Inhibition of Hepatitis B Virus DNA Polymerase by Enantiomers of Penciclovir Triphosphate and Metabolic Basis for Selective Inhibition of HBV Replication by Penciclovir

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The deoxyguanosine analog penciclovir (PCV; 9-[4-hydroxy-3-hydroxymethyl-but-1-yl]guanine), has shown potent antiviral activity against herpes viruses and hepadnaviruses. Efficacy against chronic hepatitis B virus (HBV) infection has been demonstrated in an animal model and in recent clinical trials of famciclovir, the oral form of PCV. The antiviral activity of PCV is believed to be dependent on the intracellular formation of PCV-triphosphate (PCV-TP) which is presumed to inhibit HBV replication by interfering with viral DNA polymerase activity. The (*S*)-enantiomer is preferentially formed in herpes virus-infected cells, and is the more active against the herpes simplex virus; however, little is known about the biochemical mechanisms of PCV phosphorylation or of interference with viral replication in HBV-infected cells. Here, we report that in contrast with herpes simplex virus, the (*R*)-enantiomer of PCV-TP is a more potent inhibitor of HBV DNA polymerase-reverse transcriptase (pol-RT) *in vitro* than the (*S*)-enantiomer. In assays for HBV DNA pol-RT activity, in which purified viral core particles were the enzyme source, the IC_{50}s for (*R*)- and (*S*)-enantiomers of PCV-TP were 2.5 μmol/L and 11 μmol/L, respectively. The estimated K_is for (*R*)- and (*S*)- PCV-TP were ≈0.03 μmol/L and ≈.04 μmol/L, respectively, about 3-fold lower than the K_m for deoxyguanosine triphosphate (dGTP) in the same system. In addition, we report that PCV metabolism is similar in both control (HepG2) and in HBV-transfected (2.2.15) hepatoblastoma cells *in vitro*, indicating that cellular enzyme(s) catalyze PCV phosphorylation. Peak PCV-TP concentrations of about .4 μmol/L were reached in both cell types in less than 12 hours, and intracellular PCV-TP was exceptionally stable with a half-life of about 18 hours. These observations provide a mechanistic basis for the potent activity of PCV against HBV. (HEPATOLOGY 1996;24:996-1002.)

Abbreviations: PCV, penciclovir; HBV, hepatitis B virus; PCV-TP, penciclovir triphosphate; pol-RT, polymerase-reverse transcriptase; GdR, deoxyguanosine; ACV, acyclovir; GCV, ganciclovir; ACV-TP, acyclovir triphosphate; HPLC, high-pressure liquid chromatography; dGTP, deoxyguanosine triphosphate.

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Received March 14, 1996; accepted June 25, 1996.

Supported by the Research and Education Fund at Fairfield Hospital and by the Australian government under a syndicated research and development scheme.

This study was presented in part at the 35th interscience conference on antimicrobial agents and chemotherapy, San Francisco, CA, September 17-20, 1994; and at the First International Conference on Therapies for Viral Hepatitis, Kauai, Hawaii, December 11-15, 1994. (see references 34 and 35).

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0270-9139/96/2405-0004$3.00/0

Chronic hepatitis B virus (HBV) infection has been estimated to affect more than 300 million individuals worldwide, and those affected are unable to benefit from recently developed hepatitis B surface antigen-containing vaccines.[1] Attempts to control chronic HBV disease using antiviral chemotherapy have, until recently, been relatively unsuccessful.[2,3] Although early attempts to treat HBV infection with nucleoside analogs were often compromised by associated toxicity,[2,3] the discovery that the replication of hepadnaviral genomes involves an obligatory reverse transcription step[4,5] has stimulated renewed interest in this field. In particular, the many nucleoside analogs which have been identified as inhibitors of the human immunodeficiency virus reverse transcriptase, seem to be generally regarded as potential anti-HBV agents.[2,3] However, it is becoming increasingly clear that there are other HBV-specific enzymatic processes besides reverse transcription which are potential targets for nucleoside analogs.[6,7]

Compound screening using a variety of systems including cell-free enzyme assays, cell culture, and live animal models suggest that apart from (-)-β-L-2′,3′-dideoxy-3′-thiacytidine (lamivudine) and structurally related deoxycytidine analogs,[3,8,9] the most efficacious anti-HBV agents identified to date are deoxyguanosine (GdR) analogs.[2,3] Members of the latter group with proven anti-HBV activity include acyclovir (ACV),[10] dideoxyguanosine,[11,12] ganciclovir (GCV),[13-17] penciclovir (PCV),[18-22] and the carbocyclic analog of 2′-deoxyguanosine,[23,24] the structures of which are compared in Fig. 1. Relatively minor alterations in the structure of nucleoside analogs can produce large alterations in biological activity, so that individual analogs must be studied as separate entities, besides being seen in the context of particular groups.[2,3] This is particularly true for GdR analogs.[11,12,18,21,23,25-28] For example, PCV appears to be a more potent and specific inhibitor of hepadnaviral replication, both *in vitro*[18] and *in vivo*[15,19,20] than GCV, which is structurally identical except that PCV contains a methylene group in place of oxygen in the pentose ring (Fig. 1).

