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INTRACELLULAR METABOLISM AND ENZYMATIC PHOSPHORYLATION OF 9-(1,3-DIHYDROXY-2-PROPOXYMETHYL)GUANINE AND ACYCLOVIR IN HERPES SIMPLEX VIRUS-INFECTED AND UNINFECTED CELLS

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Abstract—The antiherpes agent 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) is a much more potent inhibitor of herpes simplex viruses *in vivo* than acyclovir, yet both are equally active *in vitro* against these viruses. To explain this difference, studies were conducted to compare the intracellular metabolism and enzymatic phosphorylation of the two compounds. In herpes type 1 and type 2 infected cells, the levels of DHPG triphosphate were only about 2-fold greater than levels of acyclovir triphosphate at virus-inhibitory concentrations (≤ 1 μM). At concentrations > 2.5 μM in herpes type 1 but not in type 2 infected cells, acyclovir phosphorylation was inhibited relative to that of DHPG. When drug was removed after 6 hr from infected cells, acyclovir triphosphate rapidly degraded to acyclovir and was excreted into the culture medium. In contrast, DHPG triphosphate persisted at 60–70% of the original level for 18 hr after drug removal, and DHPG excretion from cells was very slow. This finding could be a key factor to the superior potency of DHPG in animals, despite the fact that blood levels of both compounds fall rapidly after dosing. In uninfected cells, low levels of DHPG and acyclovir triphosphates were produced at 100 μM concentrations. Phosphorylation of DHPG to mono-, di- and triphosphates by purified viral and cell enzymes was more rapid than that of acyclovir. However, acyclovir triphosphate was a much more potent inhibitor of herpes virus and cell DNA polymerases.

The acyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG†) is structurally related to the antiherpes agent acyclovir (ACV). *In vitro*, both compounds inhibit herpes simplex virus types 1 and 2 at approximately equal concentrations [1–3]. In certain animal infection models, DHPG exhibits much more potent effects than ACV, requiring 50 to 70-fold less drug to achieve the same degree of efficacy [2, 3]. In addition, the toxicity of DHPG in terms of acute LD_{50} in mice is similar to that of ACV.

The antiviral actions of these compounds evidently involve their selective phosphorylation in herpes-infected cells [2–5] and subsequent inhibition of herpes DNA polymerase by the drug 5′-triphosphates [6–9]. Field and coworkers [3] have suggested that DHPG (referred to as 2′-NDG in their article) may be superior to ACV *in vivo* because of its more rapid phosphorylation in infected cells. This would be advantageous because more drug could be converted to the antivirally active 5′-triphosphate before blood levels of nucleoside declined. In support of this hypothesis, DHPG has been shown to be a better substrate than ACV for purified herpes type 1 thymidine kinase [2, 3] and cell guanylate kinase [3]. Cheng *et al.* [10] recently established that DHPG is metabolized to mono-, di- and triphosphates in herpes-infected cells, and to a much lesser degree in uninfected cells. No comparison to ACV was made, however. Limited comparisons performed by other investigators of DHPG and ACV phosphorylation in HSV-1 infected cells [7, 8] indicate greater triphosphate accumulation in DHPG-treated cultures.

We undertook the present work to compare the metabolism of DHPG and ACV in infected and uninfected cells under various conditions. One purpose of these studies was to determine if the rates of phosphorylation correlate with differences of drug potency *in vivo*. The major enzymes phosphorylating ACV to the triphosphate [5, 11, 12] were examined in order to determine quantitative differences in rates of phosphorylation of DHPG and ACV. The effects of DHPG and ACV triphosphates on herpes virus and cell DNA polymerase activities were also studied. A preliminary report of this work has been presented [13].

EXPERIMENTAL PROCEDURES

Materials. African green monkey kidney (Vero) and human cervical carcinoma (HeLa) cells were purchased from the American Type Culture Collection, Rockville, MD. They were passaged as described previously [2]. Herpes simplex virus type 1 (HSV-1), F strain, and type 2 (HSV-2), G strain, were obtained from Bernard Roizman, University

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† Abbreviations: DHPG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; acyclovir or ACV, 9-(2-hydroxyethoxymethyl)guanine; HSV-1 and HSV-2, herpes simplex virus types 1 and 2; PFU, plaque forming units; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; and DNAp, DNA polymerase.

