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Selective Activity and Cellular Pharmacology of (1*R*-1α,2β,3α)-9-[2,3-bis(Hydroxymethyl)cyclobutyl]guanine in Herpesvirus-Infected Cells

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SUMMARY

The cycloburtane nucleoside analog (1*R*-1α,2β,3α)-9-[2,3-bis (hydroxymethyl)cyclobutyl]guanine [(*R*)-BHCG or SQ 34,514] was recently synthesized and shown to be the active enantiomer of (±)-BHCG (SQ 33,054), a potent inhibitor of several strains of herpesviruses [*J. Med. Chem* **34**:1415–1421 (1991); *Antiviral Res.* **13**:41–52 (1990)]. In plaque reduction assays, (*R*)-BHCG was about 1000 times more active than its *S*-enantiomer on herpes simplex virus type I (HSV-1) and over 200 times more active against a thymidine kinase-deficient mutant HSV-1 and human cytomegalovirus (HCMV). We now show that both (*R*)-BHCG and (*S*)-BHCG are efficiently phosphorylated to their mono-, di-, and triphosphates by HSV-1-infected cells, in a manner similar to that of acyclovir [*Proc. Natl. Acad. Sci. USA* **74**:5716–5720 (1977)]. The uptake of both enantiomers was greatly increased upon infection; however, (*S*)-BHCG was taken up to about twice the extent of (*R*)-BHCG and accumulated primarily as the mono- and diphosphates. (*R*)-BHCG accumulated primarily as the triphosphate, and accumulation was linear with both time and added drug concentration. The triphosphate had an apparent half-life of about 10 hr. Metabolic studies using HCMV-infected cells showed only a small degree of phosphorylation of (*R*)-BHCG and none of (*S*)-BHCG. When cells were labeled with 25 μM (*R*)-BHCG, the amount of (*R*)-BHCG triphosphate formed was <0.5 pmol/10^6 cells. Interestingly, the ED_{50} value of (*R*)-BHCG is about 100-fold higher against HCMV than against HSV-1, and the relative levels of (*R*)-BHCG triphosphate formed in cells infected by the two viruses are roughly proportional to the antiviral activities.

The antiviral activity of a novel class of compounds, the cyclobutane nucleoside analogs, has recently been described (1–6). One member of this group, (±)-BHCG (SQ 33,054; cyclobut G), is of particular interest because it has been reported to exhibit broad spectrum anti-herpesvirus (1, 2, 5, 6) and anti-HIV (3, 4) activities. In plaque reduction assays in WI-38 cells against HSV-1 strains, the ED_{50} value of this drug ranged between 0.08 and 0.8 μM and compared favorably with that of acyclovir (0.4–0.8 μM) (2). (±)-BHCG was shown to be a substrate for the HSV-1 TK, and the triphosphate of (±)-BHCG was an effective inhibitor of the HSV-1 DNA polymerase (K_i = 0.004 ± 0.002 μM) (2). These studies suggested that one mode of action of the drug in cell culture could be through HSV-1 DNA polymerase inhibition by (±)-BHCG triphosphate, in a manner analogous to that previously shown for other antiviral nucleoside analogs, including acyclovir (7, 8). Acyclovir is strictly dependent upon the viral TK for activation. Examination of this TK dependence in cell culture, using *tk*⁻ mutants of HSV-1 and HSV-2, revealed a 1000-fold differential (compared with the wild-type) in the ED_{50} value. By comparison, the differential for (±)-BHCG was only 20–100-fold (2). (±)-BHCG exhibited moderate activity against HSV-1 *tk*⁻ mutants (ED_{50} = 0.8–1.9 μM) and was 10-fold more active than acyclovir against HCMV (which lacks TK activity) (9, 10) in cell culture (ED_{50} = 2–4 μM). Taken together, these results suggest that the viral TK can contribute to the antiviral effect of the compound but that the kinase is not strictly required for the antiviral activity. (±)-BHCG represents a novel and interesting compound, with an extended spectrum that includes HCMV and HSV *tk*⁻ mutants, both of which are of clinical relevance.

Recently, the chiral synthesis of BHCG was reported, and the *R*-enantiomer[1] (*R*)-BHCG was identified as the active form of the drug (11–13). Interestingly, one report (13) and data

[1] The designations of *R* and *S* used in this paper are based on the absolute determination of configuration at the 1-position of the cyclobutyl ring (12). In ref. 13, (+)-cyclobut G corresponds to (*R*)-BHCG and (−)-cyclobut G to (*S*)-BHCG.

ABBREVIATIONS: (±)-BHCG, (±)-(1α,2β,3α)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine; HSV, herpes simplex virus; HCMV, human cytomegalovirus; VZV, varicella zoster virus; TK, thymidine kinase; acyclovir, 9-(2-hydroxyethoxymethyl)guanine; ganciclovir, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; HIV, human immunodeficiency virus.

from our own laboratory (13a) showed that the inactive (S)-BHCG is phosphorylated 20–25 times better than the active (R)-BHCG by HSV-1 TK. These data cast doubt on our hypothesis that the mechanism of viral inhibition would be similar to that of acyclovir, requiring the phosphorylation of the nucleoside to the monophosphate by viral TK, followed by phosphorylation to the diphosphate and triphosphate by cellular kinases (14). Questions that arose involved (1) what the active form of the drug might be, 2) whether the target was indeed DNA polymerase, 3) whether (S)-BHCG was actually phosphorylated to the triphosphate in virus-infected cells, and 4) whether (S)-BHCG triphosphate was capable of inhibiting the viral DNA polymerase. In the study reported here, we utilized radiolabeled (±)-BHCG, (R)-BHCG, and (S)-BHCG to determine whether these compounds would serve as substrates for the kinase activities present in mock-infected, HSV-1-infected, and HCMV-infected cells *in vitro*. Our results show that the active isomer, (R)-BHCG, is very poorly phosphorylated to the monophosphate, relative to (S)-BHCG, by HSV-1-infected cells. However, once formed, (R)-BHCG monophosphate is efficiently anabolized to the triphosphate, where its long half-life facilitates its accumulation to inhibitory concentrations in HSV-1-infected cells. In HCMV-infected cells, (S)-BHCG was not metabolized, and only the di- and triphosphates of (R)-BHCG were detectable. In a separate report,[2] we also show that (R)-BHCG triphosphate but not (S)-BHCG triphosphate is a potent inhibitor of purified HSV-1 DNA polymerase.