In addition, it is becoming apparent that different isomers of the same structure may differ in specific activities.[3] The intracellular phosphorylation of PCV can generate two different enantiomers of polymerase-reverse transcriptase PCV-TP[29] which differ substantially in antiviral activity.[6,27,28,30,31] The (*S*)-enantiomer of PCV-TP is preferentially formed by a virally encoded deoxynucleoside kinase in herpes symplex virus-infected cells and has been found to be a more effective inhibitor of herpes symplex virus DNA pol than the (*R*)-enantiomer.[30] However, the enzyme which catalyzes the phosphorylation of PCV in HBV-infected cells is unknown, but it is unlikely to be a virally encoded deoxynucleoside kinase.[2,3] In addition, gene sequence data show only limited homology between HSV DNA pol and HBV DNA pol-RT, which belong to different polymerase families.[32,33] It is not yet known whether one or another enantiomer of PCV is preferentially

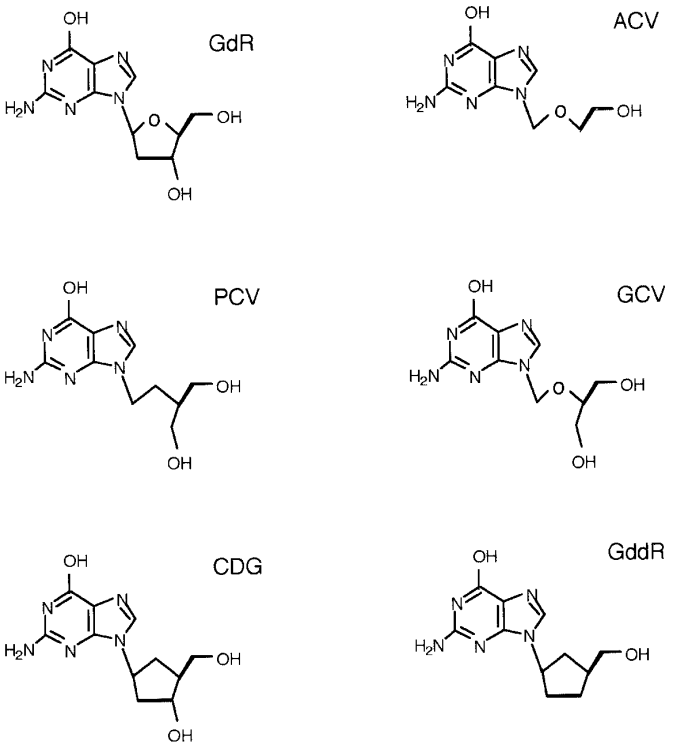

FIG. 1. Structures of GdR and five of its analogs that have proven anti-HBV activity. CDG, carbocyclic 2′-deoxyguanosine; GddR, 2′,3′-dideoxyguanosine.

formed in HBV-infected cells or whether the enantiomers differ in potency as inhibitors of HBV replication. Accordingly, our aims were to investigate the activity of PCV-TP against partially purified HBV DNA pol-RT and to investigate the metabolism of PCV in uninfected and HBV-transfected human hepatoblastoma cells.

MATERIALS AND METHODS

Radiochemicals

Racemic PCV-TP, purified (*R*)- and (*S*)- PCV-TP enantiomers, ACV-TP, PCV, and [4′-^{3}H]PCV (specific activity 1.3 Ci/mmol) were supplied by SmithKline Beecham Pharmaceuticals (Harlow, Essex, UK). The purity of each PCV-TP enantiomer was >90%. [2′-^{3}H]ACV (specific activity 31 Ci/mmol) and [8-^{3}H]GCV (specific activity 17.6 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). [8-^{3}H]GdR (specific activity 8.4 Ci/mmol) was purchased from ICN Pharmaceuticals Australasia (Seven Hills, New South Wales, Australia). [α-^{32}P]dGTP and [α-^{32}P]deoxycytidine triphosphate (specific activity of each ≈3,000 Ci/mmol) were from Amersham International (Amersham, Buckinghamshire, UK). Calf intestinal alkaline phosphatase and *Crotalus durissus* phosphodiesterase were obtained from Boehringer Mannheim Australia (Castle Hill, New South Wales, Australia). All other chemicals and reagents were purchased from local suppliers and were analytical grade or high-pressure liquid chromatography (HPLC) grade.

Cell Lines

The 2.2.15 cell line was originally produced by transfecting a human hepatoblastoma cell line (HepG2) with a plasmid containing two head-to-tail dimers of human HBV.[36] Both cell lines, which were obtained initially from Dr. Allison Jilbert of IMVS Adelaide, South Australia, were cultured in Dulbecco's modification of Eagle's medium supplemented with 10% vol:vol of fetal calf serum (Commonwealth Serum Laboratories, Victoria, Australia), ampicillin and gentamicin (40 μg/mL of each) at 37°C in a humid atmosphere containing 5% vol:vol of CO_2.

