determinations, except for (*S*)-BHCG-TP, which was determined in two independent experiments.

	DNA polymerase inhibition, K_i	
	HSV-1	α
	μM	
(*RS*)-BHCG-TP	0.015 ± 0.006	0.42 ± 0.01
(*R*)-BHCG-TP	0.017 ± 0.003	0.36 ± 0.02
(*S*)-BHCG-TP	400	480
ACV-TP	0.025 ± 0.017	3.3 ± 1.5

was then prepared with guanylate kinase, nucleoside-5′-diphosphate kinase, and a creatine phosphate/ATP-regenerating system (3). Attempts to phosphorylate (*S*)-BHCG-DP with nucleoside-5′-diphosphate kinase (and the creatine phosphate/ATP-regenerating system) were unsuccessful, leading us to believe that most of the (*R,S*)-BHCG-TP reported previously (3) was actually (*R*)-BHCG-TP.

Inhibition of DNA polymerase α by (*R*)-BHCG-TP and (*S*)-BHCG-TP. (*R*)-BHCG-TP was also an inhibitor of HeLa DNA polymerase α, with an inhibition constant of 0.36 μM (Table 1). In contrast, (*S*)-BHCG-TP was a poor inhibitor of DNA polymerase α (K_i = 480 μM). The inhibition constant determined for (*R,S*)-BHCG-TP was similar (K_i = 0.42 μM) to that observed for (*R*)-BHCG-TP, suggesting that (*R,S*)-BHCG-TP was not an equimolar mixture of the two enantiomers. Inhibition patterns indicate that inhibition of DNA polymerase α by (*R*)-BHCG-TP was linear mixed-type (18), suggesting that DNA polymerase α inhibition was different than inhibition of HSV-1 DNA polymerase. Indeed, as shown below, (*R*)-BHCG-TP is a substrate for HSV-1 DNA polymerase but apparently is not a substrate for DNA polymerase α.

Incorporation of (*R*)-BHCG-TP into DNA. Tritiated (*R*)-BHCG-TP and (*S*)-BHCG-TP were prepared, and the ability of HSV-1 DNA polymerase to incorporate these analogs into the well defined DNA template 5′-TCGCAGCTC/3′-AGCGTCGAGCGATTCCCAAA was measured (Fig. 2). (*R*)-BHCG-TP was incorporated into the primer/template by HSV-1 DNA polymerase (Table 2), whereas (*S*)-BHCG-TP incorporation was below levels of detection (<1%). ACV-TP and DHPG-TP were also incorporated to similar extents, compared with the natural substrate dGTP.

Incorporation of dCTP into DNA with (*R*)-BHCG-MP at the 3′ terminus. Once (*R*)-BHCG-MP was incorporated into the primer/template, we wished to see whether the DNA chain could be extended by the addition of subsequent dNTPs. [8-^{3}H]dGTP, (*R*)-[8-^{3}H]BHCG-TP, [8-^{3}H]DHPG-TP, or [8-^{3}H]ACV-TP was incorporated into primer/template by HSV-1 DNA polymerase at 25°. Tritiated primer/template was isolated by HPLC and reacted with HSV-1 DNA polymerase plus [α-^{32}P]dCTP, dTTP, and dATP. Samples were run on DNA-sequencing gels, and bands were localized by autoradiography. The dGMP-terminated primer was extended four nucleotides and terminated when a guanine should be the next base incorporated (note that dGTP was not present). (*R*)-BHCG-MP and DHPG monophosphate termini were extended by one nucleotide (dCMP), and a very small percentage of each incorporated the next nucleotide (dTMP). The ACV monophosphate-primer/template had no visible bands in the autoradiograph. All bands larger than 10 nucleotides were excised from the gel and quantitated by liquid scintillation counting. The average of two independent experiments is shown in Fig. 6. Only 10% of the (*R*)-BHCG-MP-terminated primers were extended during the time course of the experiment.