Experimental Procedures

Materials. The synthesis of (+)-BHCG, (R)-BHCG, and (S)-BHCG has been described (1, 12). Acyclovir was prepared according to the procedure described in Netherlands Patent 7709458 (1978) issued to The Wellcome Foundation Ltd. (London, UK). Ganciclovir was prepared according to the method of Martin *et al.* (15). (±)-[^{3}H]BHCG, (R)-[^{3}H]BHCG, (S)-[^{3}H]BHCG, [^{3}H]ganciclovir, and [^{3}H]acyclovir were prepared from the 8-bromo derivatives of the respective nonradioactive compounds by catalytic exchange of tritium into the C-8 position of the guanine ring. (±)-BHCG was prepared by P. Egli (Bristol-Myers Squibb Company, Princeton, NJ) and the others by Amersham Corp. (Arlington Heights, IL). (±)-[^{3}H]BHCG, (R)-[^{3}H]BHCG, (S)-[^{3}H]BHCG, [^{3}H]ganciclovir, and [^{3}H]acyclovir were prepared to specific activities of 15.4, 19.9, 20.7, 12.0, and 6.0 Ci/mmol, respectively, and were stored at ≈1 mCi/ml in 90% ethanol at −20°. All radioactive compounds were taken to dryness and resuspended in PBS just before use. Bacterial alkaline phosphatase and snake venom phosphodiesterase were from Sigma Chemical Co. (St. Louis, MO). The sources of other reagents have been described (2).

Cells and viruses. WI-38 (CCL75) and Vero (CCL81) cells were obtained from the American Type Culture Collection and were grown as described previously (2). HSV-1 strains Schooler and KOS and HSV-2 strain 186 were prepared as extracts from infected Vero cell cultures. HCMV strain AD169 and VZV strain Ellen were prepared as suspensions of infected WI-38 cells. HSV-1(KOS)TK$^-$ was isolated from the parent KOS by selection with the thymidine analog 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil. It was found to lack all detectable viral TK activity, when assayed under the conditions described earlier (2).

Plaque reduction assay. Viruses were adsorbed to WI-38 monolayers in six-well culture plates (Costar, Cambridge, MA) for 1–2 hr, followed by addition of test compounds diluted into maintenance medium (2). Inhibition of plaque development was evaluated on monolayers, which were stained after 4–6 days of incubation at 37°. ED_{50} values were determined from the drug concentration that conferred at least a 50% reduction in plaque number/plate, compared with virus controls. All titrations were done in duplicate and expressed as the range in repeat assays.

Cell growth inhibition assay. WI-38 cells were plated at 1.2 × 10^5 cells/well in 12-well culture plates (Costar). After overnight incubation, cultures were re-fed with fresh medium containing serial dilutions of a compound of interest and were incubated at 37° for 3 days. Quadruplicate cultures were harvested by trypsinization and counted for viable cells by staining with trypan blue. Untreated control cells increased in number approximately 3–5-fold.

Culture conditions and preparation of cell extracts for nucleotide analysis. WI-38 cells were grown to 30–50% confluence in six-well culture plates. The medium was removed and the cells were infected in 0.5 ml with virus. For experiments involving HSV-1, cells were infected with HSV-1(KOS), at a multiplicity of infection of 10, for 1–2 hr, re-fed with 1.5 ml of fresh medium, and incubated for various periods of time in the presence of tritiated compounds at 37°. For experiments involving HCMV, cells were infected at a multiplicity of 0.2–1.0, re-fed with 2.0 ml of fresh medium, and then incubated for 24 hr before the addition of tritiated compounds. Labeling of HCMV-infected cells was for 24 hr or longer. For the experiments described below, specific activities (final concentrations) of radiolabeled compounds were 0.5 Ci/mmol (25 μM), 1.0 Ci/mmol (12.5 μM), 2 Ci/mmol (2.5 μM), and 20 Ci/mmol (0.25 μM).

To prepare extracts from radiolabeled cells, the medium was removed and the cells were washed three times with 2 ml of PBS, to remove extracellular radioactivity. Soluble intracellular radioactivity was extracted through two successive applications of 1 ml of ice-cold 60% methanol for 1 hr at 4° (16, 17). The methanol extracts were pooled, taken to dryness, and then redissolved in distilled water. Extracts were clarified in an Airfuge (Beckman Instruments, Palo Alto, CA) before analysis by HPLC.

HPLC analysis of intracellular nucleotide pools. Clarified extracts were analyzed on a Vydac C18 reverse phase column, using a linear gradient of 0–25% acetonitrile in 10 mM potassium dihydrogen phosphate, 2 mM tetrabutylammonium phosphate. The flow rate was 1.5 ml/min, and all runs were cycled through a 50% acetonitrile wash before column re-equilibration. Radioactivity was detected on-line, using a Radiomatic A250 radioactivity detector (Radiomatic Instruments, Tampa, FL). Raw counts were converted to picomoles using an average counting efficiency of 38%. Data are expressed as pmol/10^6 cells, based on an estimate of 1.5 × 10^6 cells/well (9.6 cm^2) at 100% confluence. All data are the average of duplicate or triplicate determinations, where the average variation between samples is <10%.