Inhibition of HBV DNA Polymerase-RT

HBV DNA Pol-RT Assays. Methods for polymerase assay were based on previously published methods.[10,24] Human HBV core particles were isolated and partially purified from cultures of 2.2.15 cells through a sucrose density gradient centrifugation. Partially purified cores were pelleted by ultracentrifugation (3 hours at 40,000 rpm in a Beckman SW 41 rotor (Beckman Industries, Palo Alto, CA) at 4°C). The pellet was resuspended in 10 mmol/L Tris-HCl (pH 7.4) containing 1.5% vol:vol of Triton-X100 and 0.1% vol:vol of 2-mercaptoethanol. Small (50 μL) aliquots of HBV core preparations were frozen at −70°C until used as the enzyme source in subsequent assays. For IC_{50} determinations, assays were performed at 37°C in a total volume of 15 μL containing final concentrations of: 30 mmol/L Tris-HCl (pH 7.4), 3 mmol/L $MgCl_2$, 10 μmol/L deoxyguanosine triphosphate (dGTP), thymidine triphosphate, and deoxycitidine triphosphate; .01 μmol/L [α-^{32}P]dGTP and varying concentrations of PCV-TP or ACV-TP. For determination of the Michaelis Constant (K_m) for dGTP and K_is for PCV-TP, assay conditions were identical except that the labeled deoxynucleotide was [α-^{32}P]deoxycitidine triphosphate and the dGTP concentration was varied. Preliminary experiments established that the reaction rates were linear for at least the first hour, which was the routinely used reaction time. The controls in which the enzyme was added at the end of the incubation period were included in each set of assays, and the assay results were corrected for background (control) activity. Reactions were stopped by spotting 10-μL aliquots of each reaction mix onto a glass fiber disc that was presoaked in 10% wt:vol of trichloroacetic acid. The discs were dried before being washed in ice-cold 10% trichloroacetic acid containing 10 mmol/L sodium pyrophosphate. Three further 10 minute rinses in ice-cold 5% trichloroacetic acid followed. The washed discs were finally rinsed in absolute ethanol, air-dried, and counted for trichloroacetic acid-insoluble radioactivity by liquid scintillation counting. Data were analyzed by using EnzymeKinetics version 1.2 (Trinity Software, Campton, NH).

Metabolism of [^{3}H] Penciclovir in 2.2.15 or HepG2 Cells

Cell Cultures and Treatment. Plastic 25 cm^2 tissue culture flasks were seeded with ≈2.0 × 10^6 HepG2 or 2.2.15 cells in 10.0 mL of culture medium. When the cell monolayers were almost confluent (after approximately 3 days), the medium was aspirated and replaced with 2.0 mL of fresh medium containing 2.0 μCi/mL [^{3}H]nucleoside tracer and unlabeled nucleoside at final concentrations of 1.0 μmol/L for PCV, GCV, and ACV or of 5.0 μmol/L for GdR. Incubation with radiolabel was continued for 24 hours. For determination of the intracellular half-life of PCV-TP, incubation was continued for up to 3 days. In some experiments, combinations of two compounds, one [^{3}H]labeled with a 100-fold excess of the second (unlabeled) nucleoside, were used. Specific details appear in figure captions and with results. After incubation for the indicated times, duplicate cultures were processed by removing the medium (which was saved for subsequent analysis), quickly rinsing the cells with 20 mL of phosphate-buffered saline, then extracting *in situ* with two separate 1.0-mL

TABLE 1. Separation of Guanine Nucleoside and Guanine Nucleoside Analog Metabolites Using Different Chromatographic Conditions

	Approximate retention times (min)		
	Reversed-phase	Ion-pairing (TBAP)	Anion exchange
Guanine	5.9	4.8	
Guanosine	11	6.9	
Gdr	14	7.4	5.0
GCV	7.9	5.5	
ACV	9.7	6.5	
PCV	15.2	7.5	5.0
GMP	6	7.2	8.0
dGMP	7	8.7	9.0
GTP		15.0	20
dGTP		18.9	24
GCV-TP		17.0	
ACV-TP		18.2	
PCV-TP		20.2	26

Abbreviations: TBAP, tetrabutylammonium phosphate; GMP, guanosine monophosphate; dGMP, deoxyguanosine monophosphate; GTP, guanosine triphosphate; GCV-TP, ganciclovir triphosphate; PCV-TP, penciclovir triphosphate.

