ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1995, p. 1802–1808 Vol. 39, No. 8
0066-4804/95/$04.00+0

Mode of Action of (*R*)-9-[4-Hydroxy-2-(Hydroxymethyl)Butyl]Guanine against Herpesviruses

D. M. LOWE,[1]* W. K. ALDERTON,[1] M. R. ELLIS,[1] V. PARMAR,[1] W. H. MILLER,[2] G. B. ROBERTS,[2] J. A. FYFE,[2] R. GAILLARD,[2] P. ERTL,[1] W. SNOWDEN,[1] AND E. LITTLER[1]

Wellcome Research Laboratories, Beckenham, Kent, United Kingdom,[1] and Burroughs Wellcome Company, Research Triangle Park, North Carolina[2]

Received 27 February 1995/Returned for modification 19 April 1995/Accepted 2 June 1995

The activity, metabolism, and mode of action of (*R*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (H2G) against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) and varicella-zoster virus (VZV) were studied. Compared to acyclovir (ACV), H2G has superior activity against VZV (50% inhibitory concentration of 2.3 μM) and Epstein-Barr virus (50% inhibitory concentration of 0.9 μM), comparable activity against HSV-1, and weaker activity against HSV-2. The antiviral effect on HSV-1 showed persistence after removal of compound. H2G was metabolized to its mono-, di- and triphosphate derivatives in virus-infected cells, with H2G-triphosphate being the predominant product. Only small amounts of H2G-triphosphate were detected in uninfected cells (1 to 10 pmol/10^6 cells), whereas the level in HSV-1-infected cells reached 1,900 pmol/10^6 cells. H2G was a substrate for all three viral thymidine kinases and could also be phosphorylated by mitochondrial deoxyguanosine kinase. The intracellular half-life of H2G-triphosphate varied in uninfected (2.5 h) and infected (HSV-1, 14 h; VZV, 3.7 h) cells but was always longer than the half-life of ACV-triphosphate (1 to 2 h). H2G-triphosphate inhibited HSV-1, HSV-2, and VZV DNA polymerases competitively with dGTP (K_i of 2.8, 2.2, and 0.3 μM, respectively) but could not replace dGTP as a substrate in a polymerase assay. H2G was not an obligate chain terminator but would only support limited DNA chain extension. Only very small amounts of radioactivity, which were too low to be identified by high-performance liquid chromatography analysis of the digested DNA, could be detected in purified DNA from uninfected cells incubated with [^{3}H]H2G. Thus, H2G acts as an anti-herpesvirus agent, particularly potent against VZV, by formation of high concentrations of relatively stable H2G-triphosphate, which is a potent inhibitor of the viral DNA polymerases.

(*R*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (H2G) is an acyclic guanosine analog with structural similarities to acyclovir (ACV){9-[(2-hydroxyethoxy)methyl]guanine}, the established treatment for herpesvirus infections (6), and penciclovir (PCV) [9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine], a compound recently licensed in some countries for the treatment of herpes zoster (29). The racemic mixture of (*RS*)H2G [previously abbreviated (±)2HM-HBG (1, 3, 17)] was originally shown to have good activity against herpes simplex virus (HSV) (18) and varicella-zoster virus (VZV) in vitro (3) and in vivo (17), and subsequent studies with the separated isomers showed that this activity resided predominantly with the *R* isomer (1, 25), now called H2G (25, 27). H2G has activity against HSV type 1 (HSV-1) and type 2 (HSV-2) (1) and has been reported to have activity against human herpesvirus type 6 (4).

Such a broad-spectrum anti-herpesvirus activity makes H2G an interesting candidate for clinical development, particularly for the treatment of VZV infection, where its impressive activity could lead to improved efficacy over current therapies.

Strains of VZV that are deficient in thymidine kinase (TK) are resistant to H2G (1, 3), suggesting a mechanism of activation involving selective phosphorylation by herpesvirus-induced TKs, as is the case for ACV and PCV. This is supported by in vitro data on the phosphorylation of (*RS*)H2G by the VZV TK (3). Further anabolism to the triphosphate derivative of H2G (H2G-TP) is thought to occur by the action of cellular enzymes, and (*RS*)H2G-TP has been shown to be a competitive inhibitor of VZV DNA polymerase (2).

H2G exhibits a persistent anti-VZV effect in tissue culture after removal of compound (1, 3) similar to that seen with PCV but not shown by ACV (12).

In this paper we extend the published data on the in vitro antiviral activity of H2G and report studies on the metabolism of H2G in HSV-1-, HSV-2-, and VZV-infected cells and in two uninfected cell lines. The kinetics of phosphorylation of H2G by the three herpesvirus TKs and inhibition of the viral DNA polymerases by H2G-TP are also presented. This information provides us with a biochemical rationale for the profile of antiviral activity displayed by H2G.

MATERIALS AND METHODS

Radiochemicals. [8-^{3}H]H2G (10.6 Ci/mmol) was prepared by direct tritiation of unlabeled H2G by Moravek Biochemicals (Brea, Calif.) and had a radiochemical purity of 99.9% by high-performance liquid chromatography (HPLC) analysis. [8-^{3}H]ACV (18 Ci/mmol), [8-^{3}H]PCV (20 Ci/mmol), [5-methyl-^{3}H]thymidine (dThd) (53 Ci/mmol), [8-^{3}H]2′-deoxyguanosine (dG) (4.2 Ci/mmol) and [8-^{14}C]2′-dG (56 mCi/mmol) were also obtained from Moravek Biochemicals. Radiochemical purities were >98% by HPLC analysis. [^{3}H]Thymidine was also obtained from Amersham International Plc. (Little Chalfont, United Kingdom). [8-^{3}H]dGTP, [methyl-^{3}H]TTP, and [γ-^{32}P]ATP were obtained from DuPont NEN (Stevenage, United Kingdom, and Boston, Mass.).