of Chicago, IL, and from the American Type Culture Collection respectively. They were propagated to > 10^8 plaque forming units (PFU) per ml in Vero cells. [^{3}H]DHPG (16 Ci/mmole) and [^{3}H]ACV (1.6 Ci/mmole), both labeled on the terminal carbon of the side chain, were produced by Howard Parnes at Syntex Research, Palo Alto, CA. The radiochemical purity of these substances, as determined by thin-layer chromatography, was > 99%. [^{3}H]Thymidine (10 Ci/mmole) and [^{32}P]NaH_2PO_4 (300 mCi/mmole initially) were from ICN Chemical and Radioisotope Division, Irvine, CA. [^{3}H]dGTP (12 Ci/mmole) and [^{3}H]ATP (13 Ci/mmole) were bought from New England Nuclear, Boston, MA. Herpes thymidine kinases were affinity purified by the methods of Fyfe *et al.* [5], except that desalting and thymidine removal were accomplished by Sephadex G-25 column chromatography instead of ammonium sulfate precipitation. HSV-1 and HeLa cell DNA polymerases were purified according to the procedure of Ostrander and Cheng [14]. Guanylate kinase was purified from human erythrocytes by the methods of Miller and Miller [11]. Phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and human erythrocyte nucleoside diphosphokinase were products of the Sigma Chemical Co., St. Louis, MO. Other materials required for enzyme assays (buffers, substrates, etc.) were purchased from Sigma. Activated calf thymus DNA was made by Worthington Diagnostics, Freehold, NJ. DE-81 chromatography paper and 25 mm paper discs used in radioactivity assays were from Whatman, Clifton, NJ, and Schleicher and Schuell, Keene, NH, respectively. PEI-cellulose thin-layer chromatography (TLC) plates were purchased from EM Laboratories, Elmsford, NY.

Preparation of nucleotides. DHPG monophosphate [a racemic (*R*, *S*) mixture of what we refer to as the 3′- and 5′-phosphates] and ACV monophosphate were chemically prepared by Ernie Prisbe at Syntex. DHPG monophosphate was used to produce (*R*, *S*)-DHPG di- and triphosphates using purified guanylate kinase and phosphoglycerate kinase alone or in combination. Small amounts of chiral DHPG mono- and triphosphates were prepared using purified HSV-1 thymidine kinase and a dialyzed extract of HSV-1 infected HeLa cells respectively. It was not possible to produce enough chiral DHPG mono- and diphosphates for kinetic analysis because of the low yield of thymidine kinase and its slow reaction rate using DHPG as substrate. Nucleotide species were separated from each other using high pressure liquid chromatography (HPLC) as described later. The compounds were desalted by absorption to an AG1X8 anion exchange resin (formate form, Bio-Rad Laboratories, Rockville, NY) or to activated charcoal. The concentration of each final product was determined using tritiated reference standards. ACV triphosphate was a gift from Y-C. Cheng, University of North Carolina, Chapel Hill.

Preparation of cell extracts for nucleotide analysis. Infected Vero cell cultures received 10 PFU/cell of HSV-1 or HSV-2 for 1.5 hr, following which medium was removed and radioactive drug was applied. Uninfected cells received radioactive drug only. DHPG assays were run with cells in T-25 flasks, whereas ACV studies used 80 mm tissue culture dishes. More cells were required in the ACV experiments to obtain enough counts of the various ACV nucleotides. In assays where drug was removed part way through the experiment, cells were washed three times to take off residual drug and then were refed culture medium devoid of compound. After further incubation at 37°, medium was aspirated, and cells were trypsinized and pelleted. The dry pellets were stored at −80° prior to HPLC analysis. Nucleotide extracts were prepared by precipitating the pellets in 300 μl of 3.5% perchloric acid. After 30 min at 4°, samples were neutralized with 1 N KOH containing 0.2 M imidazole. These procedures were similar to those used by other investigators [7, 8, 10, 15]. [^{3}H]-DHPG and [^{3}H]ACV samples from 0.5 to 5 μM contained 1680 and 1460 cpm/pmole of radioactivity, respectively. [^{3}H]DHPG and [^{3}H]ACV samples from 10 to 100 μM contained a fixed amount of radioactivity regardless of drug concentration. The specific activity of [^{3}H]DHPG ranged from 3130 (10 μM) to 313 (100 μM) cpm/pmole in these assays, and [^{3}H]-ACV from 1160 to 116 cpm/pmole. Micromolar concentrations were calculated based on an estimated intracellular volume of 1.5×10^{-6} l/10^6 cells.