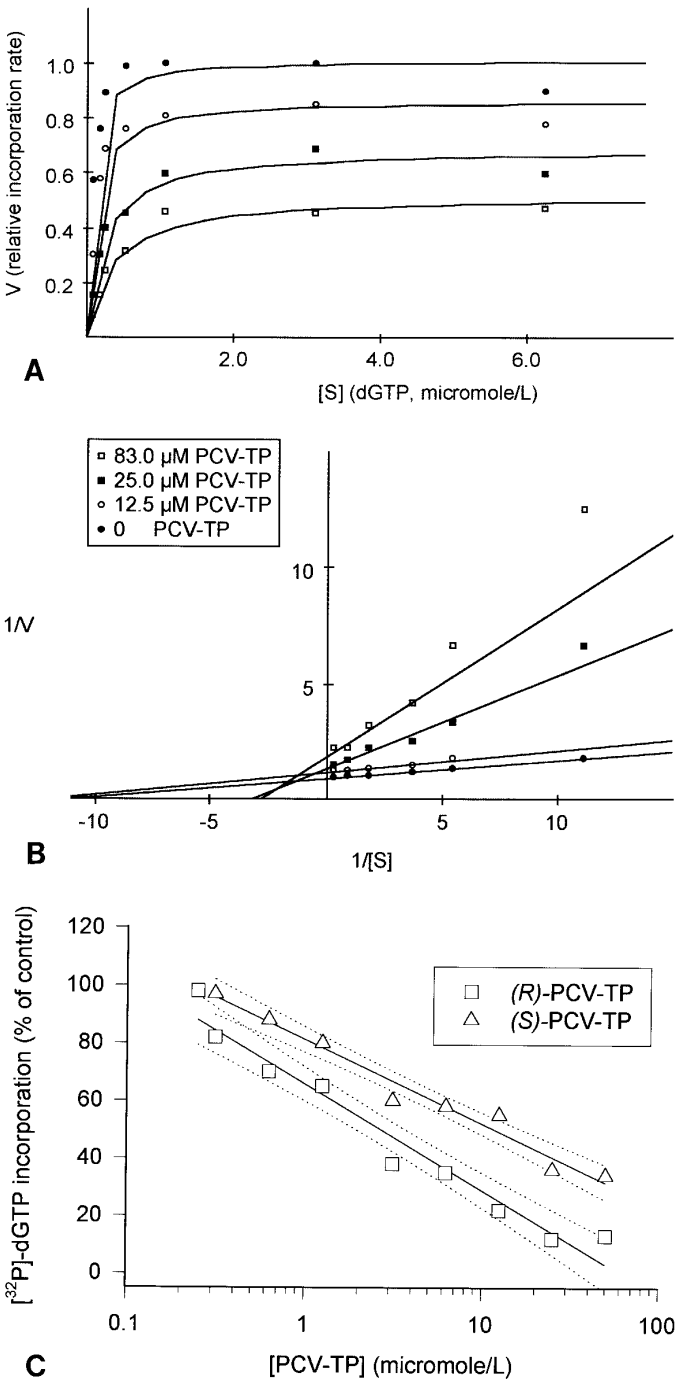

FIG. 2. Inhibition of HBV DNA polymerase by PCV-TP. Pooled data from two experiments in which polymerization rates were standardized relative to apparent V_{max} = 1.0. Plots were generated and analyzed with the aid of EnzymeKinetics version 1.2 (see Materials and Methods). (A) Plot of dGTP concentration vs. deoxynucleotide polymerization rate in the presence of different concentrations of (*R,S*)-PCV-TP. Incorporation did not follow strict Michaelis-Menten kinetics. (B) Double reciprocal plot of the same data as in (A), omitting all points corresponding to 6.25 μmol/L dGTP. Alteration of both V_{max} and K_m in the presence of PCV-TP indicates mixed inhibition (see discussion in text). (C) Preferential inhibition of HBV DNA polymerase/reverse transcriptase activity by the (*R*)-enantiomer of PCV-TP. Pooled data from three experiments, with each point representing the average of at least two determinations; (· · ·) 95% confidence limits for the regression analyses are shown.

aliquots of distilled water:acetonitrile:ethanol (80:70:50 vol:vol:vol) containing 10 mmol/L Tris-HCl (pH 7.5) for 10 minutes on ice. The cell extracts were pooled, and the cell debris was removed by centrifugation before lyophilization to dryness. Lyophilized extracts were stored at −70°C until immediately before analysis. After extraction, the residual cell layer was dissolved in 1.0 mL of 0.2% wt:vol of sodium dodecyl sulfate and 20 mmol/L Tris-Cl (pH 8) to check the extraction efficiency.[37] Cell viability was monitored in replicate cultures by neutral red uptake[38]; further replicate cultures were trypsinized, and cells were counted and sized using a Coulter STKS particle counter (Coulter Electronics, Miami, FL).

HPLC Methods. Analyses were performed at room temperature under 5 psi positive helium pressure using a BAS 200A liquid chromatograph (Bioanalytical Systems, West Lafayette, IN) equipped with an inbuilt variable wavelength ultraviolet-visible detector operating at 254 nm. Three different analytical methods were used. Table 1 shows the retention times for various nucleoside and nucleotide standards using the different systems. The peaks of interest were identified where possible by coelution with known standards, by comparison of chromatographic profiles obtained using different systems, and in the case of nucleoside phosphates by peak shifts after digestion with alkaline phosphatase and/or phosphodiesterase.[27]

For analyses of culture media, protein was precipitated with an equal volume of 7% wt:vol of perchloric acid and removed by centrifugation. Aliquots of the supernatant (20 μL) were injected onto a pH-stable reversed phase C_{18} column (4.6 × 250 mm) (Inertsil, SGE Australia) (Ringwood, Victoria, Australia) that was protected by a guard column containing the same stationary phase, and were eluted with 3.0% acetonitrile in 70 mmol/L ammonium phosphate (pH 8.0). The solvent reservoir was sparged with helium; the flow rate was .7 mL/min, and .35 mL fractions of eluant were collected and mixed with 4 mL of liquid scintillation cocktail (OptiPhase 'HiSafe') (Wallac, Turku, Finland) before assay by scintillation counting.

The same system was used to analyze lyophilized cell extracts except that the ion-pairing agent tetrabutylammonium phosphate was included in the eluant. The addition of tetrabutylammonium phosphate (final concentration, 5mmol/L) reversed the normal elution sequence for nucleosides and their phosphorylated derivatives (Table 1). Lyophilized cell extracts were redissolved in 100 to 200 μL of distilled water, and 20-μL aliquots, (equivalent to cytosol from ≈1.0×10^6 cells) were injected onto the column.

In some experiments, peak identities were confirmed by anion exchange HPLC using a strong anion exchange column (4.6 × 250 mm) (SGE, Australia). In this case, analytes were eluted with an ammonium phosphate gradient from 5 to 500 mmol/L (pH 5.5) over 20 minutes followed by a 10 minute elution with 500 mmol/L ammonium phosphate. The column was reequilibrated with 5 mmol/L ammonium phosphate for 20 minutes between analyses.

