ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1992, p. 2747–2757
0066-4804/92/122747-11$02.00/0

Vol. 36, No. 12

Mode of Antiviral Action of Penciclovir in MRC-5 Cells Infected with Herpes Simplex Virus Type 1 (HSV-1), HSV-2, and Varicella-Zoster Virus

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Received 23 July 1992/Accepted 5 October 1992

The metabolism and mode of action of penciclovir [9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine; BRL 39123] were studied and compared with those of acyclovir. In uninfected MRC-5 cells, low concentrations of the triphosphates of penciclovir and acyclovir were occasionally just detectable, the limit of detection being about 1 pmol/10^6 cells. In contrast, in cells infected with either herpes simplex virus type 2 (HSV-2) or varicella-zoster virus (VZV), penciclovir was phosphorylated quickly to give high concentrations of the triphosphate ester. Following the removal of penciclovir from the culture medium, penciclovir-triphosphate remained trapped within the cells for a long time (half-lives, 20 and 7 h in HSV-2- and VZV-infected cells, respectively). In HSV-2-infected cells, acyclovir was phosphorylated to a lesser extent and the half-life of the triphosphate ester was only 1 h. We were unable to detect any phosphates of acyclovir in VZV-infected cells. (*S*)-Penciclovir-triphosphate inhibited HSV-1 and HSV-2 DNA polymerases competitively with dGTP, the K_i values being 8.5 and 5.8 μM, respectively, whereas for acyclovir-triphosphate, the K_i value was 0.07 μM for the two enzymes. Both compounds had relatively low levels of activity against the cellular DNA polymerase α, with K_i values of 175 and 3.8 μM, respectively. (*S*)-Penciclovir-triphosphate did inhibit DNA synthesis by HSV-2 DNA polymerase with a defined template-primer, although it was not an obligate chain terminator like acyclovir-triphosphate. These results provide a biochemical rationale for the highly selective and effective inhibition of HSV-2 and VZV DNA synthesis by penciclovir and for the greater activity of penciclovir than that of acyclovir when HSV-2-infected cells were treated for a short time.

Penciclovir, through its triphosphate ester (Fig. 1), is a potent and selective antiherpesvirus agent, particularly against herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) and varicella-zoster virus (VZV) (9, 3, 4). Previous studies (3, 4, 9) showed that penciclovir has a spectrum of antiviral activity similar to that of acyclovir, but that penciclovir has an antiviral effect that is longer lasting than that of acyclovir. This may relate to the efficient trapping of the active metabolite, the triphosphate ester of penciclovir, within virus-infected cells (22). Penciclovir and its well-absorbed oral form, famciclovir (23), are undergoing clinical trials for their efficacies not only against HSV-1 infections but also against HSV-2 and VZV infections. There may be appreciable quantitative differences between the rates of metabolism of penciclovir in HSV- and VZV-infected cells. The uptake and phosphorylation in VZV-infected cells has been reported for a pyrimidine analog, 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (25), and for acyclovir at 250 μM (1), but we are unaware of any reports of similar work with acycloguanosine analogs at clinically relevant concentrations. Only recently (2) have we been able to provide a clear indication that penciclovir has prolonged antiviral activity in VZV-infected cells. Therefore, it was of particular interest to determine whether penciclovir-triphosphate (PCV-TP) is formed and then remains at high concentrations within VZV-infected cells following treatment of the cell culture for a short period.

In this report, we describe the continuation of our studies of the mode of action of penciclovir in comparison with that of acyclovir. We investigated the phosphorylation of the acyclonucleosides in HSV-2- and VZV-infected human cells and the stability of PCV-TP in these cells. Also, we report results of our initial studies in which we investigated the effect of PCV-TP on HSV and VZV DNA polymerases. The phosphate esters of penciclovir, unlike those of acyclovir, are chiral with the possibility that the (*R*) and (*S*) enantiomers of the triphosphate ester are formed, although the (*S*) enantiomer is the predominant form in HSV-1-infected cells (11). Also, because of the availability of a hydroxyl group corresponding to the 3′-hydroxyl of the 2′-deoxyribose ring, penciclovir is not an obligate DNA chain terminator as is acyclovir (18, 19). We compared racemic and (*S*)-PCV-TPs as inhibitors of viral and cellular DNA polymerases and investigated their effects on DNA chain extension.

MATERIALS AND METHODS

Radiochemicals. [4′-^{3}H]penciclovir (27.8 GBq/mmol; 27.0 GBq/mmol after allowing for purity) was prepared by SmithKline Beecham Pharmaceuticals, and [2′-^{3}H]acyclovir (925 GBq/mmol) was obtained from NEN Research Products, Du Pont (UK) Ltd., Stevenage, United Kingdom. [*methyl*-^{3}H]thymidine 5′-triphosphate (1.63 TBq/mmol), [8-^{3}H]deoxyguanosine 5′-triphosphate (603 GBq/mmol), and adenosine 5′-[γ-^{32}P]triphosphate (110 TBq/mmol) were obtained from Amersham International plc., Little Chalfont, United Kingdom.

Cells and viruses. MRC-5 cells were grown by standard cell culture techniques. HSV-1 strain SC16 (10) was pro-

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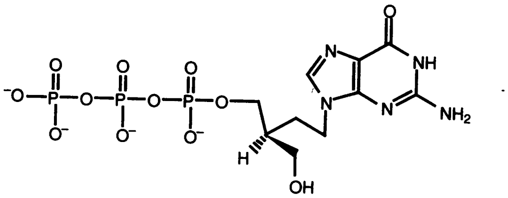

FIG. 1. Structure of (*S*)-PCV-TP.

vided by H. J. Field, Department of Pathology, University of Cambridge, Cambridge, United Kingdom, and HSV-2 strain MS and VZV strain Ellen were obtained from the American Type Culture Collection.

Formation and stability of penciclovir and acyclovir phosphates. With HSV-2-infected cells, experiments were performed essentially as described previously (22). For comparison with earlier work (see reference 22 and references therein), the rates of formation of the triphosphate esters [in picomoles/(minute · gram of cells)] were calculated from the slope of the line in the graph, multiplying by 250, to convert 10^6 cells to 1 g of cells (22), and dividing by 60 to convert hours to minutes.

Cell-associated VZV was prepared in MRC-5 cells. When the cytopathic effect was estimated to be approximately 80%, the cell monolayer was treated with trypsin, and the cells were resuspended in growth medium containing 10% dimethyl sulfoxide and stored at −196°C.

For phosphorylation experiments, MRC-5 cells were grown to near confluency under normal conditions (medium supplemented with 10% fetal calf serum) in 25-cm^2 flasks; the medium was then poured off and the monolayer was infected with cell-associated virus in fresh medium (10 ml, 2% newborn calf serum 1% penicillin-streptomycin). Infections were allowed to proceed for a minimum of 48 h, at which point the first signs of a cytopathic effect became visible.

Once the desired extent of the cytopathic effect was reached, cell monolayers were incubated with fresh maintenance medium (3 ml) containing 10 μM [^{3}H]penciclovir or [^{3}H]acyclovir (each at 28 GBq/mmol). At appropriate times after acyclonucleoside addition, intracellular phosphates were extracted as described previously (22). The stability of PCV-TP was studied by adding fresh maintenance medium (3 ml) containing 10 μM [^{3}H]penciclovir (28 GBq/mmol) approximately 100 h after infection, incubating for a further 18 h before removal, and replenishing with fresh medium (50 ml). Harvesting of the remaining intracellular phosphates, at the times indicated in the Results, was as described above for HSV-2 infected cells.