Nucleotide analysis. Nucleotide extracts (100 μl volumes from HSV infected cells or 425 μl from uninfected cells) were analyzed by HPLC with a Whatman Partisil PXS 10/25 SAX column. The column was eluted at a flow rate of 1 ml/min with a linear gradient of KH_2PO_4, 0.01 to 1.0 M (pH 3.5). A 30-min gradient was followed by 15 min at 1 M buffer to elute the triphosphates of DHPG and ACV. Fractions (1 ml) were collected directly into scintillation vials, and radioactivity was determined. In the HPLC assay, DHPG mono-, di- and triphosphate peaks eluted at 11, 19 and 32 min respectively; ACV mono-, di- and triphosphates eluted at 11, 21 and 35 min respectively. The concentration of each nucleotide was calculated in terms of pmoles/10^6 cells using the specific activities for [^{3}H]DHPG and [^{3}H]-ACV stated earlier, and in micromolarity.

Enzyme assays. Thymidine kinase activity was assayed by incubating each enzyme in a 100-μl reaction mixture containing 100 mM Tris–HCl (pH 7.5), 20 mM NaF, 2 mM $MgCl_2$, 50 mM KCl, 1 mg bovine serum albumin/ml, 2 mM ATP, and appropriate concentrations of ^{3}H-labeled nucleoside. Reactions at 37° were initiated with addition of enzyme and terminated after 30–60 min by spotting duplicate 15-μl samples on DE-81 paper squares. The squares were washed for 10 min in water followed by three washes for 5 min each in methanol. After drying, squares were placed into scintillation vials containing 1 ml of 1.0 M KOH to release nucleotide into solution. Radioactivity was determined using Aquasol scintillation fluid (New England Nuclear). Kinetic binding constants were expressed as either K_m values for enzyme–substrate interactions obeying classical Michaelis–Menten kinetics or $S_{0.5}$ values (the substrate concentration yielding $0.5\,V_{max}$) for non-Michaelis–Menten interactions.

Guanylate kinase activity was determined spectrophotometrically as described by Agarwal *et al.* [16], or radioisotopically as described by Miller and Miller

[11]. Pyruvate kinase was assayed according to the procedure described by Miller and Miller [12]. Phosphoglycerate kinase was assayed as described by Miller and Miller [12], except that [^{32}P]NaH_2PO_4 was used to produce [^{32}P]−1,3-diphosphoglycerate and, in the subsequent reaction, [^{32}P]-labeled nucleoside triphosphates. DNA polymerases were assayed as described by Derse *et al.* [6] using [^{3}H]dGTP as competitor of DHPG and ACV triphosphates. Activated calf thymus DNA was used to prime DNA synthesis.

Nucleoside diphosphokinase activity was determined radioisotopically by measuring the rate of conversion of [^{3}H]ATP to [^{3}H]ADP which occurs concomitantly with the phosphorylation of nucleoside diphosphates. The assay mixtures contained 50 mM Tris acetate (pH 7.5), and 5 mM $MgCl_2$, 3 mM [^{3}H]ATP, and appropriate amounts of nucleoside diphosphate and enzyme, in a volume of 50 μl. All ingredients except nucleoside diphosphate were preincubated for 15 min at 30°. The reaction was begun by adding nucleoside diphosphate and incubating for 6 min. The reaction was terminated by spotting 5-μl aliquots of the reaction mix on PEI-cellulose TLC plates which had been prespotted with 50 nmoles each of ATP and ADP. The plates were developed in 0.7 M formic acid containing 0.7 M LiCl. Spots corresponding to ADP were identified by u.v. absorbance and quantified for associated radioactivity.

RESULTS

Effect of exogenous drug concentration on nucleoside phosphorylation. DHPG and ACV nucleotide formation in HSV-1 and HSV-2 infected cells was dependent upon the concentration of drug in the medium (Fig. 1). At DHPG concentrations of 0.5 to 5 μM, a linear dose–response relationship of nucleotide synthesis was achieved after 6 hrs of incubation with virus. ACV, on the other hand, exhibited a differential dose–response pattern of phosphorylation in HSV-1 infected cells. A linear relationship occurred at 0.5 to 2.5 μM, but inhibited phosphorylation was evident at 5–20 μM. The pattern of ACV nucleotide anabolism in HSV-2 infected cells was similar to that of DHPG with respect to its dose–response character and to pmoles of triphosphate produced. At drug concentrations causing inhibition of HSV-1 and HSV-2 plaque formation (0.5 to 1 μM), the amount of DHPG triphosphate produced was no greater than 2-fold that of ACV triphosphate. The amounts of DHPG mono- and diphosphates produced in these infected cells were approximately 1 and 9% of triphosphate levels respectively. Equal amounts of ACV mono- and diphosphates were present and were about 9% of the triphosphate concentrations.