RESULTS

Inhibition of HBV DNA Polymerase-RT by* (R,S)-*PCV-TP and by Its Separate Enantiomers. The rate of DNA polymerization by HBV DNA pol-RT in the presence of different concentrations of dGTP did not follow strict Michaelis-Menten kinetics, either in the presence or absence of PCV-TP, presumably because both the enzyme and inhibitor were mixed. In addition, the concentration of one substrate, the endogenous nucleic acid template, may become limiting if PCV-TP acts as a chain terminator (see below). HBV core-associated enzymatic activities include DNA pol, RT, RT-primase, and RNAse H, each of which may show a different susceptibility to each PCV-TP enantiomer, complicating the interpretation of results. However, it was clear that (*R,S*)-PCV-TP increased the apparent K_m for dGTP and decreased the apparent maximum polymerization rate. At the lowest concentration of PCV-TP (12.5 μmol/L) the K_m was affected more than the maximum velocity (V_{max}), whereas at the highest PCV-TP concentration (83 μmol/L), the opposite was observed (Figs. 2A and 2B). These results suggest that at lower concentrations, PCV-TP may act as a competitive inhibitor with respect to dGTP for HBV DNA pol binding sites, but at high concentrations it behaves more like a noncompetitive inhibitor. Such a phenomenon might be expected if higher concentrations of PCV-TP caused DNA chain termination, effectively reducing the amount of available enzyme and endogenous substrate. From 4 experiments, the K_i for HBV DNA pol for (R,S) PCV-TP was estimated to be .04 ± .01 μmol/L, about half that of the K_m for dGTP, which was estimated as .09 ± .03 μmol/L in the same system. Using the same assay system,