Enzymatic digestion of tritiated metabolites. Aqueous solutions of cellular metabolites were prepared as described above. For enzymatic digestion, samples (100 μl) were adjusted to 20 mM Tris·HCl, pH 7.5, 80 mM NaCl, 1.6 mM $MgCl_2$ (for snake venom phosphodiesterase), or 40 mM diethanolamine, pH 9.3, 1.6 mM $MgCl_2$, 0.08 mM $ZnCl_2$ (for bacterial alkaline phosphatase). Reactions were initiated by the addition of 0.5 unit of snake venom phosphodiesterase or 1.5 units of bacterial alkaline phosphatase. Samples were incubated at 25° for 30 min to 24 hr and then quick-frozen on dry ice. Samples were analyzed by HPLC, as described above, immediately upon thawing.

Results

Antiviral activity of (R)-BHCG and (S)-BHCG in tissue culture studies. The racemic compound (±)-BHCG was previously shown to inhibit several strains of herpesviruses in plaque reduction assays (1), with almost all of the activity attributable to the *R*-enantiomer, (R)-BHCG (6, 12). When evaluated in plaque reduction assays, (R)-BHCG was generally more potent than (±)-BHCG on six different herpesvirus strains, including an HSV-1 KOS strain lacking all TK activity (Table 1). In contrast, (S)-BHCG was 3–4 orders of magnitude

TABLE 1

Antiviral efficacy of (*R*)-BGCG and (*S*)-BHCG in WI-38 cell culture

Data are expressed as the mean (range in parentheses) of two to five determinations.

Virus (strain)	ED_{50}				
	(±)-BHCG (racemic)	(*R*)-BHCG	(*S*)-BHCG	Acyclovir	Ganciclovir
			μM		
HSV-1(KOS)	0.4 (0.08–0.8)	0.06 (0.02–0.2)	126 (94–190)	0.5 (0.2–0.9)	0.06 (0.04–0.08)
HSV-1[(KOS)TK⁻]	8 (4–18)	2 (0.2–4)	>377	350 (220–440)	ND[a]
HSV-1(Schooler)	0.1 (0.04–0.2)	0.04 (0.008–0.08)	190 (94–370)	0.5 (0.2–0.9)	0.06 (0.04–0.08)
HSV-2(186)	0.07 (0.02–0.08)	0.04 (0.02–0.08)	>377	0.4 (0.2–0.9)	0.06 (0.04–0.08)
HCMV(AD169)	3 (2–4)	3 (2–4)	>377	30 (20–40)	1 (0.4–2)
VZV(Ellen)	0.2 (0.08–0.4)	0.05 (0.02–0.08)	>377	2 (1–4)	1 (0.8–2)
Cytostatic ID_{50} (WI-38)	180 (48–380)	150 (30–270)	>600	Variable (>800)	530 (390–640)
Cytostatic ID_{50}(Vero)	36 (10–62)	24	ND	>800	ND

[a] ND, not determined.

less potent than (*R*)-BHCG[2]. When compared with two established drugs, acyclovir and ganciclovir, (*R*)-BHCG was about as potent as ganciclovir on HSV-1, HSV-2, and HCMV and 10-fold more potent on VZV. It was also 10-fold more potent than acyclovir on wild-type strains of HSV-1, HSV-2, and HCMV and 40-fold more potent on VZV and HSV-1(KOS)TK⁻.

When assayed for inhibition of growth of WI-38 cells, (*R*)-BHCG showed a large variability in its cytostatic activity (Table 1). The range observed, 31–270 μM, was somewhat lower than that of (±)-BHCG. In contrast, the inactive enantiomer, (*S*)-BHCG, was noncytostatic up to at least 600 μM. Using an average ED_{50} of 0.03 μM for the antiviral activity on HSV-1 and HSV-2, the calculated therapeutic index for (*R*)-BHCG ranged between 1000 and 9000.

Phosphorylation of the racemic compound (±)-BHCG by mock- and HSV-1-infected cells. In order to better understand the mechanism by which (±)-BHCG and its enantiomers exert their effects in cells, we prepared tritiated derivatives of these compounds. In our initial experiments, mock- and HSV-1-infected cells were labeled for 6 hr with 25 μM (±)-[^{3}H]BHCG, washed, and extracted for HPLC analysis, as described in Experimental Procedures. Similar to earlier studies performed with [^{3}H] acyclovir (7), the HPLC profile of (±)-BHCG metabolism by mock-infected cells revealed a single major peak of radioactivity (Fig. 1A). When extracts were coinjected with authentic (±)-BHCG, a single symmetrical peak was seen. The profile from HSV-1-infected cells contained (±)-BHCG plus three additional peaks (Fig. 1B). These additional peaks were assigned to the monophosphate, diphosphate, and triphosphate, based on the following criteria: 1) degradative treatment with alkaline phosphatase and snake venom phosphodiesterase gave results consistent with those previously found with acyclovir (7), 2) single-peak HPLC profiles were produced by mock-infected cells and cells infected with HSV-1(KOS)TK⁻ (data not shown), and 3) previous enzymatic data showed purified HSV-1 TK to be capable of phosphorylating