In uninfected cells, a concentration-dependent phosphorylation of DHPG and ACV was evident (Fig. 1). The amount of each nucleoside triphosphate produced in uninfected cells at 100 μM was approximately equal to that produced with 0.5 μM in HSV infected cells. The levels of DHPG diphosphate in these cells was 20% of the triphosphate concentration. DHPG mono-, ACV mono-, and ACV diphosphate amounts relative to the triphosphates were similar to those in HSV infected cells.

Effect of time of exposure of DHPG and ACV on phosphorylation. DHPG at 5 μM was incubated with HSV-1 and HSV-2 infected cells through 24 hr (Fig. 2). In these cells, phosphorylation was linear through

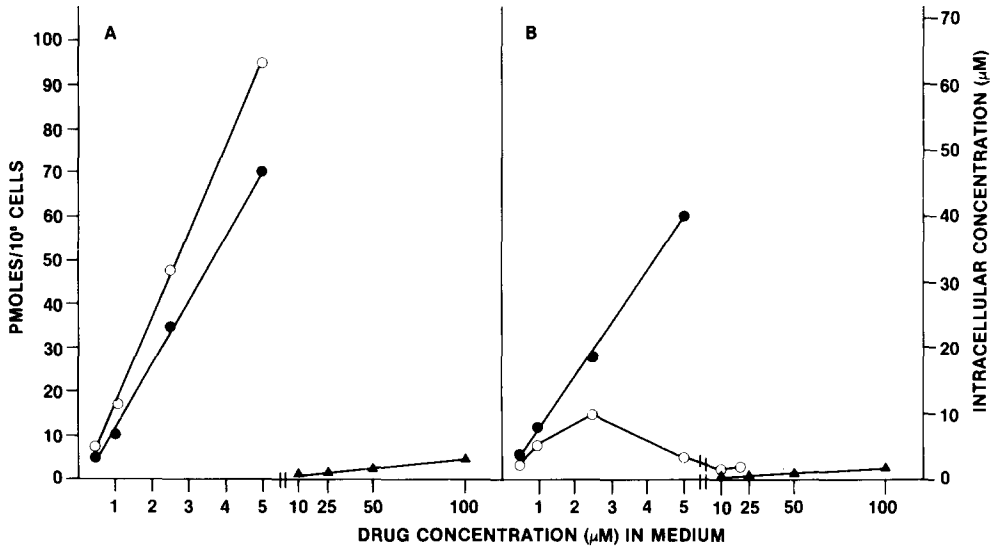

Fig. 1. Effect of extracellular drug concentration on DHPG (A) and ACV (B) triphosphate levels in HSV-infected and uninfected cells. Infected cultures were radiolabeled with 1.6 to 16 μCi [^{3}H]DHPG/ml or 0.8 to 8 μCi [^{3}H]ACV/ml, depending upon the micromolarity applied. These samples contained a fixed ratio of unlabeled to labeled nucleoside. Uninfected cultures received a constant amount of [^{3}H]-DHPG (48 μCi/ml) or [^{3}H]ACV (16 μCi/ml) and variable concentrations of unlabeled compounds. HPLC analysis was performed with extracts from 5×10^5 DHPG-treated infected cells, 1×10^6 ACV-treated infected cells, 2×10^6 DHPG-treated uninfected cells, or 4×10^6 ACV-treated uninfected cells. Incubation with drug was for 6 hr. Symbols: (○) HSV-1; (●) HSV-2; and (▲) uninfected.

