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Conversion of 1-[((*S*)-2-Hydroxy-2-Oxo-1,4,2-Dioxaphosphorinan-5-yl)Methyl]Cytosine to Cidofovir by an Intracellular Cyclic CMP Phosphodiesterase

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Cidofovir (HPMPC) {1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]-cytosine} is an acyclic nucleotide analog with potent and selective activity against herpesviruses. The prodrug, cyclic HPMPC (cHPMPC) {1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine}, has antiviral activity similar to that of the parent compound but exhibits reduced toxicity in animal models. cHPMPC is converted to cidofovir by a cellular cyclic CMP phosphodiesterase (EC 3.1.4.37) which hydrolyzes a variety of substrates, including adenosine 3′,5′-cyclic monophosphate (cAMP) and cytidine 3′,5′-cyclic monophosphate (cCMP). The K_m and V_{max} values for hydrolysis of cHPMPC by cCMP phosphodiesterase purified from human liver are 250 μM and 0.66 nmol · min^{-1} · unit^{-1}, respectively. These values are similar to the K_m and V_{max} values for cAMP (23 μM and 1.16 nmol · min^{-1} · unit^{-1}, respectively) and cCMP (75 μM and 2.32 nmol · min^{-1} · unit of enzyme^{-1}, respectively). The catalytic efficiency (V_{max}/K_m ratio) of this enzyme for the cHPMPC substrate is only 10- to 20-fold lower than those for the natural cyclic nucleotides, indicating that cHPMPC is a viable intracellular substrate for the human enzyme. Kinetic analysis indicates that cHPMPC, cAMP, and cCMP are competitive with respect to each other and that they are hydrolyzed by the same enzyme. cHPMPC is hydrolyzed to cidofovir in all primary human cell systems tested, including those derived from target organs that might be infected in patients with human cytomegalovirus (HCMV) disease. Importantly, hydrolysis of cHPMPC is not diminished in cells infected with HCMV.

Cidofovir (HPMPC) {1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine} is an acyclic nucleotide analog of dCMP (Fig. 1) with potent in vitro and in vivo activity against a broad spectrum of herpesviruses, including cytomegalovirus (CMV) (4, 5, 13). Cidofovir diphosphate, the active metabolite of cidofovir, exerts its antiviral effect by functioning as a competitive inhibitor and alternative substrate, with respect to dCTP, of the human CMV (HCMV) DNA polymerase (28). Cidofovir is metabolized to its active form by cellular enzymes (9, 10) and thus differs from antiherpetic nucleoside analogs such as ganciclovir which are initially phosphorylated to the monophosphate form by virus-encoded enzymes. This difference in metabolism explains why cidofovir can confer prophylaxis to uninfected cells (26). An additional benefit of cidofovir is that it can be administered infrequently due to the long intracellular half-lives of the diphosphate form and of the phosphocholine adduct, which has been postulated to function as an intracellular reservoir (1, 19).

Cidofovir has recently been approved for the treatment of CMV retinitis in AIDS patients. In clinical trials, cidofovir has shown dose-dependent anti-HCMV activity, as measured by HCMV levels in urine and semen samples from patients (21, 26), and has been shown to delay the progression of HCMV retinitis (20). Nephrotoxicity, the dose-limiting toxicity in humans and in animal models, is due to accumulation of the compound in proximal convoluted tubule (PCT) cells (18, 26). The nephrotoxicity can be mitigated by the coadministration of probenecid and hydration (26). Pharmacokinetic and toxicology data indicate that cidofovir is concentrated in the PCT cells through the action of the organic anion transporters in these cells (3).

Cyclic HPMPC (cHPMPC) is a prodrug of cidofovir which is stable in plasma but is readily converted to cidofovir in cells (3) (Fig. 1). Because of the efficient intracellular conversion of cHPMPC to cidofovir, cells incubated with cHPMPC or cidofovir accumulate similar levels of the active metabolite, cidofovir diphosphate. Consistent with this observation, cHPMPC and cidofovir have similar activities against herpesviruses in vitro and in vivo (3). However, in animal models, cHPMPC is less nephrotoxic than cidofovir (18), most likely due to its reduced charge altering its rate of transport by the anion transporters in the basolateral and/or luminal membranes of the PCT cells (3). The observation that, in vitro, cHPMPC has an efficacy similar to that of cidofovir, with substantially reduced toxicity in animals, has suggested that cHPMPC might provide a greater therapeutic index for treatment of viral diseases in patients.

In this report, conversion of cHPMPC to cidofovir is demonstrated in several primary human cell lines derived from tissues that are known to be infected in patients with HCMV disease. It is also demonstrated that infection with HCMV does not compromise the ability of cells to convert cHPMPC to cidofovir. In addition, the human cellular enzyme which converts cHPMPC to cidofovir is identified, and its kinetic interaction with cHPMPC is characterized.

MATERIALS AND METHODS

Materials. cHPMPC was synthesized as described previously (3). [5-^{3}H]cHPMPC (11.5 Ci/mmol), [2,8-^{3}H]adenosine 3′,5′-cyclic monophosphate (30 Ci/mmol), and [5,6-^{3}H]cytosine 3′,5′-cyclic monophosphate (10 Ci/mmol) were from Moravek Biochemicals (Brea, Calif.). Q Sepharose Fast Flow ion-exchange resin and Sephacryl S-100 HR gel filtration medium were from Pharmacia (Piscataway, N.J.). All nucleotides, cAMP-agarose (C-8 linkage), and gel filtration standards were from Sigma Chemical Co. (St. Louis, Mo.). Pefabloc SC protease