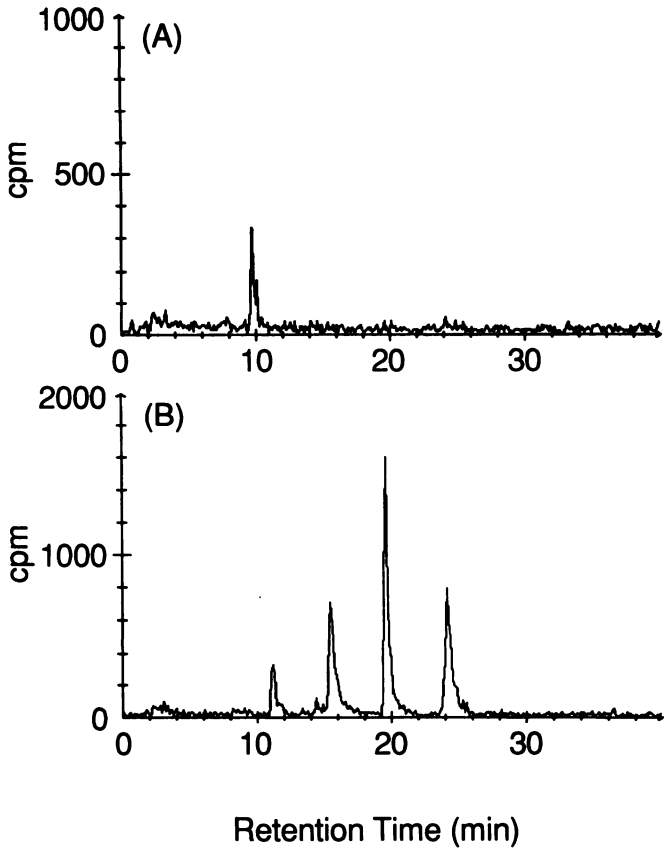

Fig. 1. HPLC profile of extracts of mock- (A) and HSV-1(KOS)-infected (B) WI38 cells incubated with 25 μM (±)-[^{3}H]BHCG for 6 hr.

(±)-BHCG (2). Furthermore, compounds having one, two, and three phosphate groups interact with increasing strength with the ion-pairing reagent (tetrabutylammonium phosphate) in our buffer system, and their elution occurs at progressively later times in our profiles.

Phosphorylation of (*R*)-BHCG and (*S*)-BHCG by mock- and HSV-1-infected cells. When experiments similar

[2] The small amount of activity seen with this compound was possibly due to contamination from (*R*)-BHCG, which could have been present at up to 0.07%.

to the one shown in Fig. 1 were performed using the purified enantiomers of (±)-BHCG, neither isomer was noticeably metabolized in mock-infected cells (Fig. 2, A and B). The HPLC profiles occasionally revealed a radioactive peak eluting at the retention time for the triphosphate; however, this peak was not consistently seen, nor did it ever account for >2% of the total radioactivity. In HSV-1-infected cells, both enantiomers were phosphorylated, but with strikingly different profiles (Fig. 2, C and D). (*R*)-[^{3}H]BHCG was effectively converted to the triphosphate, with increased accumulation at each phosphorylation step. In contrast, (*S*)-[^{3}H]BHCG was phosphorylated primarily to the monophosphate and diphosphate, accumulating to over 4 times the level of these derivatives of (*R*)-BHCG, but only half the level of the triphosphate. A minor unidentified peak was sometimes seen in (*S*)-BHCG profiles, appearing just before the monophosphate peak (Fig. 2D). A comparable peak was not seen in profiles from (*R*)-[^{3}H]BHCG-labeled cells.

The effect of nucleoside concentration on the metabolism of (*R*)-BHCG and (*S*)-BHCG by HSV-1-infected cells is shown in Table 2. Each compound was added to cultures at 0.25, 2.5, or 25 μM, and the samples were incubated for 6 hr before analysis by HPLC. Total accumulation of radioactivity for both compounds varied proportionally with the concentration of nucleoside added to the infected cell culture. However, phosphorylated derivatives accumulated to varying extents at the different concentrations examined. The overall phosphorylation efficiency of (*R*)-BHCG to the triphosphate increased with increasing concentrations of added nucleoside and ranged from about 30 to 70% of the total intracellular pool. In contrast, (*S*)-BHCG triphosphate levels were all <20%. At the lowest concentration, 0.25 μM, the level of (*S*)-BHCG triphosphate was <1% of the nucleotide pool and below our limit of quantitation. Thus, (*R*)-BHCG was more efficiently phosphorylated to the triphosphate than (*S*)-BHCG, especially at low micromolar concentrations of added nucleoside.

A time course for the metabolism of (*R*)-[^{3}H]BHCG and (*S*)-[^{3}H]BHCG by HSV-1-infected cells is shown in Fig. 3. As shown in Fig. 3A, there was very little accumulation of nucleoside, monophosphate, or diphosphate for cultures labeled with (*R*)-[^{3}H]BHCG. The triphosphate, however, increased almost linearly during the entire 22-hr incubation. In a similar experiment involving (*S*)-[^{3}H]BHCG, all three phosphorylated derivatives increased for at least 10 hr, after which they declined (Fig. 3B). Unlike (*R*)-BHCG, (*S*)-BHCG has no antiviral activity; thus, the decline in intracellular concentration between 10 and 22 hr may represent virally induced cell lysis.

Intracellular lifetime of (*R*)-[^{3}H]BHCG nucleotides in HSV-1-infected cells. To better understand the mechanism for (*R*)-BHCG triphosphate accumulation, its relative rate of breakdown was measured in a pulse-chase experiment. Infected cells were labeled for 6 hr with (*R*)-[^{3}H]BHCG, washed three times with PBS, and re-fed with fresh medium. Samples were taken at 3 and 16 hr after the wash, and the intracellular nucleotide pools were analyzed. As shown in Fig. 4, free (*R*)-BHCG disappeared within 3 hr, whereas the phosphorylated derivatives decreased much more gradually. The triphosphate appeared extremely long-lived, having an apparent half-life of about 10 hr. A similar experiment was not carried out with (*S*)-BHCG, because this compound has no antiviral activity and virally induced cell lysis would prevent an accurate interpretation of the data.