HPLC analysis of penciclovir and acyclovir phosphate extracts. Phosphate-buffered ethanol extracts were dried under vacuum and resuspended in one-fifth the original volume prior to analysis by one of two high-pressure liquid chromatographic (HPLC) methods to resolve and quantitate nucleoside mono-, di-, and triphosphate esters. Samples from the penciclovir and acyclovir phosphate formation comparison in HSV-2-infected cells were dissolved in 5 mM K_2HPO_4–1 mM heptyltriethylammonium phosphate and were analyzed by using a Waters Nova-Pak C_{18} column and a linear elution gradient from 95% buffer A (5 mM KH_2PO_4–1 mM heptyltriethylammonium phosphate [pH 5])–5% buffer B (15 mM KH_2PO_4 in 70% methanol) to 60% buffer A–40% buffer B. Samples from all other experiments were resuspended in 50 mM K_2HPO_4–KH_2PO_4 (pH 6.8) and analyzed with a Phase Sep C_{18}/C_3NH_2 column by using isocratic conditions (150 mM K_2HPO_4, KH_2PO_4 [pH 6.8], 6% methanol). Flow rates of 0.5 ml/min were used for elution in both HPLC methods; the tritiated nucleosides and nucleotides were monitored with an ISOFLO detector and peak areas calculated by using either an Apple computer and a Nuclear Enterprises Ltd. program or a Walters PC AT computer and RAYTEST RAMONA Radio-Chromatographic system program. Concentrations of nucleosides and phosphate esters were calculated from the corresponding peak areas of [^{3}H]acyclonucleoside standards chromatographed under identical conditions. All other equipment and methodologies were as described previously (22), with the additional use of an LKB 2156 solvent conditioner.

DNA polymerase preparations. The following procedures were carried out at 4°C. HSV-1 and HSV-2 DNA polymerases were extracted from infected MRC-5 cells (multiplicity of infection, 0.01 PFU per cell, incubated for 40 h at 37°C) by treatment with high salt concentrations essentially as described previously (17). After dialysis versus buffer C (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.8]; 0.1 mM EDTA; 0.1 mM dithiothreitol; 20% [vol/vol] glycerol; protease inhibitors pepstatin A, leupeptin, soybean trypsin inhibitor [each at 0.5 μg/ml]), DNA polymerase extracts were chromatographed on a DEAE-Sepharose column. Fractions containing DNA polymerase activity were pooled and then frozen at −40°C until they were required for K_i determinations. VZV DNA polymerase from infected cultures showing a cytopathic effect of about 80% was prepared as described above, except that the extract was chromatographed on a phosphocellulose column. MRC-5 cell DNA polymerase α from uninfected cells was purified by DEAE-Sepharose chromatography. In order to perform DNA chain elongation assays, HSV-2 and MRC-5 DNA polymerases needed further purification to remove contaminating DNase activity. HSV-2 DNA polymerase was chromatographed on a Whatman P11 phosphocellulose column; the later part of the DNA polymerase peak (fraction 2) was nuclease-free. MRC-5 DNA polymerase α was further purified on an anti-DNA polymerase immunoglobulin G-agarose column (modified from a previously described method [15]); the monoclonal immunoglobulin G antibody was from hybridoma line SJK 287-38 (CRL 1644; American Type Culture Collection).

DNA polymerase assays. HSV DNA polymerase activity in column fractions was measured in reaction volumes (25 μl) containing 50 μM dATP, 50 μM dCTP, 50 μM dGTP, 37 KBq of [^{3}H]dTTP (1.63 TBq/mmol), 50 mM Tris-HCl (pH 7.5), heat-treated bovine serum albumin (250 μg/ml), activated calf thymus DNA (5 μg) (20), 1 mM dithiothreitol, 3 mM $MgCl_2$, 150 mM $(NH_4)_2SO_4$, and enzyme fraction (2.5 μl). Assays for MRC-5 DNA polymerase α were similar, but the assay mixture contained 10 mM $MgCl_2$ and additional bovine serum albumin (250 μg/ml) in place of $(NH_4)_2SO_4$. The conditions used for the determination of K_i values were those described above, except that a suitable range of [^{3}H]dGTP concentrations (all at 120 GBq/mmol) and 50 μM dTTP were substituted.

All incubations were for 30 min at 37°C. Reactions were stopped by the addition of an equal volume of 20% (wt/vol) trichloroacetic acid in 20 mM sodium PP_i. After reaction mixtures were left on ice for 15 min, they were spotted onto glassfiber filter mats (type 1205-404; Pharmacia-LKB, Milton Keynes, United Kingdom), washed three times for 15 min each time in ice-cold 5% (wt/vol) trichloroacetic acid, rinsed for 30 s in ethanol, dried, and counted by using an LKB 1205 Betaplate scintillation counter.

Preparation of PCV-TP and ACV-TP. Racemic (*R,S*)-PCV-TP and acyclovir-triphosphate (ACV-TP) were chemically synthesized by SmithKline Beecham Pharmaceuticals. (S)-PCV-TP (enantiomeric purity, >95%) (11) was prepared as follows. MRC-5 cells, infected 24 h previously with HSV-1 SC16 at 0.01 PFU per cell, were incubated for 24 h in medium containing 100 μM penciclovir. PCV-TP was extracted with phosphate-buffered ethanol as described above. All acyclonucleoside triphosphate preparations were purified by HPLC by using a PhaseSep C_{18}/C_3NH_2 column with conditions as described above; this was followed by ammonium formate elution from DEAE-Sepharose and finally repeated lyophilization (each preparation was subsequently demonstrated by HPLC to be 96% pure PCV-TP).

DNA chain elongation assays. (i) Assays with [^{32}P]oligonucleotide primer. A 17-mer oligonucleotide of sequence 5'-TGTGAAATTGTTATCCG-3' was synthesized by using an Applied Biosystems 380A machine (Applied Biosystems Ltd., Warrington, United Kingdom) and was end-labeled by using [γ-^{32}P]ATP and a 5'-terminus DNA labeling kit containing T4 polynucleotide kinase (GIBCO Bethesda Research Laboratories, Paisley, Scotland). In a total assay sample volume of 7.5 μl, labeled 17-mer singly annealed to single-strand M13mp18 positive-strand DNA (0.2 μg); 50 mM Tris-HCl (pH 7.5); 3 mM $MgCl_2$; 150 mM $(NH_4)_2SO_4$; 50 μM (each) dATP, dCTP, and dTTP; 1 mM dithiothreitol; bovine serum albumin (250 μg/ml); nuclease-free HSV-2 DNA polymerase (fraction from phosphocellulose chromatography, 2 μl); and various dGTP-inhibitor concentrations were incubated at 37°C for 60 min. After incubation and addition of formamide-bromophenol blue, samples were electrophoresed on a urea-denaturing polyacrylamide gel in Tris-borate buffer (pH 8.3). Autoradiography of dried gels allowed visualization of discrete DNA elongation products.

(ii) Assays with [^{3}H]dNTPs and 17·26-mer primer-template. Assays with [^{3}H]deoxynucleoside triphosphates (dNTPs) and 17·26-mer primer template were done in a total assay volume of 12.5 μl containing the components listed above, except for the following modifications. The 17·26-mer primer-template (1 μg), [^{3}H]dNTP (dATP, dCTP, or dTTP, each at 2.5 μl) of the appropriate dilution, PCV-TP (2.5 μl), and HSV-2 DNA polymerase (1.25 μl) were incubated at 37°C for 60 min. Reactions were stopped by the addition of an equal volume of 40 mM EDTA; and the reaction mixture was spotted onto DEAE filter mats (type 1205-405, Pharmacia-LKB), washed three times in 2× SSC buffer (1× SSC is 0.015 M NaCl plus 0.015 M sodium citrate), dried, and counted in a LKB 1205 Betaplate counter.