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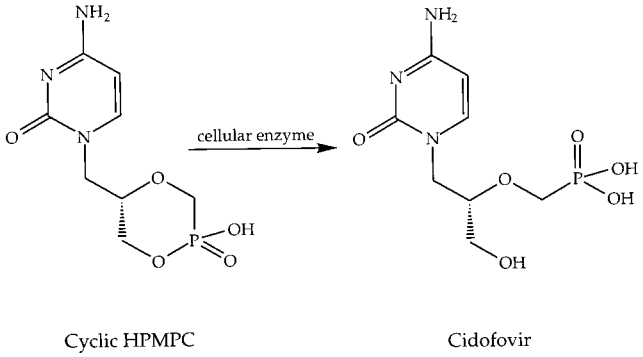

FIG. 1. Structures of cHPMPC and cidofovir.

inhibitor was from Boehringer Mannheim (Indianapolis, Ind.). Polyethylenimine cellulose thin-layer chromatography (PEI TLC) sheets were from Alltech (Deerfield, Ill.).

Cells and viruses. MRC-5 human embryonic lung fibroblast cells and Caco-2 human colorectal carcinoma cells were from American Type Culture Collection (Rockville, Md.) and were grown in Eagle's minimum essential medium containing Earle's salts and 10% fetal bovine serum. Normal human renal proximal tubule epithelial cells, normal human epidermal keratinocytes, and normal human bronchial/tracheal epithelial cells were from Clonetics (San Diego, Calif.) and were grown according to the conditions specified by the supplier. Stocks of HCMV (Towne strain; American Type Culture Collection) were prepared from MRC-5 cells infected at a low multiplicity of infection and harvested when the cells were fully cytopathic. Cells were disrupted by sonication, and cellular debris was removed by centrifugation at 2,000 × *g*. Viral stocks, with titers of approximately 10^7 PFU/ml when tested on MRC-5 cells, were stored frozen at −70°C until use.

Intracellular metabolism of cHPMPC. Metabolism of cHPMPC in tissue culture cells was measured as previously described (3, 8). Briefly, cells at 75% confluency in 75-cm^2 flasks were given 10 ml of fresh medium containing 5 μM [^{3}H]cHPMPC (specific activity, 1 Ci/mmol). Twenty-four hours later, cells were washed twice with phosphate-buffered saline, harvested following trypsinization, and counted by using hemacytometer, and the cell pellets were disrupted by repeated freeze-thaw cycles in 60% methanol. The methanol-soluble fraction was dried under vacuum, dissolved in 50 μl of water, and 10-μl aliquots were resolved on PEI TLC strips in 0.5 M LiCl. After chromatography, the strips were cut into 1-cm-thick slices, the radioactivity in each slice was quantitated, and the levels of cHPMPC, cidofovir, and its phosphorylated metabolites were determined. This solvent system does not resolve cidofovir mono- and diphosphate.

To investigate cHPMPC metabolism in HCMV-infected cells, MRC-5 cells were infected with HCMV at 0.2 PFU/cell. Seventy-two or 120 h later, fresh medium containing 5 μM [^{3}H]cHPMPC (1 Ci/mmol) was added to the cells. Twenty-four hours after the radioactive compound was added, the cells were harvested, and the intracellular metabolites were measured as described above. Mock-infected cells were analyzed in parallel.

Purification of cHPMPC-converting enzyme. Unless indicated otherwise, all procedures were carried out at 4°C.

(i) Preparation of liver cytosol. Samples (10 to 15 g) of frozen human liver (IIAM; Keystone Skin Bank, Exton, Pa.) were homogenized in 4 volumes of buffer A (50 mM Tris [pH 7.5], 250 mM sucrose, 5 mM benzamidine, 0.1 mM EGTA, 0.2 mM dithiothreitol [DTT], 50 μg of Pefabloc SC per ml, 2.5 μg of leupeptin per ml). The homogenate was centrifuged for 15 min at 25,000 × *g*, and the harvested supernatant was centrifuged for 1 h at 100,000 × *g*. The supernatant of the second centrifugation was used as the crude cytosol. Two volumes of ammonium sulfate (3 M, pH 7.9) were mixed with the crude cytosol, and the mixture was left on ice for 1 h. The sample was then centrifuged to pellet precipitated proteins, and 240 mg of solid ammonium sulfate was dissolved in each milliliter of the supernatant to produce a 90% saturated ammonium sulfate solution. This mixture was left on ice for 1 h and then centrifuged. The pelleted material was dissolved in a volume of buffer A equal to that originally used to disrupt the tissue and dialyzed extensively against buffer A lacking leupeptin and Pefabloc SC. Following dialysis and removal of insoluble material by centrifugation, the supernatant was used as the purified cytosol.

(ii) Ion-exchange chromatography. Purified cytosol was applied to a Q Sepharose Fast Flow column (1.5 by 14 cm) equilibrated with buffer B (20 mM Tris [pH 7.5], 5 mM benzamidine, 0.1 mM EGTA, 0.2 mM DTT, 10% glycerol). The column was washed with buffer B until protein levels in the effluent returned to baseline levels. Bound proteins were eluted with a linear NaCl gradient (final concentration, 300 mM NaCl; total volume, 100 ml) in buffer B and collected in 1.5-ml fractions. Cytosol application, column washing, and protein elution were all carried out at a flow rate of 0.5 ml per min.

(iii) Gel filtration chromatography. Fractions from the ion-exchange column containing peak cHPMPC-converting activity were pooled and concentrated to 0.7 ml in a Centriprep-10 concentrator (Amicon, Beverly, Mass.). Glycerol was added to a final concentration of 15%, and the sample was applied to a Sephacryl S-100 HR column (1.5 by 45 cm) equilibrated with buffer B. Proteins were eluted at a flow rate of 0.2 ml per min and collected in 1-ml fractions. Calibration of the column was performed independently of purification of the enzyme and used bovine serum albumin (BSA) (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa) as standard proteins, along with Blue Dextran.