Preparation of H2G-TP. H2G-TP was synthesized enzymatically from H2G as follows. The reaction mixture (160 ml) contained 67 mg of H2G (0.2 mmol), 10 mM ATP, 20 mM $MgCl_2$, 25 mM K-(*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (HEPES) (pH 8), 0.64 U of VZV TK, 10 mM phosphoenolpyruvate, 9.6 U of GMP kinase (Sigma), 800 U of pyruvate kinase, and 800 U of NDP kinase (Boehringer Mannheim) and was incubated at 37°C for 6 days. H2G-TP was purified by chromatography on DEAE Sephadex A-25, using an ammonium bicarbonate gradient. Column resolution was not sufficient to separate ATP and H2G-TP, so the fractions were combined and incubated for 20 min at 37°C with 15 mM $MgCl_2$–20 mM glucose–50 mM ammonium bicarbonate (pH 8)–3 U of

* Corresponding author. Mailing address: Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, United Kingdom. Phone: 0181-639 5517. Fax: 0181-663 3645.

yeast hexokinase (Boehringer Mannheim) per ml to convert the ATP to ADP. The H2G-TP was repurified by DEAE Sephadex chromatography as above. The final H2G-TP preparation contained 0.6% ADP and 2.1% ATP, as determined by strong anion exchange (SAX) HPLC (see below). The nuclear magnetic resonance (NMR) spectra indicated that the product had the triphosphate group on the end of the butyl chain, i.e., in the "pseudo" 5′ position: ^{31}P NMR, (D_2O, 20 mM EDTA) $\delta = -10$ (d, αP), −21 (t, βP), −7.0 (d, γP); ^{1}H NMR, (D_2O) $\delta = 7.6$ (1H, H8), 3.9 (2H, -CH_2-O-P), 3.8 (2H, N-CH_2-C), 3.2 (2H, -CH_2-OH), 2.0 (1H, CH), 1.4 (2H, -CH_2-); UV spectra: 0.1 M HCl, $\lambda_{max} = 254$ nm, shoulder at 278 nm, $\lambda_{min} = 227$ nm; pH 7, $\lambda_{max} = 253$ nm, $\lambda_{min} = 226$ nm, shoulder at 274 nm; 0.1 M NaOH, $\lambda_{max} = 267$ nm, $\lambda_{min} = 231$ nm.

Cells and viruses. MRC-5 cells (human embryo lung fibroblasts, ATCC CCL 171) were grown in the Glasgow modification of Eagle's minimal essential medium (Gibco) containing 10% (vol/vol) fetal calf serum (FCS) and antibiotics. Vero cells (ATCC CCL81) were grown in the same medium except no additional nonessential amino acids were supplied and 10% newborn calf serum was used in place of FCS. HepG2 cells (human liver carcinoma) were grown in Dulbecco's modification of Eagle's medium (Gibco) containing 10% (vol/vol) FCS and antibiotics.

Virus strains HSV-1 (SC16), HSV-2 (186), human cytomegalovirus (HCMV) (AD169), VZV (G31), VZV (Ellen) and clinical isolates have all been referenced previously (12), except for the panel of clinical isolates of VZV which were obtained from H. H. Balfour (University of Minnesota Health Sciences Center).

In vitro antiviral assays. Plaque reduction assays with HSV, VZV, and HCMV were performed in 24-well plates by a modification of the method described by Crumpacker et al. (9) to determine the 50% inhibitory concentration (IC_{50}), and yield reduction assays were as described previously (12). Epstein-Barr virus (EBV) was assayed by an enzyme-linked immunosorbent assay procedure with the human B-cell line P3HR-1 (12). The effect of exposure time of HSV-1-infected cells to compound on inhibition of HSV-1 production was studied by using 100 μM compound as described previously (12).

In vitro cellular cytotoxicity assays. In vitro cellular cytotoxicity assays were performed with MRC-5 and Vero cells with a tetrazolium salt (MTT) metabolic assay (10) under the same cell culture assay conditions used for HSV replication, and a 50% cytotoxic concentration (CC_{50}) was determined. Stem cell cytotoxicity was determined from reduction in colony numbers of human multipotential myeloid progenitor cells (28).

Formation of intracellular metabolites. MRC-5 cells were grown in 60-mm tissue culture plates, to about 10^6 cells per plate, and were either mock infected or infected with HSV-1 or HSV-2 at a multiplicity of infection of about 5 PFU per cell which was allowed to adsorb for 1 h. For VZV infections, uninfected and VZV-infected (G31) MRC-5 cells, exhibiting maximum cytopathic effect, were mixed at a ratio of 1.2:0.7 and allowed to adhere for 3 h. Uninfected HepG2 cells were also grown in T-75 tissue culture flasks.

At the start of the experiment, the medium was replaced with fresh medium (4 ml) containing 3% FCS and 5 μM [^{3}H]H2G, [^{3}H]ACV, or [^{3}H]PCV (2 Ci/mmol) and the plates were incubated at 37°C. Additional duplicate plates, containing no compound, were used for cell counts. At each time point, the medium was removed and the cell monolayers were washed twice with ice-cold phosphate-buffered saline (PBS). Duplicate flasks were then analyzed for intracellular metabolites.