(iii) Assays with [^{32}P]dATP. Assays with [^{32}P]ATP were done in a total assay sample volume of 10 μl containing the components listed above for the assays with [^{32}P]oligonucleotide primer, except for the following modifications: 12 μM [^{32}P]dATP, 12 μM (each) dCTP and dTTP, 0.2 μg of unlabeled 17-mer·M13 positive-strand DNA primer-template, dGTP–PCV-TP (1.65 μl), and HSV-2 DNA polymerase (2 μl) were incubated at 32°C for 60 min unless otherwise stated. Reactions were stopped by adding an equal volume of 40 mM EDTA. Portions of the stopped reaction mixtures (5 or 10 μl) were spotted onto DEAE filter mats, washed, and counted as described above for the assays with [^{3}H]dNTPs and 17:26-mer primer-template; and portions (5 μl) were electrophoresed on a 0.8% alkaline agarose gel (30 mM NaOH, 1 mM EDTA), which was dried and autoradiographed.

Analysis of HSV-2 DNA content of drug-treated, virus-infected MRC-5 cells. Monolayers of MRC-5 cells prepared in microtiter plates were infected with HSV-2 MS at approximately 0.3 PFU per cell and treated in triplicate with either penciclovir or acyclovir. Cell lysates were prepared 24 h after infection with 1% (wt/vol) sodium dodecyl sulfate in water and transferred to nylon filters (BioTrace RP; Gelman Sciences) by using a Hybri-Dot manifold (GIBCO Bethesda Research Laboratories). The viral DNA content was then determined by hybridization with a ^{32}P-labeled probe specific for HSV-2 DNA essentially as described previously (6). The HSV-2 DNA probe pGR60 contains the HSV-2 *Bgl*II N fragment cloned in pBR322 (provided by P. O'Hare, Marie Curie Memorial Foundation, Oxted, United Kingdom).

RESULTS

Comparison of phosphorylation of penciclovir in HSV-1- and HSV-2-infected cells. During the 4-h incubation with 10 μM penciclovir, the average rates of triphosphate formation were 2,000 and 1,200 pmol/(min · g of cells) in HSV-1- and HSV-2-infected cells, respectively. Of the total phosphorylated penciclovir, the proportions of penciclovir-diphosphate and -monophosphate were noticeably greater in cells infected with HSV-1 than in cells infected with HSV-2 (10 and 2% of the di- and monophosphates, respectively, in HSV-1-infected cells compared with 6 and <1%, respectively, in HSV-2-infected cells). During the incubation with 1 μM penciclovir, the rates of formation of the three phosphate esters were generally about 10-fold less than those described above, although the average rate of PCV-TP formation was slightly greater in cells infected with HSV-1 than in cells infected with HSV-2 [200 and 98 pmol/(min · g of cells], respectively).

Phosphorylation of penciclovir and acyclovir in HSV-2-infected cells. Both penciclovir and acyclovir were phosphorylated to the triphosphate ester in HSV-2-infected MRC-5 cells. However, the rate of phosphorylation of penciclovir was much greater than that of acyclovir (Fig. 2). From 10 μM penciclovir, PCV-TP was formed at a nearly constant rate throughout the 24-h experiment [1,200 pmol/(min · g of cells) during the first 4 h; 1,000 pmol/(min · g of cells) thereafter]. The corresponding rates for incubation with 1 μM penciclovir were 140 and 100 pmol/(min · g of cells), respectively. During the incubation with 10 μM penciclovir, the concentration of the drug in the medium remained at about 10 μM for 3 h and then decreased to 8.5 μM at 4 h and 7.5 μM at 8 h and was only 3.9 μM at 24 h. Similarly, for 1 μM penciclovir, the drug concentrations decreased after 4 h, to 0.7 μM at 8 h and 0.5 μM at 24 h. For both penciclovir concentrations, the loss of compound in the medium was balanced by the increased amount of phosphorylated compound in the cells. Because the amount of drug in the cell culture medium was reduced during the incubation to about 40 to 50% of its initial value, this could easily account for the slight reduction in the observed phosphorylation rate.

ACV-TP was also formed in increasing concentrations over the 24-h incubation, although the initial rate of formation during the first 2 h was much more than that during the remainder of the incubation [for 10 μM acyclovir, 150 and 16 pmol/(min · g of cells), respectively; for 1 μM acyclovir, about 15 and 5 pmol/(min · g of cells), respectively]. However, the concentrations of acyclovir in the medium remained unchanged throughout the 24-h incubation (9.9 and 1.1 μM found at 24 h). Therefore, the decrease in the rate of ACV-TP formation could not be accounted for by any

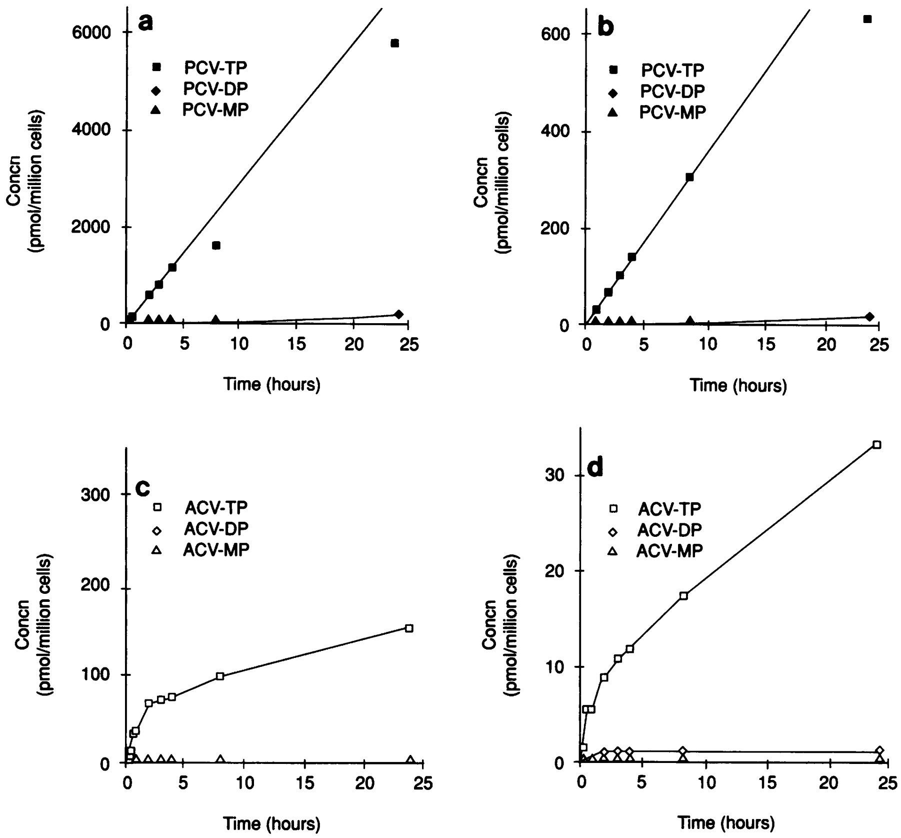

FIG. 2. Formation of acyclonucleotides in HSV-2-infected MRC-5 cells. [4′-^{3}H]penciclovir or [2′-^{3}H]acyclovir was added 20 h after infection (0.01 PFU per cell). At the indicated times, cells were extracted and the samples were assayed by HPLC as described in the text. For penciclovir at 10 μM (a) and 1 μM (b), the lines, fitted by linear regression to the values up to 4 h, are given by the equations $y = 286x - 31$ ($r^2 = 0.992$) and $y = 34x - 5$ ($r^2 = 0.982$), respectively. (c) 10 μM acyclovir; (d) 1 μM acyclovir. PCV, penciclovir; ACV, acyclovir, TP, triphosphate; DP, diphosphate; MP, monophosphate.

change in the concentration of acyclovir in the cell culture medium. Following initial treatment with the compound at 10 μM, the final intracellular amount of PCV-TP, 5,830 pmol/10^6 cells (about 1,500 μM), was much greater than that of ACV-TP, 150 pmol/10^6 cells (about 38 μM), and those of penciclovir or acyclovir in the cell culture medium (4 or 10 μM, respectively) after 24 h of treatment.