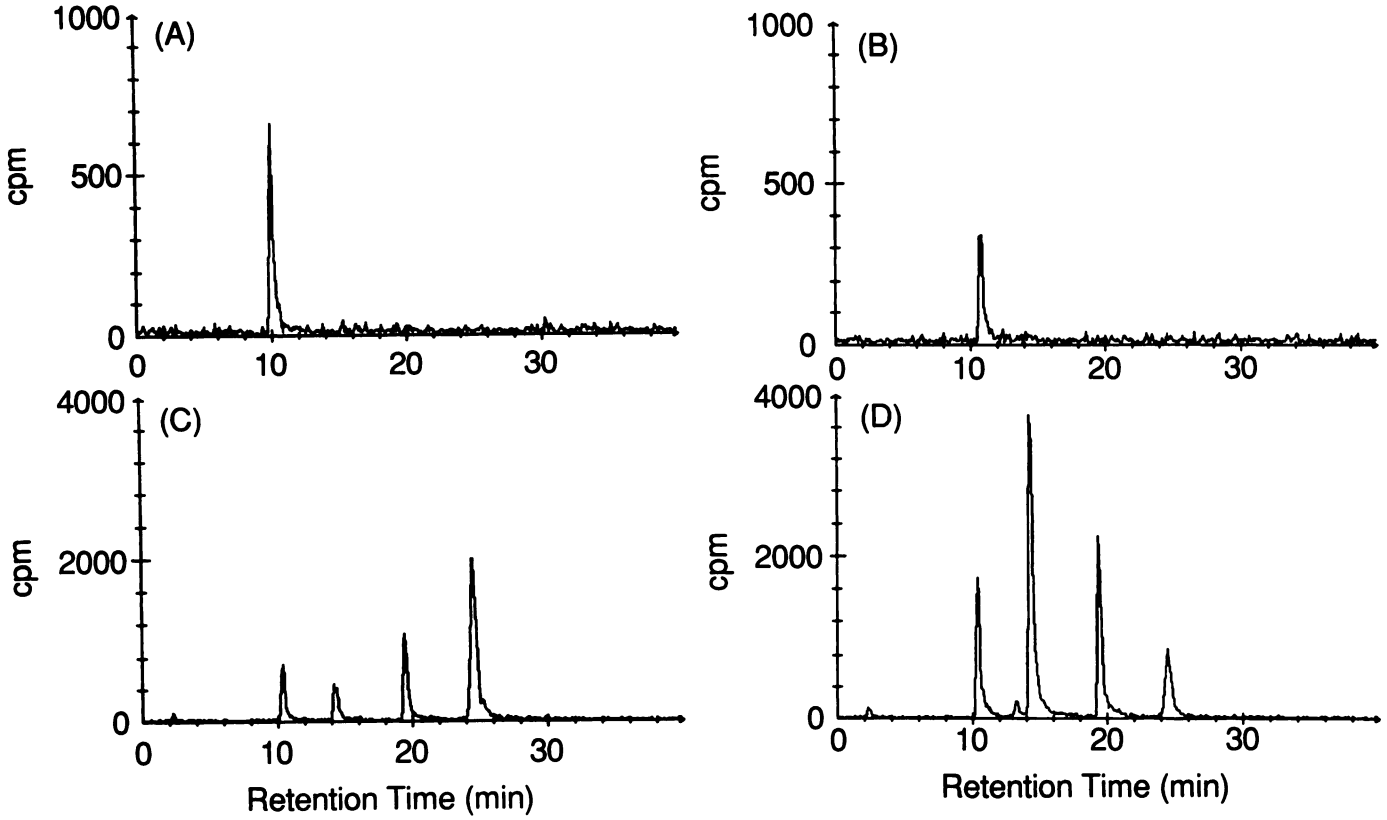

Fig. 2. HPLC profile of WI38 cell extracts incubated with 25 μM (*R*)-[^{3}H]BHCG or (*S*)-[^{3}H]BHCG for 6 hr. A, Mock-infected cells incubated with (*R*)-[^{3}H]BHCG; B, mock-infected cells incubated with (*S*)-[^{3}H]BHCG; C, HSV-1-infected cells incubated with (*R*)-[^{3}H]BHCG; D, HSV-1-infected cells incubated with (*S*)-[^{3}H]BHCG.

TABLE 2

Effect of nucleoside concentration on the accumulation and phosphorylation of (*R*)-[^{3}H]BHCG and (*S*)-[^{3}H]BHCG by HSV-1-infected cells

WI38 cells were grown to ≈50% confluence and then either mock infected or infected with HSV-1(KOS) (multiplicity of infection = 10). After a 1-hr adsorption period, cells were re-fed and then labeled with (*R*)-[^{3}H]BHCG or (*S*)-[^{3}H]BHCG at the indicated concentration. After 6 hr, cells were extracted as described in Experimental Procedures, and the radioactive products were analyzed by HPLC.

Radiolabel	Concentration	Radioactive product				
		Total uptake	Nucleoside	Monophosphate	Diphosphate	Triphosphate
	μM	pmol/10^6 cells				
(*R*)-[^{3}H]BHCG	0.25	1.4	0.16	0.22	0.66	0.41
(*R*)-[^{3}H]BHCG	2.5	11	1.2	0.5	2.7	6.7
(*R*)-[^{3}H]BHCG	25	150	15	5	22	110
(*S*)-[^{3}H]BHCG	0.25	1.9	0.10	1.3	0.54	*a*
(*S*)-[^{3}H]BHCG	2.5	16	0.6	4.8	7.5	2.7
(*S*)-[^{3}H]BHCG	25	340	18	110	160	54

[a] Peak was detectable but too small to be quantitated (<0.05 pmol/10^6 cells).