The stability of intracellularly formed phosphate esters was studied by incubating infected or uninfected cells for 24 h (20 h for VZV-infected cells) with 5 μM ^{3}H-labeled compound, then washing the cells twice with PBS, and continuing the incubation in the presence of fresh, unlabeled media (10 ml). Further changes of media (10 ml) were carried out at hourly intervals, to minimize reanabolism of diffusable acyclic nucleosides. Duplicate flasks were analyzed for intracellular phosphates as described below.

Preparation of cell extracts and HPLC analysis. Intracellular metabolites were extracted by bathing the cells in two aliquots of ice-cold, 80% (vol/vol) acetonitrile (2.5 ml) for 5 min on ice. The aliquots were combined, cell debris was removed by centrifugation, and the acetonitrile extracts were then lyophilized to dryness and stored at −70°C until analyzed. The extracts were resuspended in HPLC-grade water and analyzed by gradient HPLC using a Whatman Partisphere-5 SAX column (250 by 4.6 mm) at 1 ml/min. Elution was achieved with a two-stage linear gradient from 100% buffer A (5 mM KH_2PO_4 [pH 5]) to 50% buffer A:50% buffer B (750 mM KH_2PO_4 [pH 5]) over 10 min, continuing to 100% buffer B over a further 5 min followed by a 10-min wash at 100% buffer B.

The chromatographic eluent was analyzed for radioactivity either by collecting 1-ml fractions which were quantified by liquid scintillation counting or by continuous on-line radiochemical detection using a Berthold LB 506 C-1 monitor and mixing with Zinsser Quickszint Flow 306 scintillation fluid. The results are expressed as picomoles of nucleotide per 10^6 cells.

Nucleoside triphosphate (NTP) peaks were identified by comparison of retention times with those of authentic H2G-TP, ACV-TP, and PCV-TP. The identities of the respective mono- and diphosphate esters were confirmed by enzymatic digestion with phosphodiesterase I from *Crotalus adamanteus* Venom (Pharmacia) which cleaves internal phosphodiester bonds, converting di- and triphosphate esters to the corresponding monophosphates, resulting in peak shifting on SAX HPLC (20). Samples were also treated with calf intestinal alkaline phosphatase (Pharmacia), which reduces all the nucleotides to the corresponding nucleosides.

Nucleosides were analyzed by reverse-phase HPLC using a Whatman Partisphere C_{18} column (250 by 4.6 mm) at 1 ml/min. Elution was achieved with a two-stage linear gradient from 100% buffer A (25 mM NH_4 acetate [pH 4.25], 0.1% triethylamine) to 65% buffer A:35% buffer B (50 mM NH_4 phosphate [pH 7.2], 0.1% triethylamine, 35% acetonitrile) over 15 min, continuing to 100% buffer B over a further 10 min followed by a 10-min wash at 100% buffer B.

Incorporation of [^{3}H]-H2G, -ACV and -PCV into cellular DNA. Uninfected MRC-5 and HepG2 cells were incubated with either 5 μM (2 Ci/mmol) or 50 μM (0.2 Ci/mmol) labeled compound. At various time points, four flasks of cells were harvested with EDTA-trypsin, combined, and washed twice with PBS. Genomic DNA was isolated from the cells by using a Cell Culture DNA Isolation kit, supplied by Qiagen Inc. (Chatsworth, Calif.). Washed nuclei were lysed with the buffer supplied and proteinase K, with RNase A added as an extra modification. The released DNA was purified by using the Qiagen fractionation columns, precipitated with isopropanol, washed with 70% ethanol, redissolved in water, and quantified by measuring the A_{260}.

Viral TKs. The plasmid pTK8703, containing the HSV-2 TK gene, was a gift of W. Dallas (Burroughs Wellcome, Research Triangle Park, N.C.) and was expressed in a TK$^-$ strain of *Escherichia coli* (TX63). The plasmid pRG05, containing the VZV TK gene, was expressed in *E. coli* BL21(DE3), as previously described (22). The recombinant baculovirus, vVL106-TK1, encoding HSV-1 TK, was a gift from N. Sharpe (Wellcome Research Laboratories, Beckenham, United Kingdom). Both vVL106-TK1 and the recombinant baculovirus, vP36EBVTK, encoding EBV TK (unpublished), were expressed in *Spodoptera frugiperda* (SF-9) cells by standard methods (19). Viral TKs were purified to greater than 95% purity by ion-exchange (MonoQ) chromatography followed by affinity chromatography on a thymidine-agarose column (22).

Apparent K_i (K_i') values for ACV, PCV, and H2G with the viral TKs were determined by competitive inhibition of ^{3}H-thymidine phosphorylation, using the DEAE paper assay (23). K_i' is equal to the apparent K_m (K_m') for a competitive, alternate substrate (24, 26) and these compounds are substrates for HSV-1, HSV-2, and VZV TKs. Therefore, data are presented as K_m' values.

Relative substrate velocities with the VZV TK were determined by the phosphate transfer assay at 1 mM test compound and 1 mM Mg [γ-^{32}P]ATP (150 Ci/mol) (5), and relative velocities with the HSV-1 and HSV-2 TKs were determined by measuring phosphorylation of ^{3}H-labeled dG, dThd or acyclic nucleoside analog by the DEAE paper method (23).

Mitochondrial deoxyguanosine kinase (m-dGK). Enzyme was purified from homogenized human brain tissue (Cooperative Human Tissue Network, Birmingham, Ala.) by ammonium sulfate precipitation, anion-exchange chromatography and ATP-agarose affinity chromatography (21a).

Phosphorylation reactions, catalyzed by m-dGK, were conducted at 37°C and contained 50 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 10 mM MgATP, 0.5% Triton X-100, 25 μM [^{14}C]dG or [^{3}H]-labeled acyclic guanine nucleoside analog, and purified m-dGK. Labeled substrate and product were separated by polyethyleneimine thin-layer chromatography, cut, and quantitated by liquid scintillation analysis.