The proportions of the mono-, di-, and triphosphate esters of penciclovir and acyclovir differed. The monophosphate of penciclovir was detected only at 24 h when it was present at 0.3% of the triphosphate concentration. An increase in penciclovir-diphosphate concentrations was observed over the 24-h period, although as a percentage of the total phosphorylated derivatives, it decreased from about 25% at 1 min to about 2% at times after 40 min. For acyclovir, the proportions of diphosphate ester were comparable to those of the penciclovir diphosphate ester, but the monophosphate ester of acyclovir was present at greater concentrations, initially being the major phosphorylated derivative (55% at 5 min) but decreasing to about 1% at 3 h.

In control uninfected cells treated with 10 μM drug, very low levels of the triphosphate esters of penciclovir and acyclovir were detected. After 4 h of incubation, there was about 1 pmol/10^6 cells of triphosphate ester from penciclovir or acyclovir, and the levels remained below 2 pmol/10^6 cells for the remainder of the 24-h incubation.

Stability of intracellular triphosphate esters of penciclovir and acyclovir in HSV-2-infected cells. The stabilities of the phosphate esters of penciclovir and acyclovir following removal of extracellular drug are shown in Fig. 3. After incubation of virus-infected cells with [^{3}H]penciclovir or [^{3}H]acyclovir from 1.25 to 5.5 h postinfection and then washing of the cells, the intracellular triphosphate ester levels were 557 and 9.6 pmol/10^6 cells, respectively, and the residual extracellular concentrations of the corresponding acyclonucleosides were <1 pmol/10^6 cells. During the next 8 h of incubation, the concentrations of PCV-TP decreased slowly (Fig. 3a). In contrast, the concentrations of the phosphates of acyclovir decreased much more rapidly, falling below the detection limit within 4 h (Fig. 3b). Under these conditions, the half-lives of the triphosphates of penciclovir and acyclovir were about 20 and 1 h, respectively.

As the amounts of acyclonucleotides within the cells decreased, the resulting acyclonucleoside diffused out of the cells into the culture medium. In cell cultures treated with penciclovir, the concentrations of penciclovir in the medium increased almost linearly during the 8-h incubation period (Fig. 3c). In contrast, acyclovir concentrations initially increased rapidly but reached the maximum level after 2 h (Fig. 3d), by which time virtually all of the acyclovir phosphates had been converted to acyclovir.

Penciclovir and acyclovir phosphate formation in VZV-infected cells. Initial experiments found that, early in the cell culture infection while the cytopathic effect was low, rates of

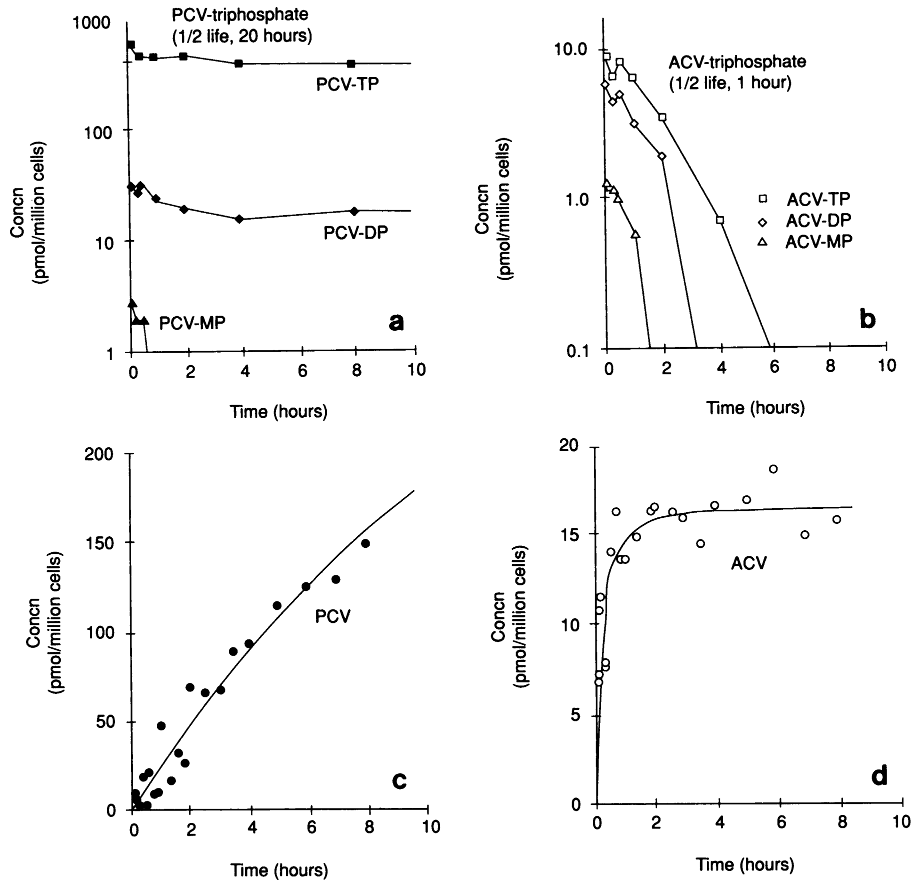

FIG. 3. Stability of intracellular penciclovir phosphates (a) and acyclovir phosphates (b) and diffusion of penciclovir (c) and acyclovir (d) into the cell culture medium. MRC-5 cells were infected with HSV-2 (1 PFU per cell), and 10 μM [4′-^{3}H]penciclovir or [2′-^{3}H]acyclovir was added from 1.25 to 5.5 h after infection. Then, the cell cultures were washed, and at the indicated times, cells were extracted and the samples were assayed by HPLC as described in the text. (a and b) The half-lives of the triphosphate esters were calculated from the lines, fitted by linear regression, given by the equations $y = 510 \times 10^{(-0.017x)}$ ($r^2 = 0.889$) and $y = 10 \times 10^{(-0.277x)}$ ($r^2 = 0.980$), respectively. (c and d) The extracellular concentrations of the acyclonucleosides were calculated from the measurement of radioactivity in a sample (50 μl) taken at each of the indicated times. In those samples also assayed by HPLC, radioactivity was present only in the peak corresponding to the acyclonucleoside. PCV, penciclovir; ACV, acyclovir; TP, triphosphate; DP, diphosphate; and MP, monophosphate.

PCV-TP formation were limited by the number of cells infected with VZV. Having determined suitable conditions for the study of phosphate formation, more comprehensive 6-h time course experiments were undertaken (Fig. 4). PCV-TP formation was nearly linear with time. At 6 h after the addition of penciclovir to a cell monolayer with a cytopathic effect of approximately 80%, the concentration of PCV-TP was 220 pmol/10^6 cells. We were unable to detect ACV-TP, even after incubation with acyclovir for 6 h in a cell monolayer with a cytopathic effect of 80%; under these conditions, the limit of detection was estimated to be 1 pmol/10^6 cells, inferring that ACV-TP levels were <0.25 μM. As in both HSV-1- and HSV-2-infected MRC-5 cells, penciclovir appears to be phosphorylated much more readily than acyclovir in VZV-infected cells, implying that the former is a better substrate for HSV- and VZV-encoded thymidine kinases. At the end of this experiment, the approximate ratio of the mono-, di-, and triphosphates were 1:5:10, respectively (Fig. 4, inset).

Stability of PCV-TP in VZV-infected cells. To ensure that sufficient concentrations of PCV-TP were formed for subsequent stability analysis, infected cell monolayers were incubated for 4 days, by which time an extensive cytopathic effect had developed, before incubating overnight with 10 μM penciclovir. The intracellular PCV-TP stability profile is depicted in Fig. 5, and from the line fitted by linear regression, a half-life of 7.2 h was derived. However, approximately 30% of the initial intracellular PCV-TP concentration was still present 24 h after drug removal.