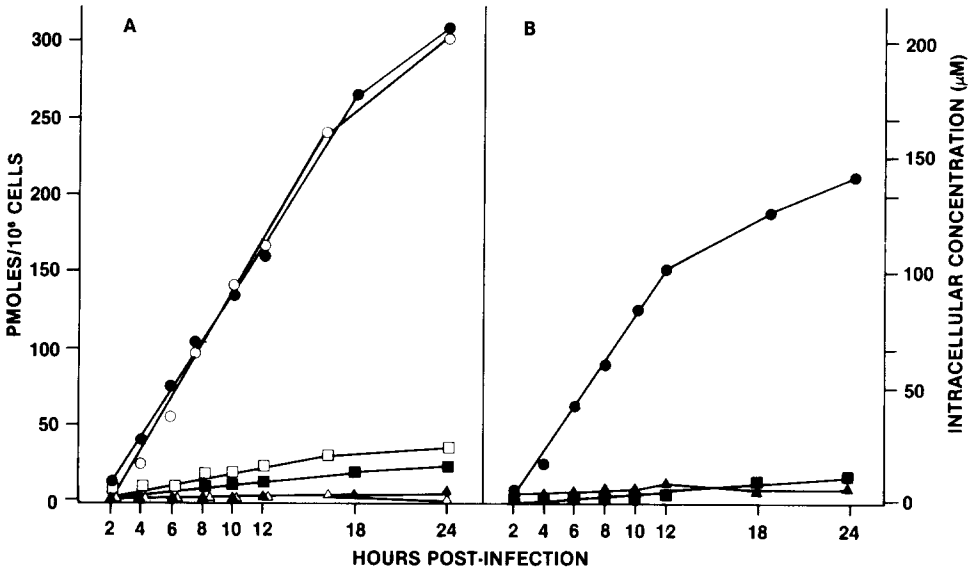

Fig. 2. Time course of DHPG (A) and ACV (B) phosphorylation in HSV-infected cells. Extracellular drug concentration was 5 μM. Other methods used here were the same as in Fig. 1. Symbols: (▲, △) monophosphate, (■, □) diphosphate; and (●, ○) triphosphate. Open symbols, HSV-1; and closed symbols, HSV-2.

16–18 hr and continued through 24 hr after infection to produce 300 pmoles triphosphate/10^6 cells. The time course of ACV phsophorylation was not examined in HSV-1 infected cells. Against HSV-2, ACV phosphorylation continued through 24 hr (Fig. 2) to produce 225 pmoles triphosphate/10^6 cells. ACV phosphorylation began to slow earlier than that of DHPG.

In uninfected cell monolayers, 10 μM DHPG was incubated up to 96 hr, with samples assayed daily to determine nucleotide levels. Results showed that intracellular DHPG triphosphate concentrations remained constant through the 4 days (< 1 pmole/10^6 cells), suggesting that an equilibrium was established between the phosphorylation and catabolism of the drug.

Effect of drug removal on nucleotide levels present in HSV infected cells. In companion studies to those presented in Fig. 2, drug was removed from cells at 6 hr, and nucleotide levels were examined at various times thereafter through 24 hr post-infection (Fig. 3). When DHPG was removed from HSV-1 or HSV-

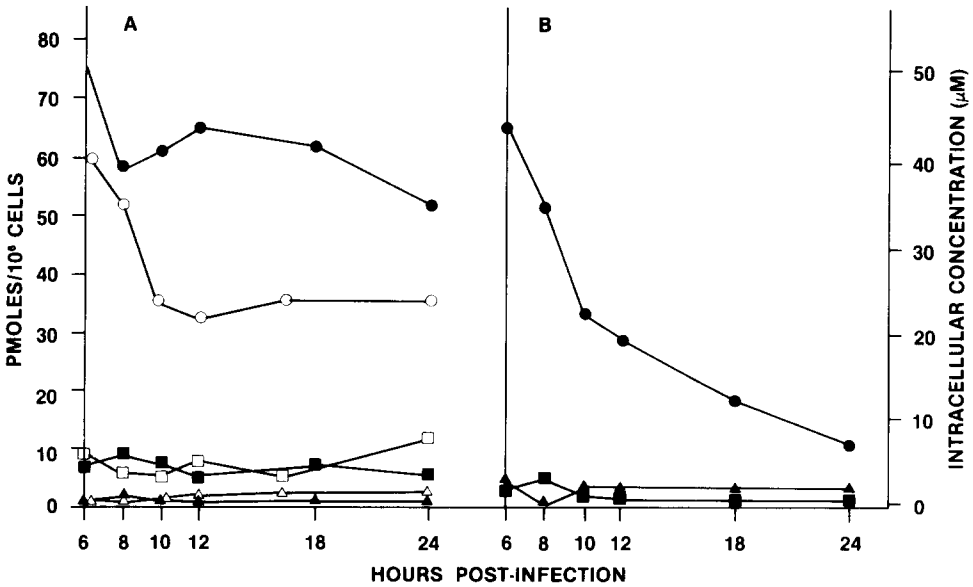

Fig. 3. Catabolism of DHPG (A) and ACV (B) nucleotides in HSV-infected cells after drug removal. Incubation with 5 μM nucleoside was for 6 hr before applying drug-free medium to cells. Other methods were the same as in Fig. 1. Symbols: (▲, △) monophosphate; (■, □) diphosphate; and (●, ○) triphosphate. Open symbols, HSV-1; and closed symbols, HSV-2.