TABLE 2

Incorporation of analog triphosphates into DNA and extension with dCTP

[8-^{3}H]dGTP, (*R*)-[8-^{3}H]BHCG-TP, (*S*)-[8-^{3}H]BHCG-TP, [8-^{3}H]DHPG-TP, and [8-^{3}H]ACV-TP were incorporated into primer/template by HSV-1 DNA polymerase at 25° (first column). Tritiated primer/template was isolated by HPLC, annealed with a 5-fold molar excess of 20-mer, and reacted with HSV-1 DNA polymerase, 10 μM [α-^{32}P]dCTP (80,000 cpm/pmol), 10 μM dTTP, and 10 μM dATP for 1 hr at 25°. Samples were electrophoresed and quantitated as described in Materials and Methods.

Substrate	Incorporation	
	Nucleotide incorporation, [^{3}H]NMP[a]	Extension, [α-^{32}P] dCMP
	pmol	
dGTP	2.39	1.74
(*R*)-BHCG-TP	2.19	0.17
(*S*)-BHCG-TP	<0.02	ND[b]
DHPG-TP	2.44	0.19
ACV-TP	1.99	0.022

[a] [^{3}H]NMP (^{3}H-nucleotide monophosphate) incorporation values have been corrected (× 0.5), because only half of the sample was electrophoresed.
[b] ND, not determined.

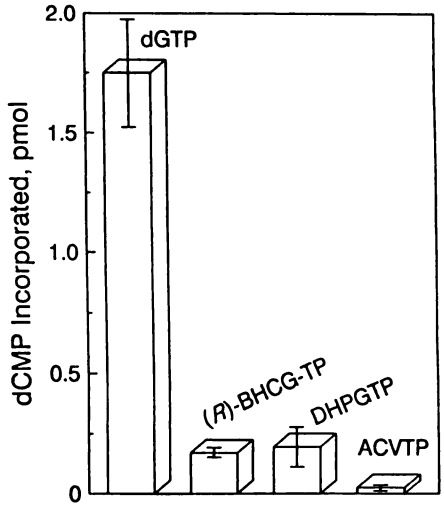

Fig. 6. Incorporation of [α-^{32}P]dCTP into DNA containing (*R*)-BHCG-MP at the 3′ terminus. Radioactive primer/template that had incorporated (*R*)-[^{3}H]BHCG-TP, [^{3}H]ACV-TP, [^{3}H]DHPG-TP, or [^{3}H]dGTP was isolated and annealed with a 5-fold molar excess of 20-mer (template). Buffer was added to give final concentrations of 50 mM Tris·HCl, pH 8, 12 mM $MgCl_2$, 1 mM DTT, 50 mM ammonium sulfate, 10 μM (each) [α-^{32}P]dCTP (74 Ci/mmol), dATP, and dTTP, 1 mg/ml BSA, and 5 μM annealed primer/template. HSV-1 DNA polymerase was added and allowed to react for 1 hr at 25°. The reaction was separated on a denaturing polyacrylamide DNA-sequencing gel. Radioactive bands were excised and quantitated in a scintillation counter. *Error bars*, data range observed.

A summary of analog incorporation and extension is presented in Table 2. Under the buffer conditions studied, HSV-1 DNA polymerase was able to extend three quarters of the primer/template reacted with dGTP (1.74 pmol/2.39 pmol), whereas only 8% of the primer/template containing (*R*)-[^{3}H]BHCG-MP or [^{3}H]DHPG monophosphate was extended by the addition of the next nucleotide (dCTP). The small amount

(1.1%) of incorporation of [α-^{32}P]dCTP observed with ACV-terminated primer (10-mer) could be due to misincorporation of dNTP in unreacted primer (9-mer) and normal extension with [α-^{32}P]dCTP.