Inhibition of isolated DNA polymerases. We determined previously (11) that, following HSV-1 infection of MRC-5 cells and incubation with penciclovir, >95% of the triphosphate ester of penciclovir formed is the (*S*) enantiomer. (*S*)-PCV-TP was synthesized biochemically via this route and was purified as described in Materials and Methods; a chemically synthesized racemate of PCV-TP was purified for these studies to give an indication of the inhibitory effect of the (*R*) enantiomer of PCV-TP. The data from representative kinetic experiments are presented as Lineweaver-Burk plots (Fig. 6), and the derived K_i values are given in Table 1. The results demonstrate that (*S*)-PCV-TP is a competitive inhibitor of HSV-1 and HSV-2 DNA polymerases with respect to the natural substrate dGTP (K_is, 8.5 and 5.8 μM, respectively). (*R*,*S*)-PCV-TP was also a competitive inhibitor of HSV DNA polymerases, but its K_i (16.0 μM) for HSV-1 DNA polymerase with respect to dGTP was almost twice as large

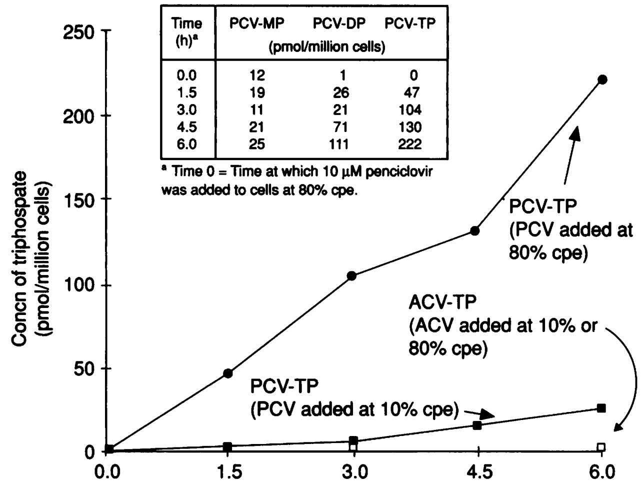

FIG. 4. PCV-TP formation in VZV-infected MRC-5 cells. [4'-^{3}H]penciclovir or [2'-^{3}H]acyclovir (each at 10 μM) was added to VZV-infected cultures showing either minimal (10%) or extensive (80%) viral cytopathic effect (cpe). At the indicated times, cells were extracted and the samples were assayed by HPLC as described in the text. PCV, penciclovir; ACV, acyclovir; TP, triphosphate; DP, diphosphate; and MP, monophosphate.

as that determined for the (*S*) enantiomer, implying that (*R*)-PCV-TP does not compete for dGTP at the same order of magnitude as the (*S*) enantiomer does. However, for HSV-2 DNA polymerase, the K_i value for (*R,S*)-PCV-TP (9.5 μM) was slightly less than twice the K_i value for (*S*)-PCV-TP (5.8 μM), allowing the possibility that (*R*)-PCV-TP could have some inhibitory activity against HSV-2 DNA polymerase.

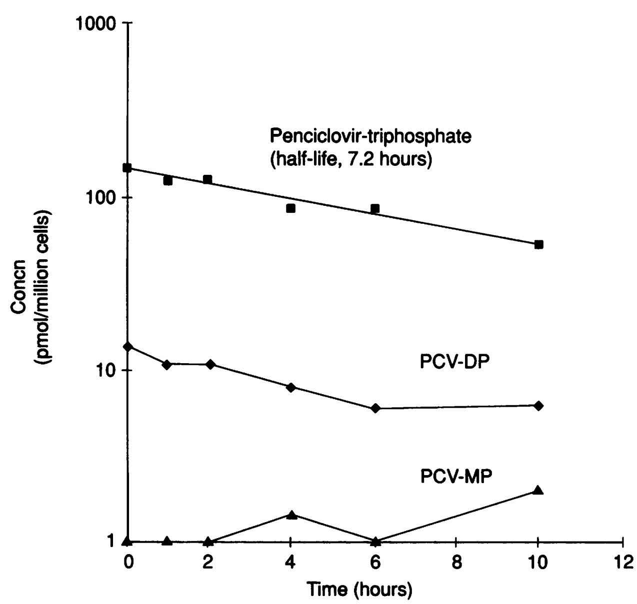

FIG. 5. Stability of PCV-TP in VZV-infected MRC-5 cells. [4'-^{3}H]penciclovir at 10 μM was added to VZV-infected cultures showing extensive viral cytopathic effect, the cells were extracted, and the samples were assayed as described in the text. The half-life of the triphosphate ester was calculated from the line, fitted by linear regression, given by the equation $y = 140 \times 10^{(-0.042x)}$ ($r^2 = 0.961$).

(*R,S*)-PCV-TP also inhibited VZV DNA polymerase, with the 50% inhibitory concentration (IC_{50}) being 75 μM, which may infer that the value for the (*S*) enantiomer would be 37.5 μM. In comparison, the value for ACV-TP was 0.88 μM. (*S*)-PCV-TP was far less inhibitory to human MRC-5 DNA polymerase α (K_i, 175 μM) than to HSV DNA polymerases (K_is, 8.5 and 5.8 μM) or than ACV-TP was to DNA polymerase α (K_i, 3.8 μM). It was surprising to find that the (*R,S*) racemate of PCV-TP was a stronger competitive inhibitor of DNA polymerase α than the (*S*) enantiomer was (K_is, 45 and 175 μM, respectively), particularly since the latter was shown to be the enantiomer mainly responsible for competitive inhibition of HSV DNA polymerases. This result implies that the K_i value for the (*R*)-PCV-TP for the cellular DNA polymerase α was about 25 μM.

DNA chain extension assays. By using the dideoxy DNA sequencing methodology with saturating concentrations of all four normal dNTPs, both MRC-5 and HSV-2 DNA polymerases were able to extend the primer hybridized to M13 DNA template, with the herpesvirus polymerase being more processive than DNA polymerase α (Fig. 7, lanes 1 and 6). In the absence of dGTP (Fig. 7, lanes 5 and 10), a small amount of misincorporation of the other three natural dNTPs was observed; HSV-2 DNA polymerase displayed a reduced fidelity of replication compared with DNA polymerase α. DNA polymerase assays in the presence of ACV-TP (Fig. 7, lanes 4 and 9) indicated that ACV-TP is readily incorporated into DNA at the first position where dGTP would normally be inserted, resulting in chain termination. PCV-TP did allow limited DNA chain extension past several presumed penciclovir-monophosphate residues (Fig. 7, lanes 2 and 7), whereas ACV-TP terminated DNA chain extension (Fig. 7, lanes 4 and 9). Inclusion of (*R,S*)-PCV-TP in DNA chain extension assays resulted in a marked decrease in DNA synthesis (Fig. 7, lanes 3 and 8) in comparison with (*S*)-PCV-TP, suggesting that the (*R*) enantiomer is a poorer substrate than the (*S*) enantiomer for both DNA polymerases.

The effects of (*S*)-PCV-TP on the incorporation of the other nucleotides were studied by using a short defined 17:26-mer primer-template:

```
3'-ACACTTTAACAATAGGCGAGTGTTAA-5'
   •••••••••••••••••
5'-TGTGAAATTGTTATCCG-3'
```

This primer-template could be extended by up to nine bases, three each of dATP, dCTP, and dTTP. Because there was no position at which dGTP would normally be inserted, the effect of (*S*)-PCV-TP on the incorporation of these other three dNTPs could be studied without hindrance from the competitive inhibition and any possible inactivation processes that would occur with the M13 DNA template.

There was no effect on the incorporation of dATP or dCTP by (*S*)-PCV-TP, but there was a clear competitive-type inhibition toward dTTP incorporation. Although this inhibition was completely reversed in the presence of 50 μM dGTP, and therefore probably does not play a major part in the inhibition of viral DNA synthesis, it is an unexpected inhibitory mechanism.