Table 1. Enzyme kinetics of DHPG, ACV, and their nucleotides using purified viral and cell enzymes*

Enzyme	K_m or $S_{0.5}$ (μM)			V_{max}			V_{max}/K_m (%)		
	Thym.	DHPG	ACV	Thym.	DHPG	ACV	Thym.	DHPG	ACV
HSV-1 thymidine kinase	0.5	11.0	375	0.5	2.2	0.3	100	22	0.09
HSV-2 thymidine kinase	0.7	15.8	305	0.3	1.3	0.4	100	20	0.33
	GMP	DHPGMP	ACVMP	GMP	DHPGMP	ACVMP	GMP	DHPGMP	ACVMP
Guanylate kinase	29	40	218	107	20	1.9	100	14	0.24
	GDP	DHPGDP	ACVDP	GDP	DHPGDP	ACVDP	GDP	DHPGDP	ACVDP
Pyruvate kinase	940	1360	2000	253	1.4	0.51	100	0.38	0.09
Phosphoglycerate kinase	340	830	2400	869	58	47	100	2.7	0.77
Nucleoside diphosphokinase	44	150	3100	303	0.085	0.083	100	0.008	0.0004

* Values for the thymidine kinases, gaunylate kinase, and nucleoside diphosphokinase are expressed as $S_{0.5}$ (the substrate concentration yielding 0.5 V_{max}) because neither DHPG nor ACV exhibited classical Michaelis–Menten kinetics with the thymidine kinases, and the other enzyme preparations represented mixtures of isozymes. V_{max} values are in units of pmoles/min for the thymidine kinases, and in units of μmoles/min/mg protein for the other enzymes. Enzyme kinetic data using ACVDP were obtained from Ref. 12.

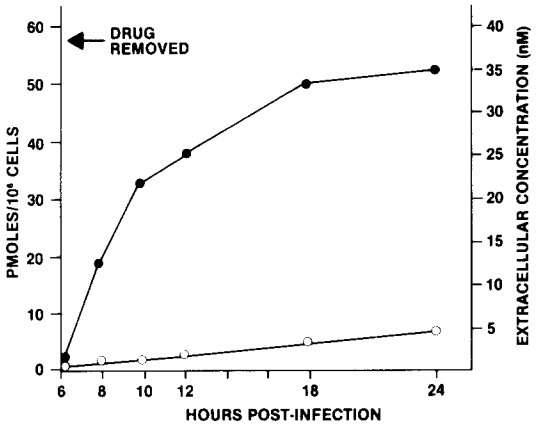

Fig. 4. Excretion of DHPG (○) and ACV (●) from HSV-2 infected cells into culture medium following drug removal. Incubation with 5 μM nucleoside was for 6 hr before applying drug-free medium to cells. At various time points 1-ml volumes of extracellular medium were counted for radioactivity. There were approximately 10^6 cells/ml of extracellular medium, and 2×10^6 DHPG-treated or 4×10^6 ACV-treated cells were employed in the test.

2 infected cultures, 60–70% of DHPG triphosphate was still present 18 hr later. In contrast, ACV triphosphate underwent a rapid and continual degradation after drug was removed from HSV-2 infected cells. Moreover, when drug was removed 6 hr postinfection, ten times more ACV than DHPG was excreted back into the culture medium by 24 hr postinfection (Fig. 4).

Phosphorylation of drug by purified viral and cellular enzymes. DHPG and DHPG monophosphate were superior to ACV and ACV monophosphate as substrates for herpes thymidine kinases and cell guanylate kinase, respectively (Table 1), based upon lower $S_{0.5}$ values and higher velocities of product formation. There were no striking differences between the rates of phosphorylation of DHPG diphosphate compared to ACV diphosphate by phosphoglycerate kinase, pyruvate kinase, or nucleoside diphosphokinase. Of these enzymes, phosphoglycerate kinase was clearly superior to the others in its activity, with nucleoside diphosphokinase being very inefficient in turning over the substrates. None of the acyclic nucleosides or nucleotides was superior to the natural substrates (thymidine, GMP and GDP) with regard to enzymatic conversion rates.