Discussion

Because most nucleoside analogs with antiherpetic activity do not inhibit HSV-1 DNA polymerase directly, these compounds are presumed to be metabolized to inhibitors of the viral DNA polymerase. Indeed, (*R*)-BHCG and (*S*)-BHCG are phosphorylated in HSV-1 (KOS)-infected cells and not in mock-infected cells.[2] This contrasts with previous reports that (*R,S*)-BHCG has antiviral activity against *tk*$^-$ strains of HSV-1 and HSV-2 (2, 3); however, the dependence of the antiviral activity of (*R,S*)-BHCG on viral thymidine kinase is only 20–50-fold, compared with approximately 1000-fold for ACV. Upon incubation with purified HSV-1 thymidine kinase, the potent antiviral (*R*)-BHCG is poorly phosphorylated (Fig. 2) (8). In contrast, (*S*)-BHCG is phosphorylated very well (V_{max} = 8 nmol/hr/μg of thymidine kinase, compared with 9.8 nmol/hr/μg for thymidine). In concert with cell culture studies,[2] (*R*)-BHCG phosphorylation illustrates that even poor HSV-1 thymidine kinase substrates such as (*R*)-BHCG can accumulate substantial phosphorylated products in virally infected cells.

Kohlbrenner *et al.* (8) have recently shown that the 3-hydroxymethyl group of (−)-BHCG [(*S*)-BHCG] was the site of phosphorylation by partially purified HSV-1 thymidine kinase; the authors stated that NMR data for (+)-BHCG were identical, but data were not presented. We have determined the site of phosphorylation of (*S*)-BHCG and agree with their assignment (data not shown). Due to the poor phosphorylation of (*R*)-BHCG by thymidine kinase, we could not determine the site of phosphorylation by NMR. However, because (*S*)-BHCG was phosphorylated at the hydroxymethyl group that most clearly overlaps the normal 5′-hydroxymethyl of 3′-deoxyguanosine by molecular modeling, we believe that (*R*)-BHCG was most likely phosphorylated at the same site. In addition, both (*R*)-BHCG-MP and (*S*)-BHCG-MP were substrates for guanylate kinase and could be quantitatively converted to their diphosphates, which suggests that initial phosphorylation occurred at the 5′-equivalent hydroxyl group.

(*R*)-BHCG-TP was a potent inhibitor of HSV-1 DNA polymerase, with an inhibition constant of 0.017 μM. Inhibition by (*R*)-BHCG-TP was competitive with respect to dGTP, implying that the analog triphosphate binds to the same site as the natural substrate. (*R*)-BHCG-TP also inhibited DNA polymerase α (K_i = 0.36 μM). The inhibition of DNA polymerase α was not strictly competitive with respect to dGTP, suggesting a different mechanism of action. The inhibition of DNA polymerase α by (*R*)-BHCG-TP may be irrelevant, because (*R*)-BHCG is not phosphorylated in uninfected cells but only in virally infected cells.[2]

The facile phosphorylation of (*S*)-BHCG by HSV-1 thymidine kinase and cellular nucleotide kinases suggests that inefficient phosphorylation to the triphosphate is not the explanation for its lack of antiviral activity. As a result, we investigated whether (*S*)-BHCG-TP was an inhibitor of HSV-1 DNA polymerase. (*S*)-BHCG-TP was a poor inhibitor of both HSV-1 polymerase and HeLa DNA polymerase α (K_i = 400 and 480 μM, respectively). This residual DNA polymerase-inhibitory activity may be due to a slight contamination (0.07%) of (*S*)-BHCG with (*R*)-BHCG; however, considering the relatively poor phosphorylation of (*R*)-BHCG by HSV-1 thymidine kinase, even less than 0.07% (*R*)-BHCG-TP would be expected to contaminate (*S*)-BHCG-TP. The absence of significant DNA polymerase-inhibitory activity for (*S*)-BHCG-TP is consistent with the fact that (*S*)-BHCG does not have antiviral activity (7, 8). A similar result was observed with the two enantiomers of DHPG-TP, where only one isomer inhibited HSV-1 DNA polymerase (19). The large difference in antiviral activity between (*R*)-BHCG and (*S*)-BHCG can be explained by their differential inhibition of HSV-1 DNA polymerase.