In an attempt to add [4'-^{3}H](*S*)-PCV-TP to the primer by using Klenow DNA polymerase, the incorporation was <1% of that with [^{3}H]dGTP. In comparison, the incorporation of [^{3}H]ACV-TP was reported to be 17% (19). Therefore, it seems that (*S*)-PCV-TP is a poor substrate for incorporation into DNA compared with ACV-TP.

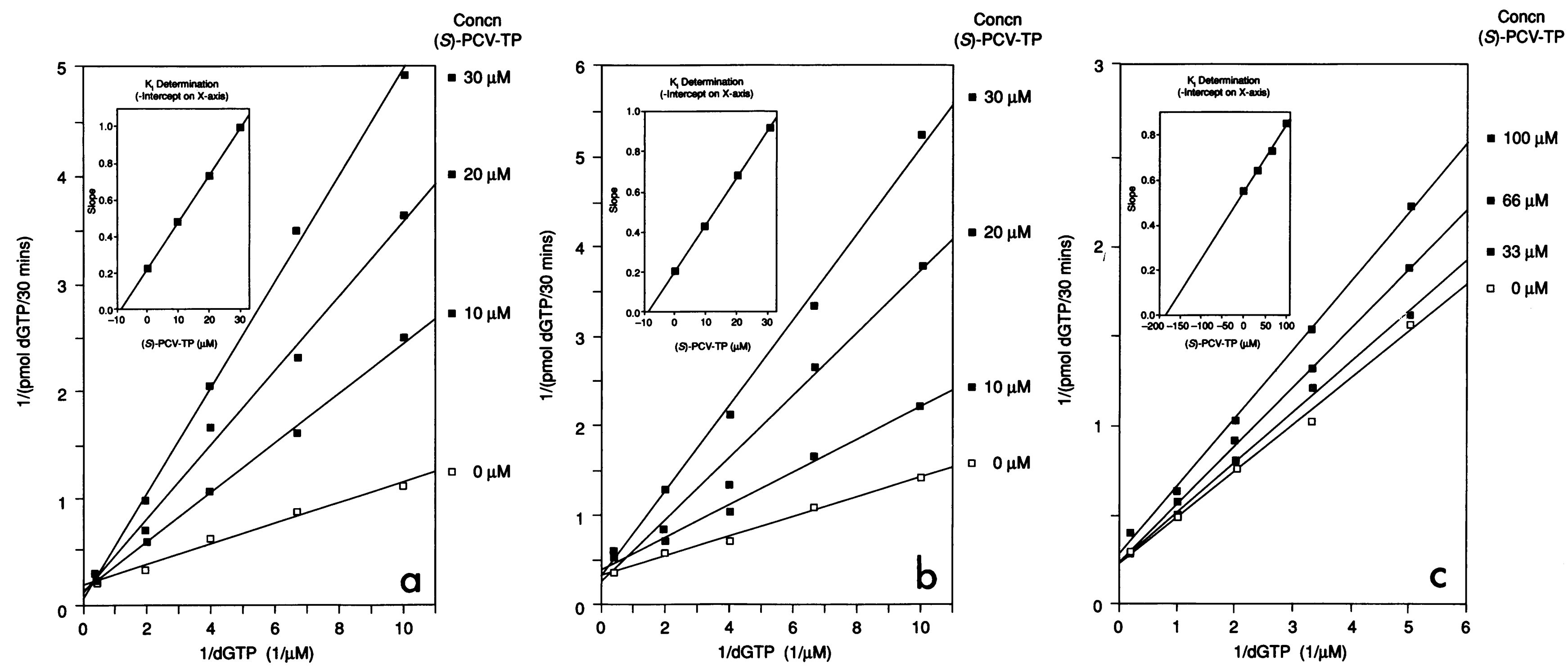

FIG. 6. Competitive inhibition of DNA polymerases by (*S*)-PCV-TP. The data are presented as Lineweaver-Burk plots for HSV-1 (a), HSV-2 (b), and MRC-5 cell DNA polymerase α (c), with dGTP as the variable substrate and activated calf thymus DNA as the template. The K_i values were calculated from a replot of the slopes (insert).

TABLE 1. Kinetic constants

DNA polymerase	Compound concn (μM)[a]			
	K_m for dGTP	K_i		
		(S)-PCV-TP	(R,S)-PCV-TP	ACV-TP
HSV-1	0.57 ± 0.2	8.5 ± 0.5	16.0 ± 2.0	0.07 ± 0.0
HSV-2	0.36 ± 0.1	5.8 ± 0.8	9.5 ± 0.5	0.07 ± 0.02
MRC-5[b]	0.97 ± 0.05	175 ± 25	45 ± 5	3.8 ± 0.7

[a] Values are the mean ± range of two determinations.
[b] DNA polymerase α.

Inhibition by (*S*)-PCV-TP under processive and nonprocessive conditions. An assay method was developed in order to size and quantitate DNA products up to 7.3 kb in length; this is the maximum length that would be obtained if the M13 positive-strand DNA was replicated completely by highly processive DNA polymerase action. DNA synthesis was quantitated by determining [^{32}P]dATP incorporation into DNA; the sizes of the synthesized DNA strands were estimated from autoradiographs of denaturing agarose gels. It had been reported previously (16) that the processivity of pure HSV-1 DNA polymerase is greatly enhanced by the addition of *Escherichia coli* single-strand DNA-binding protein. This effect was not observed with our partially purified viral DNA polymerase samples, possibly because they would have contained the viral protein encoded by the gene UL42 (7). The processivity of our HSV-2 DNA polymerase was, however, sensitive to the assay incubation temperature. DNA of almost 7 kb was synthesized at a temperature of 32°C, but only short lengths (<0.5 kb) were synthesized at 37 or 30°C. Having established the optimum conditions for processive DNA replication, dose-response curves for (*S*)-PCV-TP (Fig. 8) and (*R*,*S*)-PCV-TP were determined with 12 μM dGTP; this is about the level detected in acyclovir-treated HSV-infected human fibroblast cells (12). The IC_{50}s were calculated to be 176 ± 8 and approximately 310 μM, respectively. The ratio of IC_{50}s for (*S*)- and (*R*,*S*)-PCV-TPs (1:1.8) closely parallels the corresponding ratio of K_is for competitive inhibition with respect to dGTP (1:1.6), inferring that the inhibitory effectiveness of each enantiomer is probably directly related to its ability to compete with dGTP.

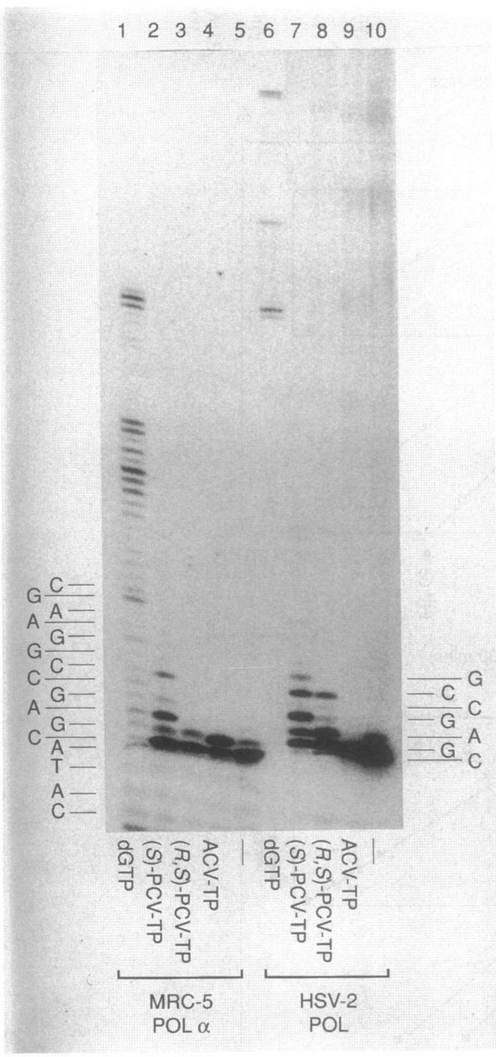

FIG. 7. Inhibition of DNA chain extension. Assays were performed as described in the text, with the following concentrations of dGTP or inhibitor (all assays contained 50 μM dATP, dCTP, and dTTP). Lanes: 1 and 6, 50 mM dGTP; 2 and 7, 50 μM (*S*)-PCV-TP; 3 and 8, 50 μM (*R*,*S*)-PCV-TP; 4 and 9, 50 μM ACV-TP; 5 and 10, no additional nucleotide. POL, polymerase.