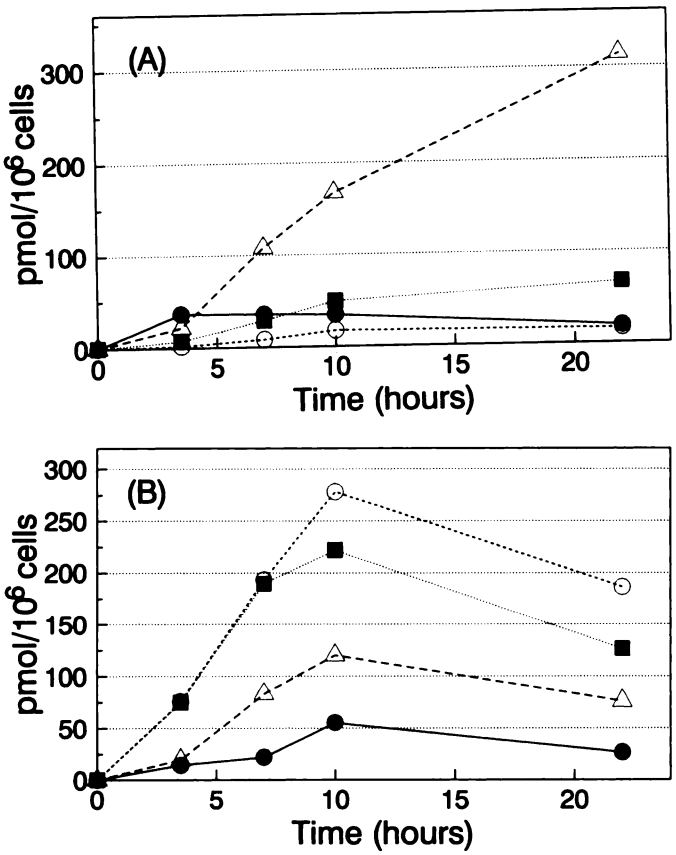

Fig. 3. Time course of phosphorylation of (*R*)-BHCG (A) and (*S*)-BHCG (B) by HSV-1-infected WI38 cells. WI38 cells were infected with HSV-1(KOS) and then continuously incubated in the presence of 25 μM (*R*)-[^{3}H]BHCG or (*S*)-[^{3}H]BHCG for the times indicated. Each *point* is the average of triplicate determinations. ●, Nucleoside; ○, monophosphate; ■, diphosphate; △, triphosphate.

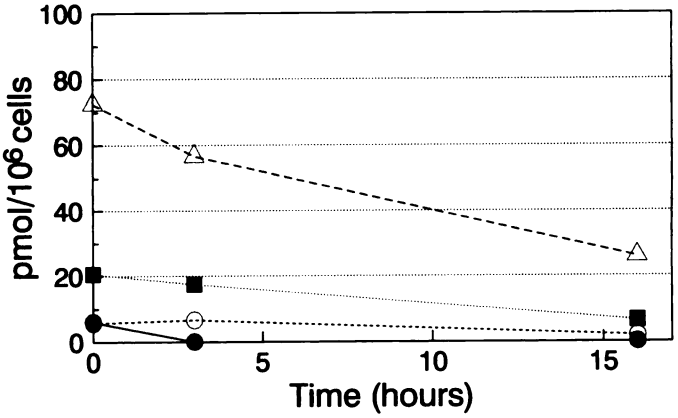

Fig. 4. Lifetime of (*R*)-BHCG and its phosphorylated derivatives in HSV-1-infected cells. WI38 cells were infected with HSV-1(KOS) and then labeled for 6 hr with 25 μM (*R*)-[^{3}H]BHCG. Labeled cells were washed three times with 2 ml of PBS and then reincubated in fresh medium (devoid of drug) for the times indicated. Each *point* is the average of triplicate determinations. ●, Nucleoside; ○, monophosphate; ■, diphosphate; △, triphosphate.

Nucleoside competition studies. Competition studies were carried out to see what effect competing nucleosides would have on the anabolism of (*R*)-[^{3}H]BHCG by HSV-1-infected cells. We first examined the effect of adding nonradioactive (*S*)-BHCG, by labeling HSV-1-infected cells for 6 hr with 12.5 μM (*R*)-[^{3}H]BHCG, either alone or in the presence of varying concentrations of (*S*)-BHCG. Low concentrations of (*S*)-BHCG stimulated the phosphorylation of (*R*)-[^{3}H]BHCG, almost exclusively at the level of the triphosphate (Table 3). The amount of (*R*)-[^{3}H]BHCG triphosphate formed in the presence of 12.5 μM (*S*)-BHCG was 60% higher than in its absence. This effect diminished at higher concentrations of (*S*)-BHCG, yet the amount of (*R*)-[^{3}H]BHCG triphosphate formed in the presence of 100 μM (*S*)-BHCG was no lower than that in the total absence of competing enantiomer. Other guanine compounds had little, if any, effect on uptake and phosphorylation.

In contrast to the guanine derivatives, thymidine was a potent inhibitor of (*R*)-[^{3}H]BHCG phosphorylation. When HSV-1-infected cells were labeled with 25 μM (*R*)-[^{3}H]BHCG, 2.5 and 25 μM thymidine inhibited total accumulation by 50 and 80%, and inhibited phosphorylation to the triphosphate by 70 and 94%, respectively.