DNA polymerases. HSV-1, HSV-2, and VZV DNA polymerases were prepared and assayed as described previously (12). DNA polymerase α was purified from H9 cells (human lymphocyte) as described by Parker et al. (21). For the study of H2G-TP as an alternative substrate for DNA polymerases, ^{3}H-TTP was used instead of ^{3}H-dGTP.

DNA chain extension assay. The DNA chain extension assay used the following defined sequence template primer (d60:d23mer), which has G as the first residue to be added to the extending primer: 3′-GCGCATATATCGACCGTA GCATGCTTTGAAAAATTCATATCCAACCCACGTCCAAGTAAA-5′ and 5′-CGCGTATATAGCTGGCATCGTAC-3′. The primer was labeled at the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP. Reaction mixtures (10 μl) contained 50 mM Tris-HCl (pH 8), 200 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 μM d60:[5′-^{32}P]d23mer, 5 μM (each) dATP, dCTP, and dTTP, and/or either dGTP or inhibitor at 10× K_m or K_i with this substrate. Reactions were started by the addition of VZV DNA polymerase and incubated at 37°C for 30 min before stopping by addition of formamide-bromophenol blue. Samples were electrophoresed on a 15% polyacrylamide-urea gel in Tris-borate buffer, and the dried gel was autoradiographed.

RESULTS

In vitro activity against herpesviruses. The antiviral activity of H2G was assessed in vitro using a panel of clinical isolates of HSV-1, HSV-2, and VZV in a standard plaque reduction assay in MRC-5 cells. In agreement with previously published data (1), the IC_{50} for H2G against HSV-1 (0.62 ± 0.3 μM) was similar to that seen for ACV (12), whereas the IC_{50} against HSV-2 (5.8 ± 1.2 μM) was significantly higher than that seen for ACV and more like the values determined for PCV (12). Against 20 clinical isolates of VZV, the average IC_{50} for H2G was 2.3 ± 0.8 μM, compared to 46.8 μM for ACV and 78.7 μM

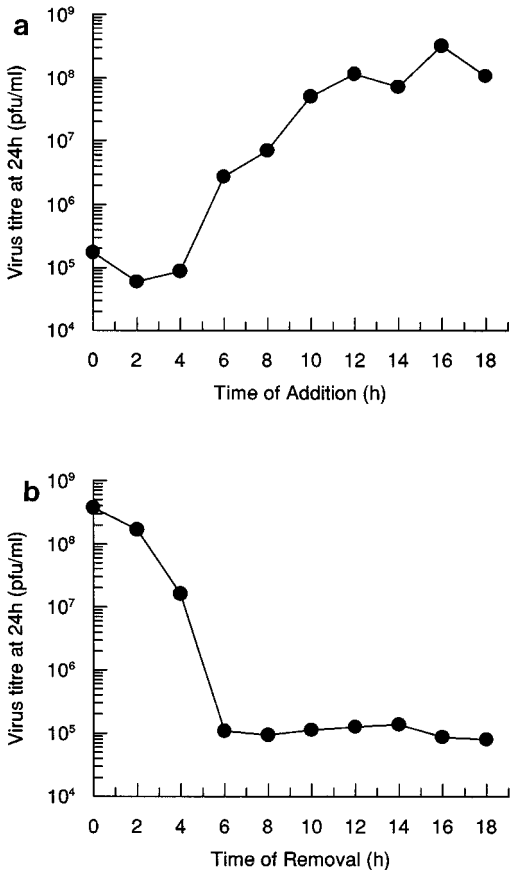

FIG. 1. Effect of time of exposure to H2G on the virus yield at 24 h postinfection with HSV-1. MRC-5 cells were infected at 5 PFU per cell for 1 h, after which time the inoculum was removed and replaced with medium or medium containing 100 μM H2G. (a) Compound was added to the medium at the times shown. (b) Compound was removed at the times shown by thorough washing of the monolayers and replacement with fresh medium. The monolayers were harvested at 24 h postinfection, and the virus yields were determined by titration in Vero cells.

for PCV, showing it to have far superior activity against this virus. Furthermore, in virus yield reduction experiments, H2G had reproducibly superior activity, with IC_{50}s of 0.57 and 0.72 μM for VZV Ellen and 0.16 and 0.54 μM for VZV G31, set up at ratios of infection of 1:10 and 1:1 (infected:uninfected MRC-5 cells), respectively.

H2G had no significant activity against HCMV up to 200 μM, but showed good activity against EBV, with an IC_{50} of 0.9 μM.

In cellular cytotoxicity studies, H2G gave a CC_{50} of >500 μM in MRC-5 cells, >2,000 μM in Vero cells, and >50 μM in human hemopoietic stem cells, results comparable to those obtained with ACV (data not presented).

To produce full antiviral effect, H2G had to be added to HSV-1-infected cells within the first 4 to 5 h of infection (Fig. 1a). Time of removal experiments, however, showed that exposure of the infected cells to H2G for the first 6 h of infection was sufficient to produce a full antiviral effect (Fig. 1b).

Metabolism in infected and uninfected cells. The anabolism of H2G to its mono-, di- and triphosphate derivatives was studied in cultured MRC-5 cells by using [^{3}H]H2G. The radioactivity elution profile from a SAX column, obtained with an extract from HSV-1-infected cells, gave retention times for H2G and its mono-, di- and triphosphates of about 4.5, 12, 19, and 25 min, respectively. A minor metabolite, with retention time of 17 min, was also detected.