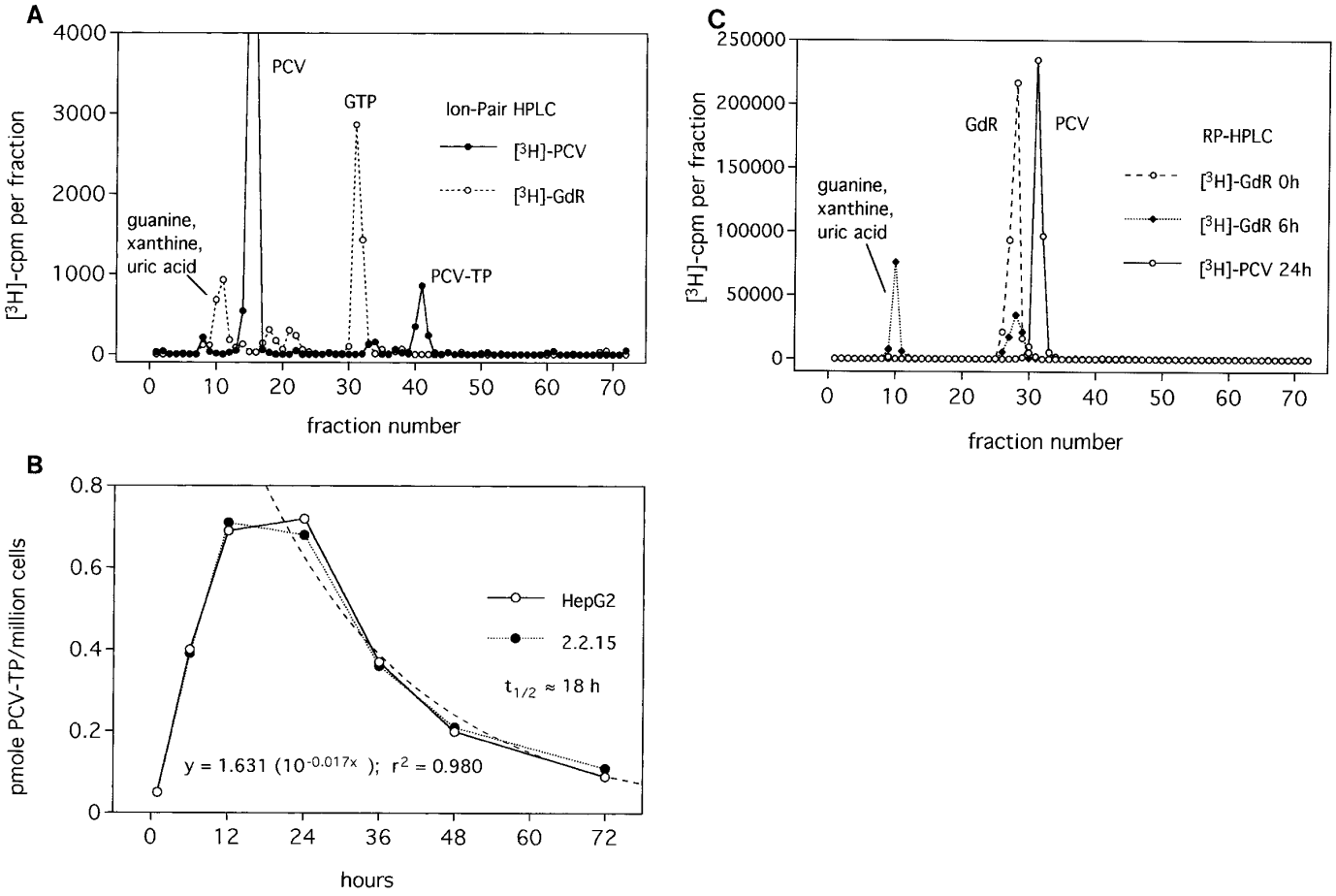

FIG. 3. (A) Penciclovir and GdR metabolism in HepG2 cells. Cells were cultured in the presence of [^{3}H]PCV (2.0 μCi/mL; 1.0 μmol/L). At the indicated times, duplicate flasks were harvested and distribution of radiolabel was analyzed by ion-pair reversed phase HPLC. Radiochromatograms from 2.2.15 cytosolic extracts were similar (not shown). In neither case was the phosphorylation of the [^{3}H]labelled compound affected by the presence in the culture medium of excess (100 μmol/L) unlabelled ACV or GCV (data not shown). (B) Phosphorylation of PCV in 2.2.15 and HepG2 cells. Cells were incubated in the presence of radiolabeled PCV (2.0 μCi/mL [^{3}H], 1 μmol/L PCV). After 24 hours, culture medium was replaced with a drug-free medium. At the indicated times, duplicate cultures were harvested and distribution of radiolabel was analyzed by ion-pairing reversed-phase HPLC. Cell-doubling times in these experiments were 4.9 and 4.0 days for 2.2.15 and HepG2 cells, respectively. After adjusting for cell numbers, similar rates of phosphorylation, plateau concentrations, and half-lives were found for PCV-TP in both cell types. The half-life of PCV-TP was 18 ± 3 hours (estimated from dotted curve), and the peak intracellular concentration of PCV-TP was ≈.7 ± .1 pmol/10^6 cells. (C) Analysis of radioactivity in cell culture medium during incubation of HepG2 cells with [^{3}H]GdR or [^{3}H]PCV. Culture medium was removed from cells either immediately or 6 hours after the addition of [^{3}H]GdR (2.0 μCi/mL, 5.0 μmol/L), or 24 hours after addition of [^{3}H]PCV (2.0 μCi/mL, 1.0 μmol/L), and radiolabel distribution was analyzed by reversed-phase HPLC. Recovery of [^{3}H]GdR in culture medium after 24 hours was negligible, with recovered [^{3}H] coeluting, with known catabolites guanine, xanthine, and uric acid. By contrast, the peak corresponding to [^{3}H]PCV remained stable during incubation; catabolites were not detected in the medium; and the fraction of total [^{3}H]PCV taken up by cells from the medium was negligible. [^{3}H]GCV and [^{3}H]ACV were similarly stable during incubation. At a starting concentration of 5 μmol/L, extracellular GdR had a half-life of ≈110 minutes (data not shown).

the K_is for (*R*)- and (*S*)-enantiomers of PCV-TP were estimated to be .03 and .04 μmol/L (the average of duplicate determinations), respectively (data not shown).

Results from 3 separate experiments, designed to compare the inhibitory effects of (*R*)- and (*S*)-PCV-TP on HBV DNA pol activity, are presented graphically in Fig. 2C. In each experiment the (*R*)-enantiomer was the more effective inhibitor. From the regression analysis of pooled data, estimated IC_{50}s for (*R*)- and (*S*)-enantiomers were 2.5 and 11.0 μmol/L, respectively. Under the same conditions, IC_{50}s for racemic PCV-TP and ACV-TP were ≈8 and 10 μmol/L, respectively.

Metabolism of [^{3}H]Penciclovir in 2.2.15 or HepG2 Cells. When 2.2.15 or HepG2 cells were incubated in the presence of [^{3}H]PCV for up to 3 days, and the cytosols were extracted and analyzed by ion-pairing HPLC, 2 major peaks corresponding to penciclovir and PCV-TP were identified. In some cytosol extracts a minor peak, assumed to be penciclovir diphosphate, on the basis of its relative retention time and digestion by alkaline phosphatase and phosphodiesterase, was also present (Fig. 3A). The rates of uptake and phosphorylation of [^{3}H]PCV in 2.2.15 and HepG2 cells were virtually identical, as shown in Fig. 3B. In both cell types, the peak concentrations of PCV-TP of .7 ± .1 pmol/10^6 cells were reached within the first 12 hours. Using a Coulter STKS particle counter (Coulter Electronics), a mean single-cell volume of ≈1600 fL was estimated for both the 2.2.15 and HepG2 cells. One million cells would therefore occupy a total volume of ≈1.6 μL. Assuming uniform intracellular distribution, peak PCV-TP concentrations would be ≈.4 μmol/L, about 10-fold higher than the estimated K_i for HBV DNA polymerase.

Parallel experiments in which [^{3}H]ACV or [^{3}H]GCV were used in place of [^{3}H]PCV were performed. Concentrations of their intracellular metabolites were either at (for GCV) or below (for ACV) the reliable limit of detection, which was ≈.05 pmol/10^6 cells; i.e., the intracellular concentrations of ACV-TP and GCV-TP were about 10-fold less than those of PCV-TP. In some experiments, minor peaks of radioactivity were found eluting both before and after the position where a nucleoside triphosphate peak was expected, following label-