Table 2. Effects of DHPG triphosphate and ACV triphosphate on DNA polymerase activity

	K_i* (μM)	
Nucleotide	HSV-1 DNAp	HeLa α-DNAp
DHPGTP (racemic)	0.19	10.8
DHPGTP (chiral)	0.09	4.2
ACVTP	0.003	0.44

* Assays were run with [^{3}H]dGTP as competitive substrate.

The phosphorylation of racemic DHPG monophosphate by guanylate kinase yielded a biphasic reaction which was derived from different rates of reaction with the two stereoisomers. The faster rate reported in Table 1 proceeded to the point where nearly 50% of available substrate was consumed before the enzyme reaction slowed. The faster rate was equivalent to the rate obtained with chiral DHPG monophosphate and was about 20-fold higher than the slower rate (data not shown). Since only the chiral DHPG monophosphate is produced *in vivo*, the faster reaction rate reflects the more accurate kinetic parameter.

Inhibition of DNA polymerases. The triphosphates of DHPG and ACV were competitive inhibitors of viral and cell DNA polymerases with respect to dGTP (Table 2). ACV triphosphate was ten to thirty times more potent than chiral DHPG triphosphate against these enzymes, and chiral DHPG triphosphate was approximately 2-fold more active than racemic DHPG triphosphate. This indicates that these DNA polymerases are highly stereospecific and that (*R*)-DHPG triphosphate is inactive.

DISCUSSION

Previous reports suggested that the superior phosphorylation of DHPG compared to ACV may account for the greater potency of DHPG *in vivo* [3]. Our findings indicate that this conclusion is only partially true, and other factors such as drug triphosphate catabolism and DNA polymerase inhibition may also play a role.

In HSV-1 infected cells, DHPG was metabolized rapidly to the triphosphate at culture medium concentrations of 0.5 to 5 μM. This was clearly more efficient than ACV phosphorylation, where linear metabolism of ACV occurred at 0.5 to 2.5 μM, with decreased accumulation of triphosphate at 5–20 μM. Cheng and colleagues [10] showed a linear dose–response accumulation of DHPG triphosphate in HSV-1 infected cells but did not evaluate ACV under similar circumstances. Other investigators have demonstrated that greater amounts of triphosphate accumulate in DHPG-treated HSV-1 infected cells relative to cells treated with ACV, at extracellular concentrations of 50–100 μM [7, 8]. This suggests that, in an *in vivo* situation, DHPG would be more efficiently phosphorylated than ACV when blood levels of drug were high. In HSV-2 infected cells, DHPG and ACV were phosphorylated at approximately the same rate at all concentrations, so *in vivo* differences in rates of phosphorylation of the two nucleosides may not be evident.

A question is raised as to whether or not the HSV-1 virus used here would be less inhibited at 10 μM extracellular concentration than it would at a 1 to 2.5 μM, based upon the intracellular triphosphate levels. This conceivably could occur. One must remember, however, that the data presented here were only a 6-hr time point. ACV triphosphate accumulation at later times was not monitored. In addition, based upon the low virus-inhibitory concentration of 0.4 μM reported for this strain of virus [2], a highly potent amount of triphosphate would still be present in the cell at the 10 μM extracellular

concentration. *In vivo* where ACV levels are dynamic, an effect such as this would be even less apparent.

In uninfected cells, slightly more DHPG triphosphate was produced than ACV triphosphate. The low levels of both triphosphates may account for the relative non-toxicity of these compounds to uninfected Vero cells, where 50% inhibition of cell proliferation is evident at 850 μM [2]. It is probably that uninfected cells lack an efficient enzyme to phosphorylate DHPG and ACV to monophosphates, thus the selectivity against HSV infected cells where the nucleoside kinase is provided by the virus. The enzyme converting ACV to ACV monophosphate in uninfected cells has not been identified, although Datta and Pagano [17] provide evidence that one candidate enzyme may be cytosol deoxycytidine kinase. The enzyme(s) which phosphorylates DHPG in uninfected cells has not been investigated.