In HSV-1-, HSV-2-, and VZV-infected MRC-5 cells, high levels of the metabolites accumulated with time, reaching a maximum concentration at about 24 h after virus infection (Fig. 2). The proportions of mono-, di-, and triphosphate derivatives of H2G after 24 h were approximately 1:8:46 for HSV-1, 1:7:21 for HSV-2, and 1:4:23 for VZV. After this time the concentration began to drop with the onset of cell death caused by the viral infection (Fig. 2). The most abundant metabolite was H2G-TP, which reached a level of about 1,900 pmol/10^6 cells in HSV-1-infected cells but only half this value in HSV-2-infected cells (Table 1). The level of H2G-TP in HSV-1-infected cells was comparable to PCV-TP levels and considerably greater than ACV-TP levels, whereas in VZV-infected cells it was about twice the concentration of PCV-TP and 165× the concentration of ACV-TP (Table 1).

At least one minor metabolite of H2G was detected in virus-infected cells, eluting just ahead of H2G-diphosphate on HPLC and present at about 1% the level of H2G-diphosphate. This minor metabolite of H2G was isolated by SAX HPLC. It appears to be a phosphorylated derivative of H2G, since it could be digested to H2G-monophosphate by phosphodiesterase I, which could then be further digested by alkaline phosphatase to yield H2G (identified by retention time on reverse-phase HPLC). The original peak, eluting at 17 min, however, was resistant to alkaline phosphatase digestion, suggesting that this metabolite is a derivative of H2G-diphosphate which does not have a free terminal phosphate. The exact identity of this metabolite remains to be established; however, it is not the 3′,5′-cyclic monophosphate derivative of H2G, which is resistant to phosphodiesterase I (8).

To investigate the selectivity of phosphorylation in virus-infected cells, we also analyzed two uninfected cell lines for the presence of anabolites of H2G. In control uninfected MRC-5 cells, only small amounts of H2G mono-, di-, and triphosphates were detected and the minor metabolite, described above, was below the level of detection. H2G-TP was again the most abundant metabolite, at about 1 pmol/10^6 cells, and the ratio of mono-, di-, and triphosphate esters at 24 h was 1:3.5:6. The level of H2G-TP in uninfected MRC-5 cells was higher than that for ACV-TP and PCV-TP (Table 1). Higher concentrations of both H2G-TP and PCV-TP were detected in uninfected HepG2 cells, whereas the very low levels of ACV-TP were similar in both cell lines (Table 1).

Phosphorylation of acyclic guanosine analogs by viral TKs. The K_m' and relative V_{max} values for ACV, PCV, and H2G were determined with purified viral TKs and compared with the values for dThd and dG (Table 2). The kinetics with the HSV-1 TK showed deviation from Michaelis-Menten kinetics which was most pronounced with ACV; these effects have been described previously (15). The K_m' values for H2G and PCV with HSV-1 TK were significantly lower than the values for both dG and ACV. H2G also has a significantly higher phosphorylation rate by the HSV-1 TK than ACV and PCV (Table 2). With the HSV-2 TK, however, K_m' values for ACV, PCV, and H2G were quite similar and were all greater than that for dG, as were the rates of phosphorylation. K_m' values for dG and the acyclic nucleosides were all high with the VZV TK, but of these, H2G was the lowest, with ACV being a particularly poor substrate. The relative rate of H2G phosphorylation by the VZV TK was, however, substantially greater than for dThd, dG, ACV, and PCV (Table 2).

Viral TKs also have a thymidylate kinase activity (7, 14). However, HPLC analysis of the phosphorylation products of H2G, PCV, and ACV with the HSV-1, HSV-2, and VZV TKs