In this report, we have shown that the previously reported DNA polymerase inhibitory activity of (*R,S*)-BHCG-TP (3) is due to one enantiomer, (*R*)-BHCG-TP. This claim is based on the fact that (*R*)-BHCG-TP inhibited HSV-1 DNA polymerase as well as (*R,S*)-BHCG-TP (K_i = 0.017 and 0.015 μM, respectively). In contrast, (*S*)-BHCG-TP was a poor inhibitor of HSV-1 DNA polymerase. Also, inhibition constants determined for HeLA DNA polymerase α were similar for (*R*)-BHCG-TP and (*R,S*)-BHCG-TP but quite different for (*S*)-BHCG-TP.

(*R*)-BHCG-TP was a competitive inhibitor of dGTP incorporation into DNA catalyzed by HSV-1 DNA polymerase. This suggests that binding of (*R*)-BHCG-TP could prevent dGTP binding or that (*R*)-BHCG-TP could act as an alternative substrate (17). (*R*)-[^{3}H]BHCG-TP was incorporated into a defined primer/template in place of dGTP by HSV-1 DNA polymerase (K_m = 3.2 μM, V_{max} = 192 pmol/hr/unit); [^{3}H]ACV-TP and [^{3}H]DHPG-TP were also incorporated, as previously reported (10, 20). In contrast, (*S*)-BHCG-TP was not incorporated into DNA. Therefore, (*R*)-BHCG-TP but not (*S*)-BHCG-TP is an alternative substrate for dGTP incorporation by HSV-1 DNA polymerase.

Once (*R*)-BHCG-TP was incorporated into DNA, the DNA molecule was poorly elongated by HSV-1 DNA polymerase. The poor DNA extension by HSV-1 DNA polymerase suggests that once (*R*)-BHCG-TP has been incorporated into the DNA, the hydroxyl available for subsequent phosphodiester bond formation is not in the optimal orientation. As a result, (*R*)-BHCG-TP exerts its effect by terminating most DNA molecules after incorporation in place of dGTP. In this respect, (*R*)-BHCG-TP acts like DHPG (21) rather than the obligate DNA chain terminator ACV (10, 22). By prematurely terminating viral DNA molecules, (*R*)-BHCG-TP would exert a potent antiviral effect. We were unable to detect incorporation of (*R*)-BHCG-TP into the primer/template with DNA polymerase α (data not shown), and we believe that incorporation into cellular DNA by DNA polymerase α is unlikely. First, detectable levels of (*R*)-BHCG-TP are not formed in uninfected cells[2] and, second, in HSV-1-infected cells the affinity of the viral DNA polymerase for (*R*)-BHCG-TP is greater than that for DNA polymerase α.

The data reported here complement the cell culture metabolism studies of (*R*)-BHCG and (*S*)-BHCG by Yamanaka *et al.*.[2] They have reported that (*R*)-BHCG is converted to its triphosphate with very little monophosphate accumulating, suggesting that initial phosphorylation is rate limiting. In agreement, we have shown that (*R*)-BHCG is poorly phosphorylated by purified HSV-1 thymidine kinase. In contrast, initial phosphorylation of (*S*)-BHCG proceeds readily, and a

[2] Yamanaka *et al. Mol. Pharmacol.*, in press.

substantial amount of (*S*)-BHCG-TP is formed. Inhibition studies of HSV-1 DNA polymerase and DNA polymerase α with (*R*)-BHCG-TP and (*S*)-BHCG-TP are consistent with (*R*)-BHCG having potent virus plaque-reduction activity and poorer cytostatic activity, whereas (*S*)-BHCG is inactive. The absence of antiviral activity for (*S*)-BHCG occurs because (*S*)-BHCG-TP does not inhibit HSV-1 DNA polymerase. Whether (*R*)-BHCG-TP behaves as an alternative substrate and is incorporated *in vivo* still remains to be determined.

Acknowledgments

We wish to thank Drs. A. Kirk Field and Mary L. Haffey for helpful discussions during the course of this work. We are indebted to Dr. Greg Bisacchi for NMR analysis of (*S*)-BHCG-MP.

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Send reprint requests to: Dr. Brian Terry, Bristol-Myers Squibb, P.O. Box 4000, Princeton, NJ 08543.