(iv) Affinity chromatography. Fractions from the gel filtration column containing peak cHPMPC-converting activity were pooled and concentrated to 0.7 ml in buffer C (50 mM Tris [pH 7.0], 5 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol) in a Centriprep-10 concentrator. The sample was applied to a 1-ml cAMP-agarose column equilibrated with buffer C. The sample was allowed to bind to the column as described previously (17). The column was washed with three 1-ml aliquots of buffer C and with one 1-ml aliquot of buffer D (50 mM Tris [pH 7.5], 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mg of BSA per ml, 10% glycerol). Bound protein was eluted at room temperature in buffer D containing 10 mM cAMP. The eluted fractions were pooled, and cAMP was removed by several rounds of dilution in buffer B and concentration in a Centriprep-10 concentrator. Aliquots of the purified enzyme (final volume of 1 ml in buffer B containing approximately 2 mg of BSA per ml) were stored at −70°C.

Assay for cHPMPC- and cAMP-converting activity during purification. Typical assays for cHPMPC- or cAMP-converting activity in column fractions used 7.5 μl of the fraction in a total reaction mixture volume of 10 μl containing 10 μM [^{3}H]cHPMPC or [^{3}H]cAMP (both at 5 Ci/mmol), 1 mM DTT, 5 mM $MgCl_2$, and 10% glycerol. Samples were incubated at 37°C for 4 h or 1 h, respectively, for assaying cHPMPC- or cAMP-converting activity. These incubation times were within the linear initial rate for each of the reactions. cHPMPC reactions were terminated with the addition of 1 μl of a stock solution containing unlabeled cidofovir and cHPMPC at 100 mM; cAMP reactions were terminated with the addition of unlabeled AMP and cAMP at 100 mM. A 5-μl aliquot of each sample was then resolved on PEI TLC sheets in 0.3 M LiCl. Following chromatography, the migration position corresponding to cidofovir or AMP was visualized under UV light, cut out, and counted for radioactivity in liquid scintillation fluid. One unit of cHPMPC-converting activity is defined as the amount of enzyme needed to convert 1 nmol of cHPMPC to cidofovir per h at 37°C at a substrate concentration of 10 μM.

Determination of kinetic constants. For kinetic experiments, [^{3}H]cHPMPC, [^{3}H]cAMP, and [^{3}H]cCMP (5 Ci/mmol for all were used as substrates at the indicated concentrations. Assays were performed in 25-μl reaction mixtures containing 5×10^{-4} U of enzyme under the conditions described above. For evaluation of inhibitory constants, the indicated nonradioactive cyclic nucleotide was added into the reaction mixture as an inhibitor. Three time points within the linear rate of the reaction were used to determine reaction velocities. Kinetic constants were calculated by using the KinetAsyst program (Think Technologies), based on algorithms described by Cleland (11), to determine the best fit of the full data set from all substrate and inhibitor concentrations in a single experiment.

TABLE 1. cHPMPC metabolism in human cell lines[a]

Cell line[b]	Amt of metabolite (pmol/10^6 cells)		
	cHPMPC	Cidofovir	Cidofovir-p and -pp
MRC-5	0.46 ± 0.15	0.41 ± 0.06	0.29 ± 0.05
Caco-2	1.28 ± 0.91	1.38 ± 0.85	0.44 ± 0.04
RPTEC	0.08 ± 0.02	0.26 ± 0.03	0.15 ± 0.02
NHBEC	0.96 ± 0.58	1.11 ± 0.16	0.41 ± 0.01
NHEK	0.50 ± 0.06	0.39 ± 0.04	0.22 ± 0.01

[a] Cells were incubated with 5 μM [^{3}H]cHPMPC for 24 h, after which cell extracts were analyzed by TLC, and the levels of cHPMPC, cidofovir, and cidofovir mono- and diphosphate (cidofovir-p and -pp, respectively) were determined as described in Materials and Methods. Results are presented as averages ± standard deviations from at least two independent experiments.

[b] RPTEC, normal human renal proximal tubule epithelial cells; NHBEC, normal human bronchial/tracheal epithelial cells; NHEK, normal human epidermal keratinocytes.

RESULTS

cHPMPC metabolism in primary human cell lines. We have previously reported that cHPMPC is metabolized to cidofovir in MRC-5 cells, a human embryonic lung fibroblast cell line which is commonly used for the study of HCMV in cell culture (3). However, in vivo, HCMV does not generally replicate in fibroblasts (2). We therefore investigated the metabolism of

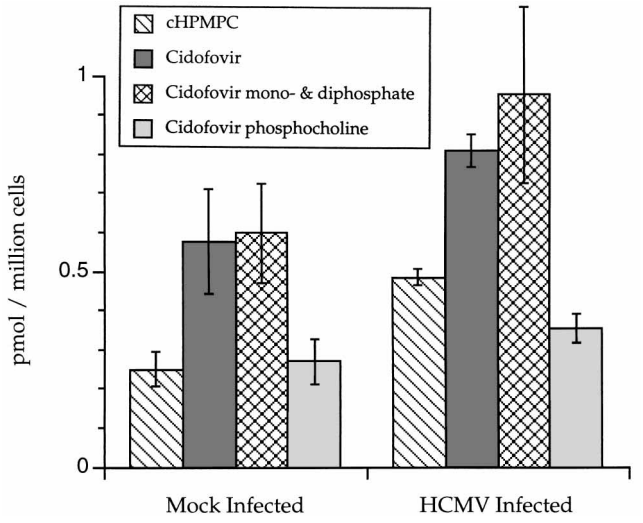

FIG. 2. HCMV infection does not inhibit cHPMPC metabolism. Intracellular levels of cHPMPC and its phosphorylated metabolites were measured in mock-infected MRC-5 cells or HCMV-infected cells. Cells were exposed to 5 μM [^{3}H]cHPMPC for 24 h beginning 72 h after infection with HCMV. Samples were processed as described in Materials and Methods. Results are presented as average values and standard deviations from two independent experiments.