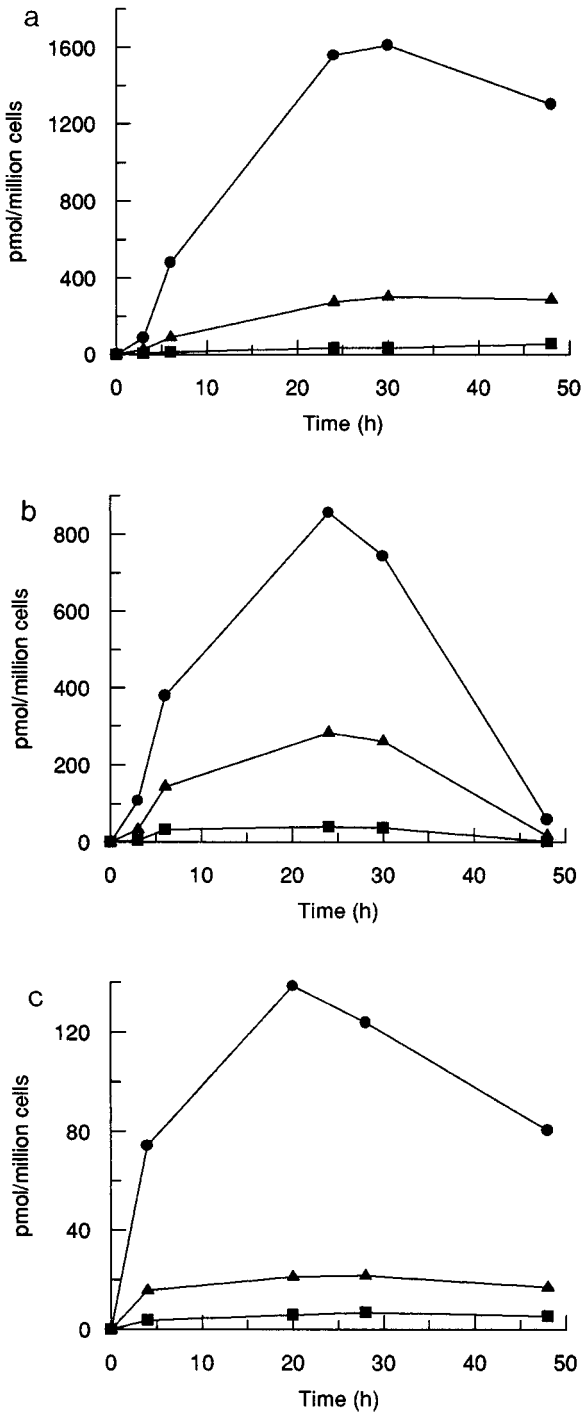

FIG. 2. Formation of phosphate derivatives of H2G in HSV-1-, HSV-2-, and VZV-infected MRC-5 cells. Cell monolayers were infected at 5 PFU/cell with HSV-1 (a) or HSV-2 (b), or VZV-infected cells were mixed with uninfected cells in a ratio of 0.7:1.2 (c). The inoculum medium was removed after 2 h, and fresh medium containing 5 μM [^{3}H]H2G (2 Ci/mmol) was added. At the times indicated, cells were extracted and assayed by SAX HPLC as described in the text. ●, H2G-TP; ▲, H2G-diphosphate; ■, H2G-monophosphate.

showed that in all cases only the monophosphate was formed (data not shown).

Finally, partially purified EBV TK from a baculovirus extract catalyzed H2G phosphorylation to the monophosphate while the control baculovirus extract did not (data not shown).

TABLE 1. Quantitation of intracellular triphosphates of H2G, ACV, and PCV formed in uninfected and infected cells after 24 h of incubation with 5 μM compound

Cell type	Triphosphates formed (pmol/10^6 cells)[a]		
	H2G-TP	ACV-TP	PCV-TP
Uninfected HepG2	10	0.005	0.67
Uninfected MRC-5	1.0	0.007	0.17
HSV-1-infected MRC-5	1,900	10	2,500
HSV-2-infected MRC-5	860	ND[b]	ND
VZV-infected MRC-5[c]	140	0.84	71

[a] Average values for duplicate samples.
[b] ND, not determined.
[c] Values determined after 20 h of incubation.

Phosphorylation of acyclic guanosine analogs by m-dGK. Previous studies have shown (*RS*)H2G to be a poor substrate for cellular cytosolic (18) and mitochondrial (3) TKs. Therefore, to explain the levels of anabolites detected in uninfected cells (Table 1), we tested H2G, ACV, and PCV with purified m-dGK from human brain. At pH 8 (physiological pH of respiring mitochondria) and 25 μM substrate, H2G and PCV were phosphorylated at 31 and 6.7%, respectively, of the rate of dG whereas, in agreement with previous reports (31), ACV was not phosphorylated.

Intracellular stability of H2G-TP. The intracellular half-life of H2G-TP was determined in HSV-1- and VZV-infected MRC-5 cells, following the removal of extracellular compound, and in uninfected HepG2 cells, where the initial triphosphate level was sufficiently high to allow a determination. In these experiments, the cells were incubated with 5 μM [^{3}H]H2G for 24 h, or 20 h in the case of VZV infection, such that the starting concentrations of triphosphate were as reported in Table 1. Comparative experiments were performed with ACV and PCV. The extracellular compound was removed by repeated changes of media throughout the remainder of the time course, such that the concentration of extracellular compound never exceeded ~5 nM. This minimized the possibility of reanabolism of the resulting diffusable acyclonucleoside, although it was not possible to control for recycling of anabolites intracellularly.

The measured half-life of H2G-TP showed considerable variation in the presence or absence of the different viruses, as has previously been shown for PCV (29). In HSV-1-infected cells, where the starting concentration of triphosphate was highest (Table 1), a half-life of 14 ± 2 h was determined for H2G-TP, whereas in VZV-infected cells, the half-life was

TABLE 2. Phosphorylation of nucleosides and acyclic guanosine analogs by viral TKs

Substrate	HSV-1 TK		HSV-2 TK		VZV TK	
	rel. V_{15} (%)[a]	K_m' (μM)	rel. V_{max} (%)	K_m' (μM)	rel. V_{max} (%)	K_m' (μM)
dThd	100	0.5	100	0.5	100	0.2
dG	0.1	20	3	25	24	530
ACV	1.2	60[b]	22	135	37	830
PCV	0.8	4.5	226	103	240	250
H2G	30	4.9	70	105	1,200	120

[a] rel., relative. For HSV-1 TK, relative velocities at 15 μM substrate (rel. V_{15}) are reported because phosphorylation kinetics deviated from Michaelis-Menten kinetics and relative V_{max} values could not be calculated.
[b] Approximate value because of significant deviation from Michaelis-Menten kinetics.

TABLE 3. Inhibition of DNA polymerases by H2G-TP

DNA polymerase	K_m (dGTP) (μM)[a]	K_i (H2G-TP) (μM)[a]
HSV-1	0.3	2.8
HSV-2	0.2	2.2
VZV	0.45	0.3

[a] All values ± 10%.

about 4 ± 1 h, with similar results determined for PCV-TP. The half-life for ACV-TP was 1 to 2 h in these experiments, in agreement with previous estimates (13, 30). The result in uninfected cells, where the influence of virus-specific anabolism is removed, was a half-life of 2.5 ± 0.1 h for H2G-TP compared to 11 ± 2 h for PCV-TP, which suggests that the catabolism rate of H2G-TP is actually significantly higher than that of PCV-TP.