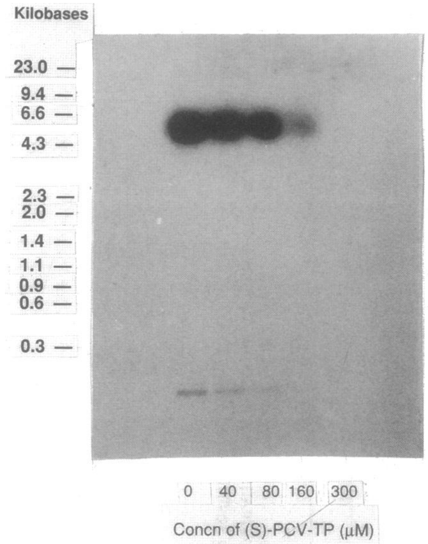

FIG. 8. Sensitivity of processive DNA replication to (*S*)-PCV-TP. With processive DNA replication at 32°C, [^{32}P]dATP was used to monitor the newly synthesized DNA which was electrophoresed on 0.8% alkaline agarose gel as described in the text.

Under nonprocessive replication conditions, inhibition of chain elongation by (*S*)-PCV-TP was not detected, confirming the observations of other workers (14) that drug activity is dependent upon polymerase processivity.

Effect of short treatment time of cells on inhibition of HSV-2 DNA synthesis. In a conventional dose-response test, in which penciclovir and acyclovir were present continuously in the cell culture medium, both compounds were almost equally active (IC_{50}s, 0.05 and 0.04 μg/ml, respectively). To examine the effect of short treatment times, compounds were added at 5 h postinfection, by which time the viral thymidine kinase should have been produced; however, this was before the start of the viral DNA synthesis at 8 h after infection. The compounds were removed at various times

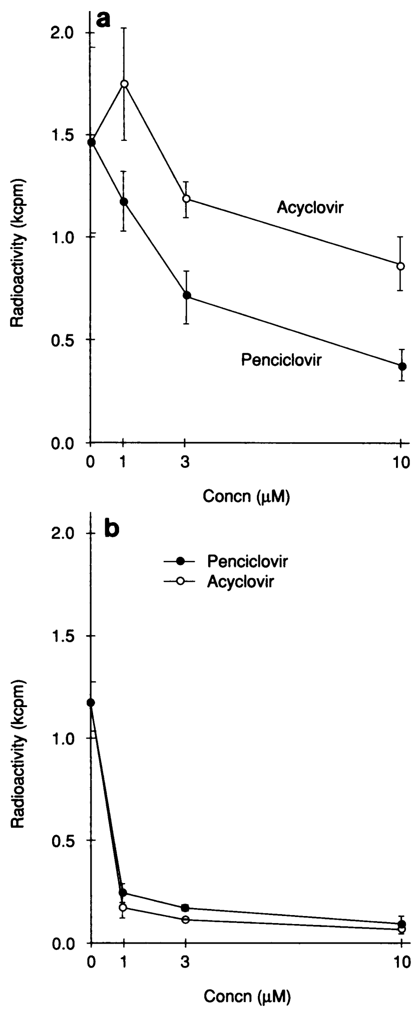

FIG. 9. Inhibition of HSV-2 DNA synthesis in MRC-5 cells following either 1.5 h (a) or 23 h (b) of treatment with penciclovir and acyclovir. MRC-5 cells were infected with HSV-2 MS at 0.3 PFU per cell. Penciclovir and acyclovir were present in the cell culture medium for 1.5 h from 5 h after infection (a) or for 23 h from 1 h after infection (b). At 24 h after infection, cell-associated HSV-2 DNA was measured by hybridization with the ^{32}P-labeled DNA probe pGR60. Each point represents the mean ± standard deviation of four replicate observations. Levels of HSV-2 DNA in cultures treated for 1.5 h were not significantly different from those in the untreated virus control cultures when penciclovir was present at 1 μM or acyclovir was present at either 1 or 3 μM ($P > 0.05$).

thereafter and replaced with drug-free medium. Levels of viral DNA were determined at 24 h postinfection by hybridization with a DNA probe specific for HSV-2. For comparison, control cultures were treated continuously from 1 to 24 h after infection.

Whereas 1 h of treatment of HSV-2-infected cells with penciclovir resulted in a slight inhibition of viral DNA synthesis, treatment for 1.5 h demonstrated the more effective inhibition of viral DNA synthesis by penciclovir than by acyclovir (Fig. 9). Significantly ($P = 0.001$) reduced levels of viral DNA were present in cells treated with 3 and 10 μM penciclovir for 1.5 h compared with the levels in cells similarly treated with acyclovir. In contrast, following continuous treatment from 1 h after infection, both compounds gave good inhibition of viral DNA synthesis.

DISCUSSION

Our initial studies (22) with HSV-1-infected cells indicated that penciclovir is phosphorylated to give high concentrations of the triphosphate ester in infected cells, but not in uninfected cells. This triphosphate of penciclovir was much more stable than that of acyclovir, the half-lives being 10 and 0.7 h, respectively. We extended these studies to include HSV-2- and VZV-infected cells. As in the previous study (22), the experimental conditions were chosen to be as relevant as possible to the clinical situation. We used human fibroblast cells (MRC-5) and penciclovir concentrations (≤10 μM) which are achieved in humans following either intravenous administration of penciclovir (5) or oral administration of famciclovir (24).

In HSV-2-infected cells, the phosphorylation of penciclovir was comparable to that in HSV-1-infected cells, but there were some small differences. The proportions of the mono- and diphosphate esters, relative to the proportion of the triphosphate ester, were slightly lower in the cells infected with HSV-2 than in the corresponding cells infected with HSV-1 and were again low in the experiment that compared penciclovir and the phosphorylation of acyclovir in HSV-2 infected cells. This may be due to a small difference in the activities of the thymidine kinases encoded by HSV-1 and HSV-2. However, the rates of formation of PCV-TP in HSV-2-infected cells treated with 1 or 10 μM penciclovir (Fig. 2) were similar to those found in HSV-1-infected cells (22).

Although we did not compare directly the phosphorylation of acyclovir in HSV-1- and HSV-2-infected cells within the same experiment, we did obtain much higher concentrations of ACV-TP with HSV-2 in this study than we did previously with HSV-1 (22). Despite an overall increased rate of phosphorylation, the levels of acyclovir-monophosphate were markedly lower than those of ACV-TP except at the earliest time points, whereas in HSV-1-infected cells, the mono- and triphosphates were present at about equal concentrations. In contrast to penciclovir, which was phosphorylated at an almost linear rate throughout the incubation, ACV-TP was formed much more slowly after the first 2 h of the incubation (Fig. 2). After 4 h of incubation in HSV-2-infected cells, the concentration of PCV-TP was about 15-fold that of ACV-TP, but this ratio was not as high, about 100-fold, as that found previously (22) in HSV-1-infected cells.