cHPMPC in primary human cell lines derived from organs (lung, kidney, and skin) which are known to be infected in patients with HCMV disease (2). The results shown in Table 1 indicate that the primary human cell lines tested metabolize cHPMPC to cidofovir and its phosphorylated metabolites. Similar results were also found for Caco-2 cells, a human cell line derived from a colon adenocarcinoma. Although Caco-2 cells are not a primary cell line, the metabolism of cHPMPC in these cells is of interest due to the high incidence of colitis in patients with systemic HCMV disease.

cHPMPC metabolism in HCMV-infected cells. Cidofovir is phosphorylated by cellular enzymes (9, 10), and therefore its phosphorylation is not dependent on viral infection of cells. Furthermore, it has been demonstrated that cidofovir metabolism is not altered in herpes simplex virus-infected cells (19). To determine whether HCMV infection alters cHPMPC metabolism, we determined the levels of intracellular metabolites in MRC-5 cells exposed for 24 h to cHPMPC beginning 72 h after infection with HCMV. The results, shown in Fig. 2, indicate that HCMV-infected MRC-5 cells metabolize cHPMPC to cidofovir and its subsequent metabolites to the same extents as mock-infected cells. The higher total cellular levels of cHPMPC and its metabolites in the infected cells may be due to increased abilities of these cells to take up cHPMPC and to phosphorylate cidofovir (10). Similar results were obtained in cells exposed for 24 h to cHPMPC beginning 120 h after infection (data not shown).

Purification of the human cHPMPC-converting enzyme. Preliminary experiments indicated that the cytosolic fractions, but not the nuclear or membrane fractions, of MRC-5 cells were able to hydrolyze cHPMPC to cidofovir. The cytosolic fractions of a human liver homogenate and of A.301 cells, a human T-cell line which we have previously shown converts cHPMPC to cidofovir (3), also converted cHPMPC to cidofovir. Although all three cytosolic fractions contained limited activity, dialysis of the liver and A.301 cytosolic fractions or crude fractionation of the cytosols with 2 M ammonium sulfate substantially increased their cHPMPC-converting activity. Ammonium sulfate-fractionated cytosol was subsequently used to determine that the cHPMPC-converting activity bound to anion-exchange resins, but not to heparin agarose, blue Sepharose, or CM Sepharose, a weak cation-exchange resin. In addition, cHPMPC-converting activity was found only in the retentate following ultrafiltration of the cytosol through a 30-kDa molecular mass cutoff filter but was detected in both the retentate and ultrafiltrate when a 100-kDa molecular mass cutoff filter was used. These results suggest a molecular mass of 30 to 50 kDa for the enzyme responsible for the cHPMPC-converting activity. Also, NaCl concentrations up to 500 mM did not affect the amount of activity in the cytosol or its behavior during ultrafiltration.

Based on these preliminary results, the cHPMPC-converting activity was purified from human liver cytosol. A summary of a representative purification is presented in Table 2. Following ammonium sulfate fractionation, the purified cytosol was applied to a Q Sepharose ion-exchange column. All of the cHPMPC-converting activity bound to the column and was eluted as a single peak at 175 mM NaCl (Fig. 3A); no other activity was eluted with buffer containing up to 1 M NaCl. Fractions from the Q Sepharose column containing peak cHPMPC-converting activity were pooled, concentrated, and applied to a Sephacryl S-100 high-resolution gel filtration column. The cHPMPC-converting activity eluted as a single, symmetric peak (Fig. 3B) corresponding to a globular protein with an estimated molecular mass of 38 kDa. After these first three steps, the cHPMPC-converting activity had been purified 500-fold.

The elution profiles for the cHPMPC-converting enzyme from ion-exchange and gel filtration columns closely resembled those of a cCMP phosphodiesterase purified from pig liver (17). Additional similarities between the two enzymes were also noted, as well as similarities in the structures of cHPMPC and cCMP. The possibility that the cHPMPC-converting activity and the cCMP phosphodiesterase are the same enzyme was therefore considered.

cCMP phosphodiesterase hydrolyzes cAMP in addition to

TABLE 2. Purification of cHPMPC-converting activity from human liver

Purification step	Amt of protein (mg)	Activity (U)[a]	Sp act (U/mg)	Fold purification	Recovery (%)
Crude cytosol	228	2.0	0.009	1	100
Purified cytosol	132	6.9	0.052	6	345
Q Sepharose	6.9	1.9	0.28	31	96
Gel filtration	0.38	1.8	4.8	533	90
cAMP-agarose flow through	0.26	1.2	4.7	522	61
cAMP-agarose eluate	ND[b]	0.03	ND	ND	1.6

[a] One unit of activity is defined as the amount of enzyme necessary to convert 1 nmol of cHPMPC to cidofovir per h at 37°C at a substrate concentration of 10 μM.
[b] ND, not determined. The protein content, and thus the specific activity and fold purification, could not be determined for the cHPMPC-converting activity eluted from the cAMP-agarose column because of the presence of added BSA (0.5 mg/ml) in the eluate.