Inhibition of viral DNA polymerases. (*RS*)H2G-TP has previously been shown to be a competitive inhibitor of VZV DNA polymerase with respect to the natural substrate, dGTP, and a very poor inhibitor of human DNA polymerase α (2). Table 3 shows the kinetic data for H2G-TP with all three herpes DNA polymerases. In all cases, H2G-TP inhibited competitively with respect to dGTP, and with the VZV DNA polymerase, the K_i for H2G-TP was slightly lower than the K_m for dGTP.

H2G-TP as an alternative substrate for viral or cellular DNA polymerases. Since H2G has a pseudo 3′ hydroxyl which might allow chain extension, we examined the ability of H2G-TP to support DNA synthesis. Figure 3 shows the polymerase activity of VZV polymerase (Fig. 3a) and DNA polymerase α (Fig. 3b) in the presence of all four nucleotides and the residual rates that were observed when dGTP was omitted. Addition of 10 μM H2G-TP in place of dGTP resulted in a decrease in this residual rate, which was more pronounced for VZV polymerase than for polymerase α. Thus, H2G-TP is behaving as an inhibitor of DNA polymerization and does not substitute for dGTP in these VZV polymerase or polymerase α reactions.

DNA chain extension assay. By using VZV DNA polymerase and a defined sequence template primer, processive DNA synthesis was observed in the presence of all four natural dNTPs (Fig. 4). When dGTP was omitted, a very small amount of misincorporation was observed and adding dGTP alone allowed incorporation at position 1, where the sequence coded for a G (see Materials and Methods). Inclusion of ACV-TP resulted in chain termination at position 1, even in the presence of the other three nucleotides, whereas H2G-TP showed incorporation at position 1 which was extendible in the presence of the other three dNTPs (Fig. 4). Particularly strong stops were observed after polymerization of one additional nucleotide beyond a H2G-monophosphate. This would suggest that H2G is not an obligate chain terminator like ACV, but rather it inhibits processive DNA synthesis by short chain termination.

Incorporation of H2G into cellular DNA. The detection of H2G-TP in uninfected cells, particularly in HepG2 cells (Table 1), and the proposed mechanism of inhibition (Fig. 4) prompted a study of incorporation into cellular DNA of cultured cells. Both uninfected MRC-5 and HepG2 cells were incubated with either 5 or 50 μM [^{3}H]H2G for periods from 7 h to 7 days. Cellular DNA was subsequently purified and analyzed for incorporated radiolabel by liquid scintillation counting. These studies were done in comparison with [^{3}H]ACV and [^{3}H]PCV, and in all cases the amount of radioactivity associated with the DNA was very low. Thus, the results at 5 μM gave 11.0 ± 3.6, 6.6, and 5.1 dpm/μg of DNA for H2G, ACV, and PCV, respectively, and at 50 μM gave 5.4 ± 0.1, 23.2, and 13.9 dpm/μg of DNA for H2G, ACV, and PCV, respectively. Subsequent digestion of the DNA to nucleosides, followed by HPLC analysis, could not confirm incorporation of the acyclic nucleosides into the DNA because of the low level of radioactivity present (results not shown).

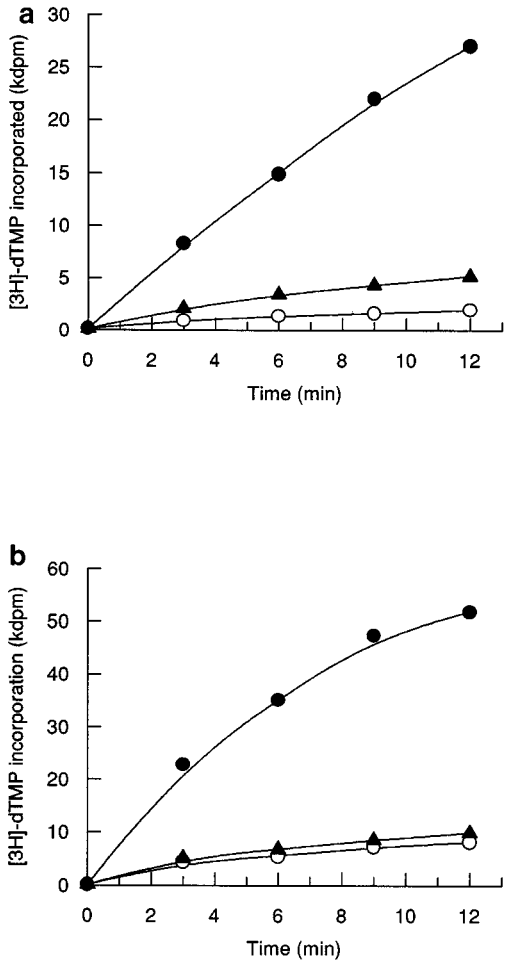

FIG. 3. VZV DNA polymerase and cellular DNA polymerase α activity with H2G-TP in place of dGTP. VZV DNA polymerase (a) and cellular DNA polymerase α (b) assays were performed as described previously (12, 21) except that dNTP concentrations were 5 μM dATP, dCTP, and [^{3}H]-dTTP and either 5 μM dGTP (●), no dGTP (▲), or 10 μM H2G-TP (○).