Metabolism of (*R*)-BHCG and (*S*)-BHCG in HCMV-infected cells. Unlike HSV-1, HCMV lacks a TK (9, 10) yet is potently inhibited by (*R*)-BHCG (Table 1). Thus, metabolic experiments similar to those described above for HSV-1 were performed. After trying a range of incubation times and multiplicities of infection, we obtained our best phosphorylation when cells were infected for 24 hr at a multiplicity of infection of 0.2 and then labeled for 40 hr in the presence of tritiated compounds (Table 4). [^{3}H]Ganciclovir, which was included as a control to monitor the degree of infection, was not appreciably metabolized by mock-infected cells but was actively phosphorylated by HCMV-infected cells. The phosphorylation to the triphosphate was generally comparable to that seen with (*R*)-[^{3}H]BHCG and (*S*)-[^{3}H]BHCG in HSV-1-infected cells (Table

TABLE 3

Effect of added nucleosides on the phosphorylation of (*R*)-[^{3}H]BHCG by HSV-1-infected cells

WI38 cells were grown to 32% confluence and then infected with HSV-1(KOS) at a multiplicity of infection of 10. After a 1-hr adsorption period, cells were re-fed and then labeled with (*R*)-[^{3}H]BHCG, either alone or in the presence of the competing nonradioactive compound. The concentration of (*R*)-[^{3}H]BHCG was 12.5 μM (experiment 1) or 25 μM (experiment 2). After 6 hr, cells were washed and extracted for nucleoside analysis, as described in Experimental Procedures.

Competing nucleoside	Radioactive product				
	Total uptake	Nucleoside	Monophosphate	Diphosphate	Triphosphate
	pmol/10^6 cells				
Experiment 1					
None	78	10	3.4	17	48
12.5 μM (*S*)-BHCG	110	11	3.1	16	77
50 μM (*S*)-BHCG	80	7.6	4.0	12	56
100 μM (*S*)-BHCG	71	7.0	3.5	13	47
25 μM Guanine	74	6.5	5.2	14	48
25 μM Guanosine	65	5.9	4.3	14	41
25 μM Deoxyguanosine	66	7.4	3.9	13	42
Experiment 2					
None	170	18	8.7	30	110
2.5 μM Thymidine	73	23	4.0	9.7	36
25 μM Thymidine	36	26	1.7	1.7	6.9

TABLE 4

Accumulation and phosphorylation of (*R*)-[^{3}H]BHCG and (*S*)-[^{3}H]BHCG by mock- and HCMV-infected cells

WI38 cells were grown to ≈30% confluence and then either mock infected or infected with HCMV(AD169) (multiplicity of infection = 0.2). After 24 hr, cells were labeled with 25 μM levels of either (*R*)-[^{3}H]BHCG], (*S*)-[^{3}H]BHCG, or [^{3}H]ganciclovir. After an additional 40 hr, cells were extracted as described in Experimental Procedures, and the radioactive products were analyzed by HPLC.

Virus	Compound	Radioactive product				
		Total uptake	Nucleoside	Monophosphate	Diphosphate	Triphosphate
		pmol/10^6 cells				
Mock	(*R*)-BHCG	14	14	0	0	0
Mock	(*S*)-BHCG	8.4	8.4	0	0	0
Mock	Ganciclovir	23	23	0	0	0
HCMV	(*R*)-BHCG	64	64	0	*a*	*a*
HCMV	(*S*)-BHCG	45	45	0	0	0
HCMV	Ganciclovir	130	70	*a*	8.8	46

[a] Peak was detectable but too small to be quantitated (<0.5 pmol/10^6 cells).

2). Surprisingly, a large increase in total accumulation was also seen, due primarily to an increased intracellular pool of free unmodified nucleoside. In HSV-1-infected cells, intracellular nucleosides, regardless of the radiolabel, never accumulated to more than 5–10% of the total radiolabel pool; most of the label accumulated as nucleotides (result not shown).

When mock- and HCMV-infected cells were labeled with (*R*)-[^{3}H]BHCG and (*S*)-[^{3}H]BHCG, the major effect of infection was also a large (4–5-fold) increase in nucleoside accumulation (Table 4). Virtually no phosphorylation was seen in mock-infected cells or in HCMV-infected cells labeled with (*S*)-[^{3}H]BHCG. The only significant and reproducible phosphorylation was seen with HCMV-infected cells labeled with (*R*)-[^{3}H]BHCG. The level was below that required for accurate quantitation (<0.5 pmol of nucleotide/10^6 cells), but the diphosphate and triphosphate of (*R*)-BHCG were readily seen in several experiments under a variety of conditions of virus input and incubation time.

Discussion

Our studies extend the work of others describing the stereospecificity for antiviral activity of the family of carbocyclic nucleoside analogs (6, 11–13, 18–20). (*R*)-BHCG is particularly interesting, because the major enzyme for its activation in HSV-1-infected cells, the viral TK, favors its inactive *S*-enantiomer by about 20-fold (13).[2] Because (*R*)-BHCG more closely resembles common nucleosides containing a D-sugar configuration, it was surprising that its *S*-enantiomer would be phosphorylated more efficiently (Fig. 2). Ganciclovir was also shown to be stereospecifically phosphorylated by the HSV-1 TK (21). This analog contains a prochiral center, and phosphorylation by the viral TK produces the *S*-enantiomer of ganciclovir monophosphate.

The activity profile of (*R*)-BHCG was quite similar to that of ganciclovir against most wild-type herpesviruses and was generally 10-fold more potent than that of acyclovir (Table 1). In contrast, (*S*)-BHCG showed almost no activity. The small amount seen was most likely due to a trace of (*R*)-BHCG contaminating the nucleoside preparation.[3] This stereospecificity for antiviral activity was also seen in the closely related analog carbocyclic 2′-deoxyguanosine (19) and in carbocyclic pyrimidine analogs (20).