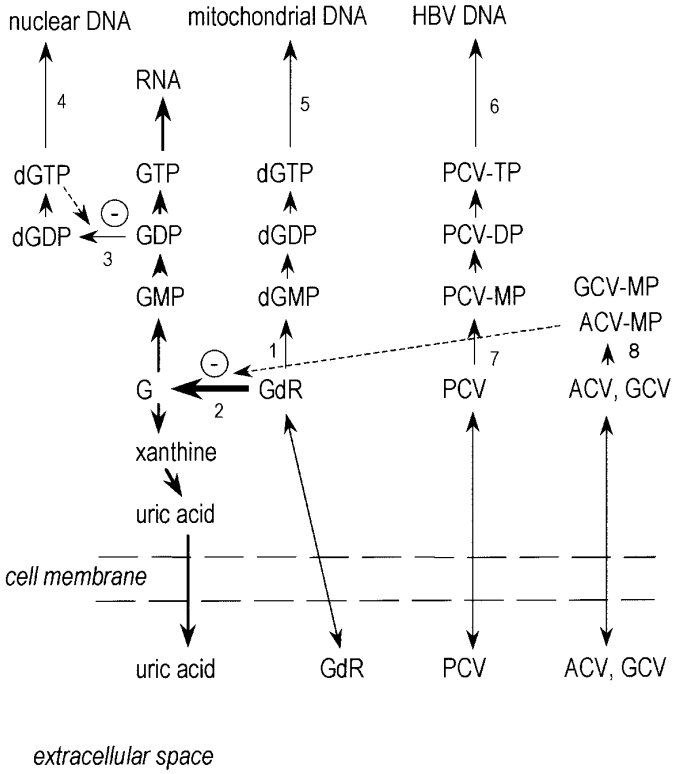

FIG. 4. Probable pathways for metabolism of GdR and its analogs in HBV-infected hepatocytes. Cellular uptake and efflux may be regulated by membrane transporters. Key enzymes are: 1) mitochondrial GdR kinase; 2) purine nucleoside phosphorylase; 3) ribonucleotide reductase; 4) DNA pols $\alpha,\beta,\delta,\epsilon$; 5) DNA pol γ; 6) HBV DNA pol/RT; 7) unknown, possibly mitochondrial GdR kinase; and 8) unknown, possibly 5′-nucleotidase. The dotted lines indicate the inhibition of purine nucleoside phosphorylase by ACV-MP and GCV-MP and of ribonucleotide reductase by dGTP. MP, monophosphate.

ing with [^{3}H]ACV or [^{3}H]GCV. These peaks were alkaline-phosphatase resistant and were not identified.

The analysis of cell culture medium by reversed-phase HPLC confirmed that ACV, GCV, and PCV were stable in cell culture for at least 3 days and were therefore subject neither to enzymatic degradation by purine nucleoside phosphorylase nor to significant enzymatic or nonenzymatic oxidation. By contrast, with [^{3}H]acyclic analogs, [^{3}H]GdR disappeared from the medium with an initial half-life of less than 2 hours (for 5 μmol/L GdR) and [^{3}H]tracer was recovered mainly as guanosine triphosphate in cytosolic extracts or as material which coeluted with the catabolites guanine, xanthine, and uric acid in the culture medium (Fig. 3C).

DISCUSSION

PCV and the related acyclic GdR analogs, GCV and ACV, were originally developed as antiherpes agents.[2,3,30] In common with all other nucleoside analogs, they are believed to be biologically active only after phosphorylation to the corresponding nucleotides,[2,3] a process which must necessarily occur within the cell because cell membranes are relatively impermeable to polar compounds, including nucleotides. In addition, the presence of ubiquitous extracellular phosphatases and nucleotidases ensures that in most tissues, exogenous nucleotides are rapidly degraded.[2] Cellular uptake and the phosphorylation of nucleoside analogs have been studied extensively and intensively in a variety of contexts, and in virtually all cases examined to date, the initial phosphorylation to the monophosphate level has been identified as the rate-limiting anabolic step.[2,39] In cells infected with herpes simplex virus or Varicella-Zoster virus, this crucial initial phosphorylation of acyclic GdR analogs is catalyzed by virally-encoded deoxynucleoside kinases.[30] Observations that noncytotoxic concentrations of acyclic GdR analogs were also active against hepadnaviruses[11-24] were therefore unexpected, because HBV genomes lack sequences that are indicative of any (deoxy)nucleoside kinase-encoding potential.[2]

The results presented here indicate that PCV phosphorylation in HepG2 and 2.2.15 cells, and by extension in HBV-infected hepatocytes, must be catalyzed entirely by cellular enzymes (Fig. 3). The identity of enzyme(s) which catalyze the initial phosphorylation of PCV in these cells is not known, a cytosolic (deoxy)nucleoside kinase[2,39] does not appear to be implicated. Although it has been reported that mitochondrial deoxyguanosine kinase can phosphorylate GCV,[40] PCV,[27] and (*R*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]-guanine (H2G),[27] the enzyme shows strong preference for purine nucleosides in which the pentose ring is complete and phosphorylates acyclic purine analogs with very low efficiency.[39,41] Furthermore, the enzyme which phosphorylates PCV is either distinct from enzyme(s) which catalyze GCV and ACV phosphorylation, or it is much more efficient with PCV as a substrate, because phosphorylation of [^{3}H]PCV-TP was unaffected by a 100-fold mol/L excess of either ACV or GCV (see legend to Fig. 3).

Phosphorylation seems to be the major, if not only metabolic fate of ACV, GCV, and PCV in HepG2 and 2.2.15 cells,

TABLE 2. *In Vitro* Metabolic and Inhibitory Characteristics of PCV, GCV, and ACV Triphosphates and Their Parent Nucleoside Analogs

Analog triphosphate	K_i for HBV DNA pol-RT (μmol/L)	IC_{50} for HBV DNA pol-RT (μmol/L)	Intracellular triphosphate concentration (pmol/million cells)	Rate of nucleoside phosphorylation by mitochondrial GdR kinase‡,§	Nucleoside IC_{50} for HBV in 2.2.15 cells *in vitro*¶ (μmol/L)	Nucleoside IC_{50} for DHBV in primary duck hepatocytes *in vitro*# (μmol/L)
(*R*, *S*)-PCV-TP	.04 ± .01*	8.0	.7	6.7	.6 ± .05	.7 ± .1
(*R*)-PCV-TP	.03†	2.5	—	—		
(*S*)-PCV-TP	.04†	11.0	—	—		
GCV-TP	nd‖	nd‖	≈.1	5.8		4 ± .2
ACV-TP	nd	10.0	<.05	1.4	>100	>10

Abbreviations: PCV-TP, penciclovir triphosphate; GCV-TP, ganciclovir triphosphate; ACV-TP, acylclovir triphosphate.