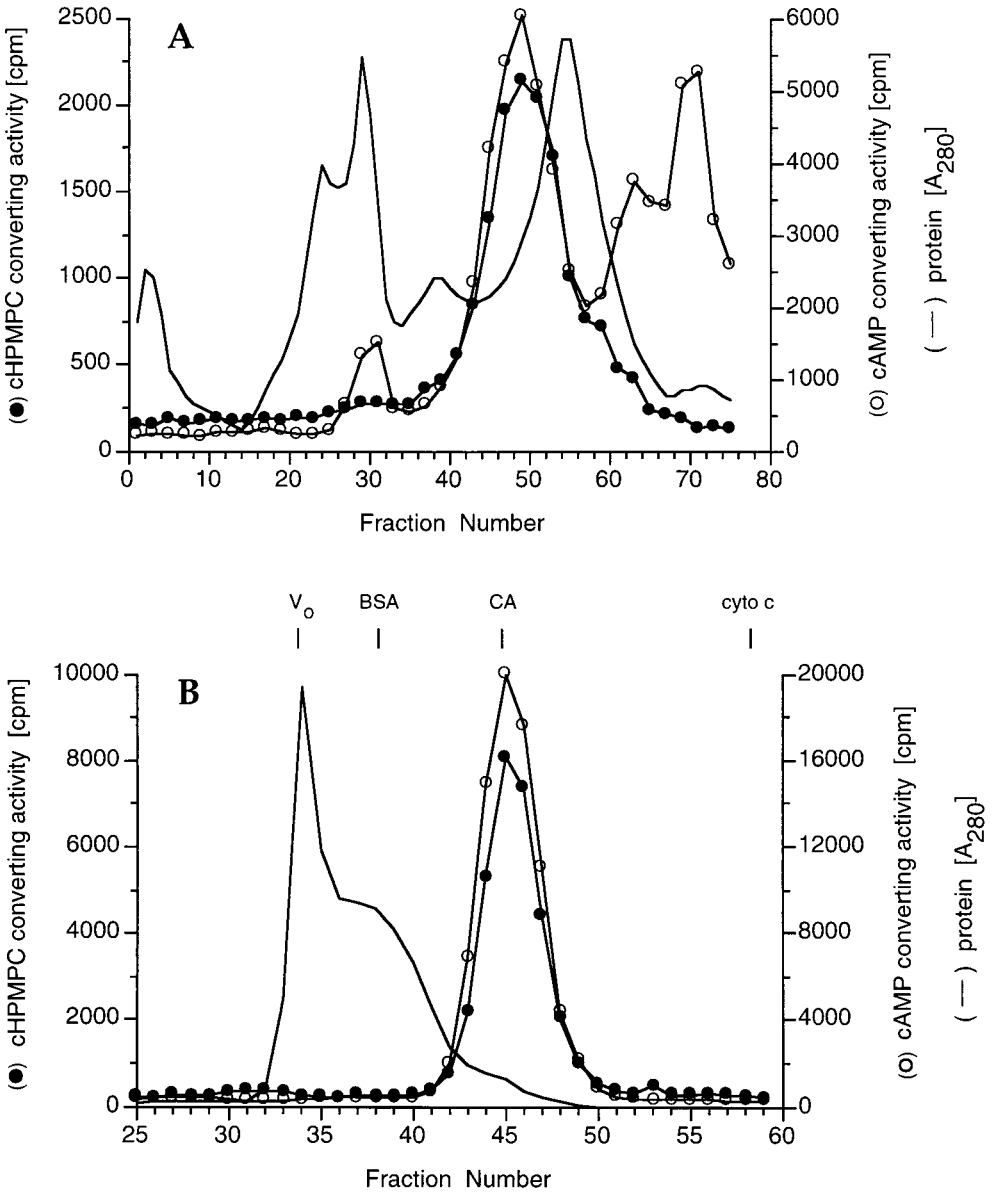

FIG. 3. Purification of cHPMPC-converting activity from human liver cytosol. (A) Purified cytosol was chromatographed on a Q Sepharose Fast Flow column. Bound proteins were eluted in fractions 15 through 75 with a linear NaCl gradient (0 to 300 mM). Odd-numbered fractions were assayed for cHPMPC- and cAMP-converting activity as described in Materials and Methods. The protein content of each fraction, based on absorption at 280 nm, is expressed in arbitrary units and is indicated by the solid line. (B) Fractions containing peak cHPMPC-converting activity from the Q Sepharose column were pooled and fractionated on a Sephacryl S-100 high-resolution column. Each fraction was assayed for cHPMPC- and cAMP-converting activity. The elution positions of the void volume (V_o) and the standard proteins (BSA, carbonic anhydrase [CA], and cytochrome *c* [cyto c]) are indicated. The protein content of each fraction is indicated by the solid line.

cCMP, and it is inhibited by inorganic phosphate but not by classic cyclic nucleotide phosphodiesterase inhibitors such as 3-isobutyl-1-methylxanthine (IBMX) (15). Therefore, fractions collected from the Q Sepharose column were assayed for cAMP-hydrolyzing activity (Fig. 3A). Although there were several distinct peaks of cAMP-converting activity, only the one which coeluted with the cHPMPC-converting activity was inhibited by inorganic phosphate, and it was not inhibited by IBMX (data not shown). The cHPMPC- and cAMP-converting activities coeluted from the gel filtration column both in terms of the peak fraction and the shape of the curve (Fig. 3B), and again the cAMP-hydrolyzing activity was inhibited by inorganic phosphate. These results provide further evidence that the cCMP phosphodiesterase and the cHPMPC-converting activity are the same enzyme.

Helfman et al. (17) used a cAMP affinity column to purify the pig liver cCMP phosphodiesterase to homogeneity. We therefore used a cAMP-agarose column as the final step to purify the cHPMPC-converting activity. The fold purification following the affinity column step could not be determined because BSA was included in the elution buffer to stabilize the protein. However, no protein bands other than those from BSA were observed on silver-stained sodium dodecyl sulfate-polyacrylamide gels of the purified enzyme (data not shown).