The persistence of DHPG triphosphate in virus-infected cells after drug removal may contribute significantly to the high potency of DHPG *in vivo*. With the persistence of DHPG triphosphate inside infected cells, virus replication may be inhibited long after blood levels have declined. This effect could also explain the results of Cheng *et al.* [18] who showed that DHPG can be removed at 8 hr from virus infected cells without a subsequent increase in virus replication. Analogous studies with ACV have not appeared in the literature. The decay of ACV nucleotides in HSV-2 infected cells following drug removal is similar to data of Furman and colleagues [15] using HSV-1.

It is interesting that the pattern of phosphorylation of ACV in HSV-1 infected cells presented here has not been mentioned elsewhere. In the report of Furman *et al.* [15], ACV metabolism was followed after doses of 0.5, 5, 100 and 500 μM, which are only partially in the range needed to observe the effect shown in Fig. 1. In the work of Elion and colleagues [4], ACV phosphorylation at 5 μM was the same as that observed at 0.5 μM, which is similar to our data. The explanation for this unusual metabolism may reside at the thymidine kinase level. Keller and coworkers [19] indicated that phosphorylation of ACV by HSV-1 thymidine kinase does not obey classical Michaelis–Menten kinetics. They reported that ACV is a substrate at one concentration and an inhibitor at another. The HSV-1 (F strain) thymidine kinase behaves similarly. We have analyzed the F strain-induced enzyme over a broad range of ACV concentrations and found concentration-dependent phosphorylation at low and moderately high levels of ACV, but at intermediate concentrations the enzyme activity was suppressed (unpublished data). Since the phosphorylating properties of thymidine kinases may vary with the virus strain [18], the pattern of ACV phosphorylation presented here may not be a universal phenomenon.

Enzyme kinetic data indicate that DHPG was phosphorylated much more rapidly than ACV by HSV thymidine kinases. Similarly, cellular guanylate kinase phosphorylated DHPG monophosphate more readily than ACV monophosphate. These differences were considerable at all substrate concentrations, but were particularly marked at low substate levels due to the K_m differences. The three major enzymes involved in the final phosphorylation step showed a small preference for DHPG diphosphate, but it is not likely that these differences are significant physiologically.

The kinetic differences observed with the purified enzymes correlated with differences in the rate of drug triphosphate accumulation which occurred in HSV-1 infected cells. In HSV-2 infected cells, however, the rates of phosphorylation of ACV and DHPG were similar, and do not reflect observed differences in enzyme kinetics. This discrepancy indicates that a simple model of drug phosphorylation, based solely on enzyme kinetic data, is not appropriate in this experimental model. *In vivo*, enzyme kinetic differences may be important to account for the high potency of DHPG. We have shown that DHPG triphosphate persists much longer than ACV triphosphate following drug removal. This could be due to a decreased rate of degradation, an increased rate of synthesis, or both. Maintaining DHPG in a highly phosphorylated state would increase its polarity and tend to inhibit leakage out of the cell. Thus, the persistence of DHPG triphosphate in infected cells may involve more efficient turnover by phosphorylating enzymes and contribute to increased *in vivo* potency.

ACV triphosphate was considerably more potent than DHPG triphosphate in inhibiting viral and cell DNA polymerases, a result recently confirmed by others using the same methodology [8, 9], although at virus-inhibitory doses the amounts of triphosphate of each nucleoside in infected cells were nearly the same. This suggests that DHPG may have an additional mechanism of action which is not reflected in results of DNA polymerase assays. Mutant viruses with altered DNA polymerases have been shown to be resistant to ACV but are still inhibited by DHPG [1, 2, 18]. DHPG may, therefore, interact with DNA polymerase by a mechanism different than ACV or, alternatively, DHPG may have an additional site of action unrelated to DNA polymerase. Frank and colleagues [8] have also shown that, once DHPG incorporates into the DNA, its potency as a terminator of further chain elongation is equivalent to that of ACV-terminated DNA. Thus, inhibition of dGTP interaction with the DNA polymerase and the inhibitory effect of DHPG or ACV following incorporation into the DNA both contribute to the overall inhibition of virus replication which is observed.

The stereospecificity of DHPG triphosphate with respect to viral and cell DNA polymerases is interesting, but probably not significant physiologically. HSV thymidine kinases apparently will phosphorylate DHPG only when the open sugar moiety is oriented like a normal deoxyribofuranose molecule [10]. Thus, only the physiologically active isomer of DHPG triphosphate will be formed *in situ*.

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