In uninfected cells, we were usually unable to detect the phosphates of penciclovir or acyclovir. However, in the control uninfected cells for experiments with HSV-2, low levels of the triphosphates were detected. With both penciclovir and acyclovir, the amount of triphosphate ester was about 1 to 2 pmol/10^6 cells at 4 h and then remained at about this level for the rest of the 24 h of incubation. Tolman (21) has indicated that it is more desirable for an acyclonucleoside to be poorly converted to a triphosphate which is an efficient inhibitor of viral replication (e.g., acyclovir) than to one which is efficiently converted to triphosphate. Efficient phosphorylation by virus-specified kinases means more phosphorylation (although orders of magnitude less) by host cell kinases in an uninfected cell. Although this may seem to be a reasonable expectation, our work has shown that this prediction does not apply to penciclovir. Penciclovir is phosphorylated much more efficiently than acyclovir in herpesvirus-infected cells, but the host cell kinases phosphorylate the two compounds to a small but comparable extent. This highly preferential metabolism of penciclovir in herpesvirus-infected cells is a major factor in its selective antiviral activity.

When it was found that penciclovir was phosphorylated in HSV-1-infected cells to give much more triphosphate ester than that of acyclovir, even though both compounds had comparable antiviral activities in standard assays, we thought that PCV-TP might be a less powerful inhibitor of viral DNA polymerase than ACV-TP. We showed that this is the case. For HSV-1 DNA polymerase, the K_i for PCV-TP is about 100-fold greater than that for ACV-TP. Therefore, although the rate of formation of PCV-TP, in molar terms, is very high, in terms of the amount needed to inhibit viral DNA polymerase and hence inhibit the virus, the rate is comparable to that of ACV-TP. Therefore, in standard plaque reduction antiviral assays in which the compounds are present in the medium throughout the test, the contrasting levels of triphosphate esters compensate for the different inhibitory activities of these triphosphate esters and so account for the comparable antiviral activities of penciclovir and acyclovir in such assays. With penciclovir, the phosphorylation to the triphosphate ester and the K_i values were similar for HSV-1 and HSV-2, thus providing a rationale for the good activities of penciclovir against both of these viruses. However, we do not know why acyclovir had comparable activities against HSV-1 and HSV-2 in view of the high levels of triphosphate ester in HSV-2-infected cells yet equal K_i values for the two viral DNA polymerases.

Hannah et al. (8) have tested penciclovir [referred to as 9-(4′-hydroxy-3′-hydroxymethyl)butylguanine] in their HSV-1 staggered enzyme assay and found that penciclovir is phosphorylated up to the triphosphate ester but that this triphosphate does not inhibit HSV-1 DNA polymerase. They suggested that the good antiviral activity of penciclovir must be expressed by a mechanism different from those of ganciclovir and other members of the acyclovir class. However, we note that in their staggered enzyme assay at the DNA inhibition step, the concentrations of the triphosphates of acyclovir and penciclovir were comparable and lower than those of the acyclonucleosides at the first step of the assay. In contrast, after 4 h of incubation with HSV-1-infected cells, we showed that the intracellular concentration of PCV-TP is 30-fold that of penciclovir in the cell culture medium. This ratio may be an underestimate because it was based on the assumption that the triphosphate is evenly distributed through the cell, whereas it may be concentrated in the cell nucleus. Even at the average intracellular concentration, PCV-TP inhibits viral DNA polymerase and so the antiherpesvirus activity of penciclovir can be accounted for by this mechanism, although this does not preclude additional modes of action.

Because penciclovir has a prochiral center with two hydroxymethyl groups, there are two enantiomers of the triphosphate ester, the (*S*) enantiomer being formed in HSV-1-infected cells (11). It is the (*S*) isomer that is structurally analogous to the natural 5′-deoxyguanosine-triphosphate. In the DNA extension assays, (*S*)-PCV-TP acted as a substrate for HSV-2 DNA polymerase. When penciclovir was added to the end of the DNA chain, the free hydroxymethyl group allowed further chain extension, albeit only inefficiently. Thus, (*S*)-PCV-TP is not an immediate chain terminator. However, when attempting to incorporate [^{3}H]penciclovir-monophosphate with Klenow DNA polymerase onto the end of the 17-mer primer, it appeared to be a very poor substrate (<1% relative to dGTP) compared with acyclovir (19). Therefore, although PCV-TP effectively inhibits DNA synthesis, it does not act in the same way as the triphosphates of acyclovir and dideoxynucleosides, which are good substrates for DNA polymerases, but their incorporation prevents any further DNA chain extension.

In VZV-infected cells, the rate of formation of PCV-TP increased as the infection proceeded, but even late in the infection, this rate was nearly 10-fold less than those in cells infected with either HSV-1 or HSV-2. This may be due, at least in part, to the slow progression of VZV infection in cell culture. However, the concentration of PCV-TP was at least 30-fold greater than that of ACV-TP, which remained below the limit of detection. The very low level of ACV-TP was to be expected from the work of Biron and Elion (1), who reported that 250 μM acyclovir is converted to the triphosphate ester (about 10 to 35 pmol/10^6 cells). Although the IC_{50} of PCV-TP was greater than that of ACV-TP, this was compensated for by the high levels of PCV-TP formed in VZV-infected cells.

The confirmation that PCV-TP inhibits herpesvirus DNA polymerases gives support to our view (22) that the entrapment of PCV-TP at high concentrations within virus-infected cells and the stability of PCV-TP accounted for the good antiviral activity against HSV-1. We showed that in HSV-2-infected cells PCV-TP is much more stable than ACV-TP, the half-lives being 20 and 1 h, respectively. Furthermore, just as we have shown good activity with penciclovir after treating HSV-1-infected cells for a short time (22), we did a similar experiment with HSV-2-infected cultures. As before, we used concentrations of penciclovir which have been achieved in plasma following oral administration of famciclovir (1,000 mg, 3.1 mmol) to healthy subjects (24). Plasma penciclovir concentrations that exceeded 10 μM (2.5 μg/ml) lasted for about 1.5 h. In contrast, after an oral dose of acyclovir (800 mg, 3.6 mmol), the maximum peak levels of acyclovir in blood were only about 7.5 μM (13). This assay with short treatment times (1.5 h) therefore represents the clinical conditions following oral dosing more closely than does the standard antiviral assay in which the compounds are present continuously at set concentrations. Whereas 3 μM acyclovir was inactive after 1.5 h of incubation (Fig. 9), penciclovir was significantly (P = 0.001) active at those concentrations (3 and 10 μM) which have been achieved in humans.

In VZV-infected cells, PCV-TP had good stability, the half-life being over 7 h (Fig. 5). We could not measure the stability of ACV-TP because the levels of the triphosphate were below the detection limit of our assay. These results indicate that penciclovir would become trapped, as its triphosphate ester, within VZV-infected cells. Hence, penciclovir would be expected to give long-lasting inhibition of VZV DNA synthesis following limited treatment times; this inhibition would be similar to that demonstrated for HSV-1- and HSV-2-infected cells. Recently (2), we have shown that the activity of penciclovir in VZV-infected cell culture is more prolonged than that of acyclovir.

In summary, the mechanism of action of penciclovir involves highly selective transformation, only in herpesvirus-infected cells, into a triphosphate which inhibits viral DNA polymerase. This inhibition is competitive with the natural substrate dGTP. Although penciclovir is not an obligate DNA chain terminator because of the availability of a hydroxyl group corresponding to the 3′-hydroxyl of the 2′-deoxyribose ring, herpesvirus DNA synthesis is effectively blocked by the high concentrations of PCV-TP found in herpesvirus-infected cells. PCV-TP is formed rapidly in HSV-1-, HSV-2-, and VZV-infected cells; accumulates in them; and has a long half-life. This accounts for the long-lasting antiviral activity of penciclovir. In the corresponding clinical infections, PCV-TP would be expected to remain trapped within the infected cells even when the concentration of penciclovir in the blood drops to low levels. Thus, it should be possible to treat each of these herpesvirus infections with famciclovir, the oral form of penciclovir, at a lower dosage frequency than is required for acyclovir.

ACKNOWLEDGMENTS

We thank R. L. Jarvest for supplying the synthetic standards of the phosphate esters of penciclovir; D. J. D. Tidy and A. Mahoney for supplying [4′-^{3}H]penciclovir; and A. G. Brown, M. R. Boyd, and M. R. Harnden for helpful discussions.

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