As shown in Fig. 2, both (*R*)-BHCG and (*S*)-BHCG were metabolized in HSV-1-infected cells, but with strikingly different patterns of phosphorylation. During a 6-hr incubation in the presence of 25 μM drug, the extent of (*S*)-[^{3}H]BHCG accumulation was almost twice that of its enantiomer. The driving force behind this difference appeared to be the much greater activity of the viral TK on the *S*-isomer. For (*R*)-BHCG, phosphorylation of the nucleoside to the monophos-

phate appeared be the rate-limiting step in the formation of the triphosphate. Subsequent phosphorylation to the triphosphate appeared to proceed with increasing efficiency, especially at high concentrations of added nucleoside (Table 2). The net result was the (*R*)-BHCG triphosphate accumulated to about twice the extent of (*S*)-BHCG triphosphate during a 6 hr incubation. The amount of (*R*)-BHCG triphosphate formed showed a linear relationship with both the concentration of nucleoside added to the medium (Table 2) and the length of incubation (Fig. 3). At 25 μM added nucleoside, the rate of (*R*)-BHCG triphosphate formation remained linear for at least 22 hr. Because this is well into the period during which cell lysis occurs in HSV-1-infected cells, (*R*)-BHCG is most likely exerting an antiviral effect during this initial round of viral infection. Indeed, analysis of two viral proteins, TK and alkaline DNase, showed about a 50% reduction in the amounts of these enzymes when HSV-1-infected cells were incubated with (*R*)-BHCG, compared with (*S*)-BHCG.[3] Furthermore, the long intracellular half-life of (*R*)-BHCG triphosphate, ≈10 hr (Fig. 4), indicates that it is not readily degraded. These data reveal an efficient mechanism for producing a high intracellular concentration of (*R*)-BHCG triphosphate during the extended course of an HSV-1 infection.

Thymidine potently inhibited the accumulation and phosphorylation of (*R*)-[^{3}H]BHCG in infected cells (Table 3). This was not unexpected, because thymidine is the natural substrate for HSV-1 TK and would be anticipated to compete with (*R*)-BHCG. The reduction in (*R*)-BHCG accumulation would be a secondary consequence of competition for phosphorylation. However, the stimulation of (*R*)-[^{3}H]BHCG phosphorylation by the addition of small amounts of (*S*)-BHCG to the incubation medium was surprising. Because (*S*)-BHCG is the preferred substrate for the HSV-1 TK, the predicted effect would be inhibition and not stimulation. The reason for the observed stimulation is not known at present. However, a likely mechanism would be a perturbation of cellular nucleotide metabolism. Cellular ribonucleotide reductase, a key enzyme in deoxynucleotide triphosphate formation (22), is known to be allosterically regulated by ribo- and deoxyribonucleotide triphosphates (23).

Neither guanine, guanosine, nor deoxyguanosine inhibited the phosphorylation of (*R*)-[^{3}H]BHCG. In agreement, Nishiyama *et al.* (6) found that a 100-fold excess of deoxyguanosine only partially reversed the antiviral activity of carbocyclic oxetanocin G [(*R*)-BHCG] against HSV-2. However, in another study, Hayashi *et al.* (4) found that deoxyguanosine readily reversed the anti-HIV activity of cyclobut-G [(±)-BHCG]. Apparently, the mechanism by which (*R*)-BHCG exerts its antiviral effects must be different against herpesviruses and HIV.

Although (*R*)-BHCG displayed a strong dependence upon a viral TK, it was moderately active against both HCMV and HSV-1(KOS)TK$^-$ mutant (Table 1). In the latter strain, it showed considerably less TK dependence than did acyclovir. As shown in Table 1, HSV-1(KOS)TK$^-$ was 1000-fold more resistant than its parent to acyclovir but only 40–50-fold more resistant to (*R*)-BHCG. Given the 10-fold greater potency of (*R*)-BHCG against the parent strain KOS, it was 200 times more effective than acyclovir against the TK$^-$ mutant.

[3] G. Yamanaka, unpublished results.

We initially tried looking at both HCMV and HSV-1(KOS)TK$^-$ for an understanding of the mechanism of TK independence of (*R*)-BHCG. However, only in HCMV-infected cells was there a difference in metabolism from mock-infected cells. The major finding was a small but specific phosphorylation of the *R*-enantiomer to the diphosphate and triphosphate in the infected cells (Table 4). Given the lack of an HCMV-encoded TK (9, 10), this was most likely due to virally induced stimulation of a cellular kinase. As noted earlier, even mock-infected cells occasionally phosphorylated (*R*)-BHCG. Thus, the detection of such stimulation would have been maximized by the conditions of the experiment, i.e., infection at a low multiplicity (0.2) and labeling for an extended period of time (40 hr).

When considered from a mechanistic perspective, the data described above for HCMV are compatible with a DNA polymerase inhibition model for antiviral activity. The amount of (*R*)-BHCG triphosphate formed when HCMV-infected cells were incubated with 25 μM (*R*)-BHCG was <0.5 pmol/10^6 cells (Table 4), which was similar to the 0.4 pmol/10^6 cells value obtained when HSV-1-infected cells were incubated with a 100-fold lower concentration (Table 2). These values approximate an intracellular concentration of <0.5 μM.[4] In both cases, the concentration of (*R*)-BHCG added to the cultures was approximately 10-fold above the ED_{50} value for that virus (Table 1); thus, it is conceivable that the intracellular triphosphate concentration at the ED_{50} would be below 50 nM. Such a value would be near the *in vitro* K_i of 17 nM for the inhibition of the HSV-1 DNA polymerase by (*R*)-BHCG triphosphate.[2] Because the HCMV DNA polymerase is closely related to the HSV-1 enzyme (24) and is similarly sensitive to the inhibitor acyclovir triphosphate (25), it is plausible that both enzymes are similarly sensitive to inhibition by (*R*)-BHCG triphosphate.

In conclusion, (*R*)-BHCG is a compound with excellent broad spectrum activity against the family of herpesviruses. It is strongly dependent upon a viral TK for activation and, in HSV-1-infected cells, undergoes an efficient phosphorylation to the triphosphate. However, even in cells infected with TK-deficient strains, (*R*)-BHCG is anabolized and appears to be potent enough to have therapeutic potential as an antiviral agent.

[4] Given the volume of a single cell to be on the order of 10^{-9} ml (7, 19), the intracellular concentration would be $<5 \times 10^{-7}$ nmol/ml or <0.5 μM.

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