* Means ± SD, 4 experiments (estimated from Dixon plots).

† Average of duplicates.

‡ % of phosphorylation rate for GdR.

§ Data from references 27 and 40.

‖ Not determined.

¶ Data from reference 21.

Previous work from this laboratory (see reference 18 and unpublished data).

in contrast to GdR, which rapidly undergoes phosphorolysis by purine nucleoside phosphorylase, an enzyme which is abundant in hepatocytes,[42] where it plays an important role in purine metabolism, being central to both the anabolic and catabolic pathways (Fig. 4). Phosphates of ACV and GCV, among other acyclic purine nucleotide analogs, are known as potent inhibitors of purine nucleoside phosphorylase, with K_is in the nanomolar range[43,44]; but this property does not appear to be shared by PCV nucleotides, because excess PCV (and by implication, its phosphates) did not inhibit GdR phosphorolysis (see legend to Fig. 3).

Our results indicate that in hepatoblastoma cells, the guanine moiety of GdR is salvaged via purine nucleoside phosphorylase, hypoxanthine-guanine phosphoribosyl transferase, and nucleotide kinases to generate guanosine triphosphate, as shown in Fig. 4. This is consistent with observations by Price et al.,[24] who found that radiolabel from extracellular [8-^{3}H]dGTP was incorporated mainly into the RNA of HepG2 and 2.2.15 cells. Together, these observations suggest that the reduction of guanosine triphosphate by ribonucleotide reductase is the main route for the generation of dGTP in hepatoma cells, and imply that it is also the source of dGTP for viral DNA synthesis in 2.2.15 cells and HBV-infected hepatocytes. Assuming that this is so, it is also possible that PCV-TP, like dGTP,[45] may inhibit ribonucleotide reductase activity, which could be expected to enhance its antiviral activity via the depletion of the endogenous deoxynucleotide triphosphate pools.

We estimated that peak intracellular concentrations of PCV-TP were $\approx .7 \pm .1$ pmole/million cells after incubation with 1 μmol/L PCV for 24 hours. By contrast, ACV and GCV triphosphates were barely detectable after incubation under similar conditions, which may account for their reduced activity against human and duck HBV compared to PCV.[14-16,18-21] Assuming uniform intracellular distribution, the intracellular plateau concentration of PCV-TP in HepG2 and 2.2.15 cells would be $\approx .4$ μmol/L, 10-fold greater than its K_i for the inhibition of HBV DNA pol-RT, but 400 to 500-fold less than the reported K_is for the inhibition of DNA pol α or DNA pol α–associated DNA primase activity.[26,28] The data summarized in Table 2 indicate that the PCV-TP concentration produced in hepatocytes is probably sufficient to cause the very efficient inhibition of HBV replication without affecting cellular DNA pol, for which data is available. Furthermore, although mitochondrial deoxyguanosine kinase may phosphorylate PCV (see above) (Table 2), the absence, to date, of reports of the impairment of respiration-dependent functions following long-term treatment with ACV, GCV, or PCV (as famciclovir)[2,46] confirm that acyclic dGTP analogs are poor inhibitors of DNA polγ[47] and support the view that long-term penciclovir treatment of HBV is unlikely to be associated with cumulative mitochondrial or cellular toxicity.

We are not able to determine whether PCV is incorporated internally into HBV DNA because of the very low levels of incorporation under our experimental conditions; so the precise mechanism by which PCV causes the inhibition of HBV replication remains unknown. The addition of PCV to nascent HBV DNA chains would not necessarily cause immediate chain termination, because PCV possesses the equivalent of a 3′ hydroxyl group, and HBV polymerases, unlike cellular DNA polymerases, lack proofreading ability[33]; however, Ilsley et al.[26] found that PCV-TP, like ACV-TP, but unlike GCV-TP, can behave as a chain terminator, which is consistent with the noncompetitive inhibition of HBV DNA pol-RT that is observed here at higher concentrations of PCV-TP (Fig. 2). The incorporation of PCV into DNA is therefore likely to result in premature chain termination; but if PCV does become incorporated internally into DNA, destabilized or nonfunctional genomes may be produced (analogous to consequences of carbocyclic 2′-deoxyguanosine incorporation into HBV DNA[24]). The latter may perhaps explain the suppression of viral RNA synthesis caused by PCV treatment of duck hepatitis B virus infection.[20] The consideration of an HBV replication strategy indicates that most DNA polymerase activity associated with cell-derived HBV core particles is RNA-dependent rather than DNA-dependent (i.e., is RT).[4,5] Interestingly, micromolar concentrations of both enantiomers of PCV-TP have been recently shown to block the priming of HBV reverse transcription,[6] with the (*R*)-enantiomer being the more effective, and (*R*)-PCV-TP has been also reported to be the more potent inhibitor of human immunodeficiency virus RT activity.[31] Collectively, these data seem to identify (*R*)-PCV as a relatively specific inhibitor of the unique processes that are associated with HBV first-strand synthesis. Further investigation of this possibility must await the availability of purified HBV DNA pol-RT, which is active on exogenous nucleic acid templates. Further work will be required to identify the enzyme(s) responsible for PCV phosphorylation and also to determine whether one or the other PCV-TP enantiomer is preferentially formed in hepatocytes.

Acknowledgment: We are very grateful to Malcolm Boyd for advice and encouragement, to Anthony Vere Hodge for reviewing the manuscript and for many constructive suggestions, and to SmithKline Beecham Pharmaceuticals (Harlow, Essex, UK and King of Prussia, PA) for their cooperation and supply of nucleoside analogs.

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