Kinetic analysis of the purified cHPMPC-converting enzyme. The kinetic constants for the hydrolysis of cHPMPC,

TABLE 3. Comparison of purified human cHPMPC-converting activity and porcine cCMP phosphodiesterase

Parameter (unit)	Value for enzyme	
	Human cHPMPC-converting activity	Porcine cCMP phosphodiesterase[a]
K_m (μM)		
cHPMPC	250 ± 23	ND[b]
cCMP	75 ± 7	182 ± 8
cAMP	23 ± 4	25 ± 3
V_{max}[c]		
cHPMPC	0.66 ± 0.03	ND
cCMP	2.32 ± 0.42	4.1 ± 0.2
cAMP	1.16 ± 0.11	1.6 ± 0.2
K_i (μM) (substrate/inhibitor)		
cHPMPC/cAMP	47 ± 9	ND
cAMP/cHPMPC	1470 ± 245	ND
cAMP/cCMP	425 ± 55	242 ± 56
Mol wt[d]	38,000	31,000
% Inhibition by[e]:		
Pi	87	90
IBMX	19	16

[a] Data from references 15 and 17.
[b] ND, not determined.
[c] V_{max} values are in nanomoles · minute^{-1} · unit of enzyme^{-1} for cHPMPC-converting activity and in micromoles · minute^{-1} · milligram^{-1} for cyclic CMP phosphodiesterase.
[d] Molecular weight estimates are based on gel filtration, assuming the proteins are globular.
[e] Inhibitors were present at 2.5 or 1 mM for cHPMPC-converting activity or cyclic CMP phosphodiesterase, respectively.

cAMP, and cCMP by the purified enzyme are shown in Table 3. The K_m value for cHPMPC was 3-fold higher than that for cCMP and 10-fold higher than that for cAMP. The V_{max} for cHPMPC was one-half to one-third that of the natural substrates, resulting in a V_{max}/K_m ratio for cHPMPC that was only 10- to 20-fold lower than for cAMP or cCMP. Since the V_{max}/K_m ratio is indicative of the catalytic efficiency of an enzyme for a specific substrate, these results indicate that cHPMPC is a viable substrate for the human enzyme.

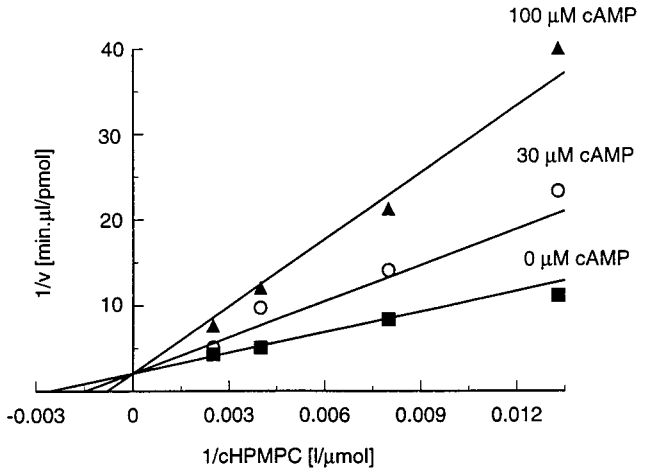

FIG. 4. Inhibition of cHPMPC hydrolysis by the purified cHPMPC-converting enzyme. Double-reciprocal plot of the inhibition by cAMP of cHPMPC hydrolysis by the purified cHPMPC-converting enzyme eluted from the cAMP-agarose column. Assays were performed and analyzed as described in Materials and Methods.

Inhibition experiments using cHPMPC as the substrate demonstrated that cAMP is a competitive inhibitor of the purified enzyme with respect to cHPMPC (Fig. 4). Competitive kinetics are also observed when cAMP is used as the substrate and either cHPMPC or cCMP are used as the inhibitor (Table 3). These results indicate that all three cyclic nucleotides are substrates for the purified enzyme and that they act competitively with respect to each other.

DISCUSSION

Cidofovir, a nucleotide analog, is the first of a new class of antiviral compounds which are active against a broad spectrum of viruses. Cidofovir has potent and selective activity against herpesviruses and offers advantages over nucleoside analogs, including its ability to confer prophylaxis to uninfected cells. cHPMPC is a prodrug of cidofovir which is stable in plasma but is converted to cidofovir in cells. We have previously reported that MRC-5 cells exposed to cHPMPC or cidofovir generate equal concentrations of cidofovir diphosphate, the active metabolite of cidofovir, thus explaining why the two compounds have similar antiviral activities in tissue culture assays (3). In the present report, we extend this observation by demonstrating that all primary human cell lines investigated, including epithelial cell lines derived from tissues which might be infected in patients with HCMV disease, efficiently convert cHPMPC to cidofovir. In addition, we show that HCMV infection does not compromise the ability of cells to metabolize cHPMPC.

The human cellular enzyme responsible for the hydrolysis of cHPMPC to cidofovir was purified 500-fold from liver cytosol following ammonium sulfate fractionation, anion-exchange chromatography, and size fractionation by gel filtration. The enzyme was subsequently chromatographed on a cAMP-agarose column. Kinetic analysis of the purified enzyme indicated that it hydrolyzes cAMP and cCMP in addition to cHPMPC and that the three cyclic nucleotides are competitive inhibitors with respect to each other. Comparison of the K_m and V_{max} values for the three substrates indicates that cHPMPC is a substrate for this enzyme, with a V_{max}/K_m ratio which is only 10- to 20-fold lower than those of the natural cyclic nucleotides cAMP and cCMP.