DISCUSSION

In the present study, we have extended the in vitro data on the *R* form of H2G to include a panel of clinical isolates of HSV-1, HSV-2, and VZV. Our data confirm the superior potency of H2G over ACV and PCV against clinical isolates of VZV while showing it to be equally potent against HSV-1 but somewhat less potent than ACV against HSV-2 and HCMV. This profile of activity against HSV-2 and HCMV is similar to that seen for PCV (12). The superior activity of H2G against VZV was also shown in virus yield reduction experiments, in which the potency of H2G was possibly less affected by the multiplicity of the infection than was the potency of ACV or PCV (12). H2G was not cytotoxic in any of the cell lines used

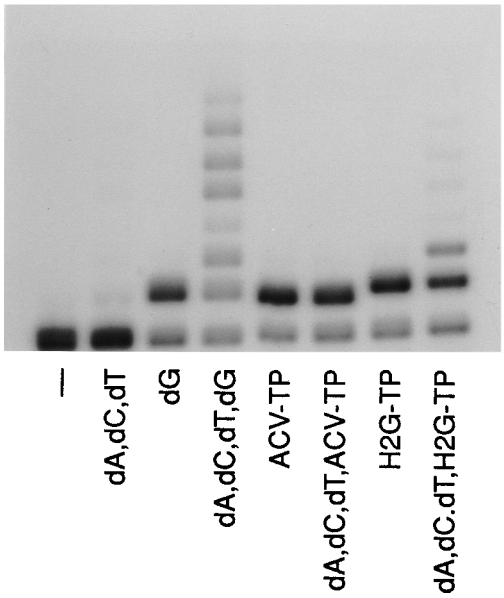

FIG. 4. DNA chain extension by VZV DNA polymerase. All lanes contained the template:primer, d60:[5′-^{32}P]d23mer. Reactions were performed as described in the text and contained 5 μM dATP, dCTP, and dTTP and/or 1 μM dGTP, 16 μM ACV-TP, or 50 μM H2G-TP, as indicated in the figure.

for this work, and the CC_{50}s determined gave a large in vitro therapeutic ratio for the compound.

The mode of action of H2G appears to be selective inhibition of viral DNA synthesis by the triphosphate metabolite. Time-of-addition studies confirm that H2G must be added to HSV-1-infected cells before the time of DNA replication at 4 to 5 h postinfection in order to achieve a full antiviral effect.

The prolonged length of action of H2G, previously observed in VZV-infected cells (1, 3), was also observed in time-of-removal experiments with HSV-1-infected cells. It has been proposed that this persistent antiviral effect could be due to formation of a large intracellular pool of relatively stable H2G-TP (1). Our measurements of the intracellular anabolites of H2G confirm that high levels of H2G-TP are formed in HSV-1-, HSV-2-, and VZV-infected cells. These triphosphate levels are similar to those seen with PCV, which also shows persistent antiviral effects (12). H2G-TP and PCV-TP levels were orders of magnitude higher than recorded for ACV-TP.

A study of the intracellular half-life of H2G-TP gave varied results depending on whether cells were infected and by which virus. This variability probably reflects the combination of triphosphate catabolism and intracellular reanabolism. In virally infected cells, the presence of viral TK would significantly increase the degree of reanabolism. The observed half-life for H2G-TP was consistently greater than that of ACV-TP but less than that reported for PCV-TP (29).

We measured the apparent K_m values for H2G with the various viral TKs and found that they correlated with the levels of triphosphates found in virally infected cells. Despite having a thymidylate kinase activity, these TKs did not catalyze any H2G-diphosphate formation, so the monophosphate must be phosphorylated further by cellular enzymes to produce the diphosphate and subsequently the triphosphate. The observation that H2G is a substrate for the EBV TK is consistent with the anti-EBV activity seen in tissue culture and may be of future clinical interest.

Since (*RS*)H2G was not detectably phosphorylated by cytosolic or mitochondrial TKs (3, 18), the presence of significant amounts of H2G-TP in uninfected cells needed further investigation. We have observed H2G- and PCV-phosphorylating activity in an m-dGK preparation. The relative phosphorylation rates of H2G and PCV correlate with triphosphate levels in uninfected cells. Therefore, m-dGK activity may contribute to H2G and PCV anabolism in these cells.

The final factor determining the antiviral activity of H2G is the potency of the triphosphate as an inhibitor of the viral DNA polymerases. We found H2G-TP to be a potent, competitive inhibitor of all three viral polymerases, particularly against the VZV polymerase which actually had a higher affinity for H2G-TP than for the competing physiological substrate, dGTP. H2G-TP is a stronger inhibitor than PCV-TP against these viral polymerases, but it is not as potent as ACV-TP (12).

H2G-TP did not act as an alternative substrate in a cellular or VZV DNA polymerase assay, indicating that, if it does become incorporated, further extension beyond an H2G-monophosphate molecule is very much inhibited. This was confirmed in a DNA chain extension assay which showed incorporation of H2G-monophosphate followed by only limited chain extension, with particularly strong stops immediately after polymerization of one additional nucleotide after a H2G-monophosphate. This property has been described previously for other nucleoside analogs (16) and is similar to that previously reported for PCV-TP (11).

Only very small amounts of radioactivity were found in cellular DNA after incubation of cells with [^{3}H]H2G, which were no greater than levels detected after similar incubations with [^{3}H]ACV, an obligate chain terminator, or PCV.

All these factors together contribute to the profile of antiviral activity and low cellular toxicity seen with H2G. The combination of a high level of H2G-TP and potent inhibition of the viral DNA polymerase results in effective inhibition of both HSV-1 and VZV replication with the possible therapeutic advantage of a prolonged length of action. The lower level of anabolites seen in HSV-2-infected cells, compared with that in HSV-1-infected cells, despite the similar sensitivity of the two DNA polymerases, probably explains its poorer activity against this virus.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of A. Walters, A. Emmerson, C. Roberts, and P. Topley and Ann Davis and Ron Crouch for their NMR work. We thank G. Darby, D. Purifoy, and J. Reardon for supporting this work. We also thank K. Biron for supplying us with the VZV clinical isolates. We acknowledge the continued cooperation of Medivir AB, which did the initial characterization of H2G prior to license to The Wellcome Foundation Ltd.

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