A ubiquitous cellular enzyme which can hydrolyze cCMP has been purified from pig liver (17) and partially characterized from rat liver (22, 27). This enzyme, referred to as cCMP phosphodiesterase, is unique among the cyclic nucleotide phosphodiesterases in that it can hydrolyze purine and pyrimidine 3′,5′ and 2′,3′ cyclic nucleotides (16). Hydrolysis of cyclic 3′,5′ nucleotides results in formation of 3′ nucleotides, while hydrolysis of cyclic 2′,3′ nucleotides results in both 3′ and 2′ nucleotides, depending on the base. This enzyme, though capable of hydrolyzing a wide variety of cyclic nucleotides, does not hydrolyze DNA or RNA and therefore is not a nonspecific phosphodiesterase. The cCMP phosphodiesterase also differs from the cAMP and cGMP phosphodiesterases, since inhibition is observed with nucleoside monophosphates and inorganic phosphate but not with IBMX (14, 15).

A comparison of the porcine cCMP phosphodiesterase and the cHPMPC-converting activity purified from human liver is presented in Table 3. Although the two enzymes were isolated from different species, they share many similarities. In particular, the two enzymes hydrolyze both cAMP and cCMP with similar relative catalytic efficiencies, and both were inhibited by inorganic phosphate but not by IBMX. Also, the two enzymes

have similar sizes, and both appear to be present in all cell types examined. The evidence therefore supports the conclusion that cCMP phosphodiesterase is responsible for the conversion of cHPMPC to cidofovir.

A second cyclic nucleotide phosphodiesterase capable of hydrolyzing cAMP and cCMP has been reported (25). This enzyme, referred to as a cCMP-specific phosphodiesterase because it has a much higher (100-fold) V_{max} for cCMP than for cAMP, has K_m values of ~10 mM for both substrates. Although we found a high K_m for the activity in the flowthrough of the cAMP-agarose column, we found similar V_{max} values for cCMP and cAMP, indicating that this activity is not the cCMP-specific phosphodiesterase. Instead, it appears that the activity in the flowthrough of the affinity column is a cCMP phosphodiesterase which has been either partially degraded or modified such that it has substantially lower affinities for the substrates.

The physiological role of cCMP phosphodiesterase, which is expressed in every cell type and tissue examined (14), is not known. It is expressed at the highest levels in liver, kidney, and intestine and is present at low levels in rapidly proliferating cells, such as regenerating liver, neonatal tissues, and leukemic cells (14). One suggestion is that cCMP phosphodiesterase, in conjunction with cytidylate cyclase (6, 7), regulates the intracellular concentration of cCMP, which may function as a second messenger system similar to cAMP and cGMP (23, 24). Alternatively, cCMP phosphodiesterase may provide a second system for regulating cAMP levels under conditions in which the more common cAMP-specific phosphodiesterases would be inhibited (22).

In summary, cCMP phosphodiesterase has been identified as the enzyme responsible for the intracellular conversion of cHPMPC to cidofovir. This enzyme appears to be ubiquitously expressed, consistent with the observations that cHPMPC is metabolized in all primary and established cell lines tested and that cidofovir is found in all organs following intravenous administration of radioactive cHPMPC to rats (12). Based on the results of this and related studies, we anticipate that cHPMPC will have comparable antiviral activity to cidofovir in patients.

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REFERENCES

1. **Aduma, P., M. C. Connelly, R. V. Srinivas, and A. Fridland.** 1995. Metabolic diversity and antiviral activities of acyclic nucleoside phosphonates. Mol. Pharmacol. **47:**816–822.
2. **Alford, C. A., and W. J. Britt.** 1990. Cytomegalovirus, p. 1981–2010. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York, N.Y.
3. **Bischofberger, N., M. J. M. Hitchcock, M. S. Chen, D. B. Barkhimer, K. C. Cundy, K. M. Kent, S. A. Lacy, W. A. Lee, Z.-H. Li, D. B. Mendel, D. F. Smee, and J. L. Smith.** 1994. 1-[((*S*)-2-Hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine, an intracellular prodrug for (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine with improved therapeutic index in vivo. Antimicrob. Agents Chemother. **38:**2387–2391.
4. **Bronson, J. J., I. Ghazzouli, M. J. M. Hitchcock, R. R. Webb II, E. R. Kern, and J. C. Martin.** 1989. Synthesis and antiviral activity of nucleotide analogues bearing the (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl] moeity attached to adenine, guanine, and cytosine, p. 88–102. *In* J. C. Martin (ed.), Nucleotide analogues as antiviral agents. American Chemical Society, Washington, D.C.
5. **Bronson, J. J., I. Ghazzouli, M. J. M. Hitchcock, R. R. Webb II, and J. C. Martin.** 1989. Synthesis and antiviral activity of nucleotide analogue (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine. J. Med. Chem. **32:**1457–1463.
6. **Cech, S. Y., and L. J. Ignarro.** 1977. Cytidine 3′,5′-monophosphate formation in mammalian tissues. Science **198:**1063–1065.
7. **Cech, S. Y., and L. J. Ignarro.** 1978. Cytidine 3′,5′-monophosphate formation by homogenates of mouse liver. Biochem. Biophys. Res. Commun. **80:**119–125.
8. **Chen, M. S., R. T. Suttman, J. C. Wu, and E. J. Prisbe.** 1992. Metabolism of 4′-azidothymidine: a compound with potent and selective activity against the human immunodeficiency virus. J. Biol. Chem. **267:**257–260.
9. **Cihlar, T., I. Votruba, K. Horska, R. Liboska, I. Rosenberg, and A. Holy.** 1992. Metabolism of 1-(S)-(3-hydroxy-2-phosphonomethoxypropyl)cytosine (HPMPC) in human embryonic lung cells. Collect. Czech. Chem. Commun. **57:**661–672.
10. **Cihlar, T., and M. S. Chen.** 1996. Identification of enzymes catalyzing two-step phosphorylation of cidofovir and the effect of cytomegalovirus infection on their activities in host cells. Mol. Pharmacol. **50:**1502–1510.
11. **Cleland, W. W.** 1979. Statistical analysis of enzyme kinetic data. Methods Enzymol. **63:**6002–6010.
12. **Cundy, K. C., A. M. Bidgood, G. Lynch, J.-P. Shaw, L. Griffin, and W. A. Lee.** 1996. Pharmacokinetics, bioavailability, metabolism, and tissue distribution of cidofovir (HPMPC) and cyclic HPMPC in rats. Drug Metab. Dispos. **24:**745–752.
13. **De Clercq, E., T. Sakuma, M. Baba, R. Pauwels, J. Balzarini, I. Rosenberg, and A. Holy.** 1987. Antiviral activity of phosphylmethoxyalkyl derivatives of purines and pyrimidines. Antivir. Res. **8:**261–272.
14. **Helfman, D. M., N. Katoh, and J. F. Kuo.** 1984. Purification and properties of cyclic CMP phosphodiesterase. Adv. Cyclic Nucleotide Res. **16:**403–416.
15. **Helfman, D. M., and J. F. Kuo.** 1982. Differential effects of various phosphodiesterase inhibitors, pyrimidine and purine compounds, and inorganic phosphates on cyclic CMP, cyclic AMP and cyclic GMP phosphodiesterases. Biochem. Pharmacol. **31:**43–47.
16. **Helfman, D. M., and J. F. Kuo.** 1982. A homogenous cyclic CMP phosphodiesterase hydrolyzes both pyrimidine and purine cyclic 2′:3′- and 3′:5′-nucleotides. J. Biol. Chem. **257:**1044–1047.
17. **Helfman, D. M., M. Shoji, and J. F. Kuo.** 1981. Purification to homogeneity and general properties of a novel phosphodiesterase hydrolyzing cyclic CMP and cyclic AMP. J. Biol. Chem. **256:**6327–6334.
18. **Hitchcock, M. J. M., S. A. Lacy, J. R. Lindsey, and E. R. Kern.** 1995. The cyclic congener of cidofovir has reduced nephrotoxicity in three species. Antivir. Res. **26:**A358.
19. **Ho, H. T., K. L. Woods, J. J. Bronson, H. De Boeck, J. C. Martin, and M. J. M. Hitchcock.** 1991. Intracellular metabolism of the antiherpes agent (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine. Mol. Pharmacol. **41:**197–202.
20. **Kupperman, B., J. Lalezari, R. Stagg, F. Kramer, G. Holland, D. Ives, E. Youle, M. Robinson, D. Northfelt, J. Eron, and H. S. Jaffe.** 1995. A phase II/III randomized study of immediate versus deferred intravenous (IV) cidofovir (CDV, HPMPC) for AIDS patients with peripheral CMV retinitis (CMV-R). *In* Program and Abstracts of the International Symposium on Experimental and Clinical Ocular Pharmacology and Pharmaceutics.
21. **Lalezari, J. P., W. L. Drew, E. Glutzer, C. James, D. Miner, J. Flaherty, P. E. Fisher, K. C. Cundy, J. Hannigan, J. C. Martin, and H. S. Jaffe.** 1995. (S)-1-[3-Hydroxy-2-(phosphonylmethoxy)propyl]cytosine (cidofovir): results of a phase I/II study of a novel antiviral nucleotide analogue. J. Infect. Dis. **171:**788–796.
22. **Lavan, B. E., T. Lakey, and M. D. Houslay.** 1989. Resolution of soluble cyclic nucleotide phosphodiesterase isoenzymes, from liver and hepatocytes, identifies a novel IBMX-insensitive form. Biochem. Pharmacol. **38:**4123–4136.
23. **Newton, R. P.** 1992. Cytidine 3′,5′-cyclic monophosphate: a third cyclic nucleotide intracellular mediator? Biochem. Soc. Trans. **20:**469–474.
24. **Newton, R. P.** 1995. Cytidine 3′,5′-cyclic monophosphate: a third cyclic nucleotide secondary messenger? Nucleosides Nucleotides **14:**743–747.
25. **Newton, R. P., and S. G. Salih.** 1986. Cyclic CMP phosphodiesterase: isolation, specificity and kinetic properties. Int. J. Biochem. **18:**743–752.
26. **Polis, M. A., K. M. Spooner, B. F. Baird, J. F. Manishewitz, H. S. Jaffe, P. E. Fisher, J. Falloon, R. T. Davey, Jr., J. A. Kovaks, R. E. Walker, S. M. Whitcup, R. B. Nussenblatt, H. C. Lane, and H. Masur.** 1995. Anticytomegaloviral activity and safety of cidofovir in patients with human immunodeficiency virus infection and cytomegalovirus viruria. Antimicrob. Agents Chemother. **39:**882–886.
27. **Worby, A., L. M. Mensah, and K. J. Murray.** 1991. Identification of the novel rat liver IBMX-insensitive phosphodiesterase as a nonspecific phosphodiesterase capable of hydrolysing cCMP. Biochem. Pharmacol. **42:**1318–1321.
28. **Xiong, X., J. L. Smith, C. Kim, E.-S. Huang, and M. S. Chen.** 1996. Kinetic analysis of the interaction of cidofovir diphosphate with human cytomegalovirus DNA polymerase. Biochem. Pharmacol. **51:**1563–1567.