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Intracellular Metabolism of the Antiherpes Agent (*S*)-1-[3-Hydroxy-2-(phosphonylmethoxy)propyl]cytosine

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SUMMARY

(*S*)-1-[3-Hydroxy-2-(phosphonylmethoxy)propyl]cytosine (HPMPC) is an antiviral phosphonate nucleotide analogue that displays activity against a range of herpesviruses. Anion exchange high performance liquid chromatography analysis of the 60% methanol extract from [^{14}C]HPMPC-treated cells reveals the formation of three major metabolites. Two of these were identified as phosphorylated forms of HPMPC, HPMPC phosphate, and HPMPC diphosphate, by liberation of HPMPC upon acid digestion and coelution with synthetic standards on high performance liquid chromatography. The third metabolite, which is resistant to alkaline phosphatase cleavage but sensitive to phosphodiesterase, is proposed to be an HPMPC phosphate adduct. In herpes simplex virus-1-infected cells the same three metabolites are detected, at concentrations comparable to those in uninfected cells. When HPMPC is removed from the medium, the concentrations of the metabolites in cells decrease slowly, with half-lives of ~6, 17, and 48 hr for HPMPC phosphate, HPMPC diphosphate, and the HPMPC phosphate adduct, respectively. HPMPC diphosphate inhibits herpes simplex virus-1 and -2 DNA polymerases with a lower K_i than that for DNA polymerase α, and enzyme inhibition is competitive in each case. The formation and the persistence of HPMPC phosphates in cells and the selective inhibition of viral DNA polymerases by HPMPC diphosphate can explain why cells pretreated with HPMPC remain refractory to viral infection even long after HPMPC is removed from the medium.

Phosphonomethyl nucleotide analogues with antiviral activity were first described in 1986 by De Clercq *et al.* (1). These were the adenine congeners HPMPA and phosphonylmethoxyethyladenine (PMEA). Antiviral activity against DNA viruses, in particular herpesviruses (1–3), and retroviruses (human immunodeficiency virus, Moloney murine sarcoma virus, etc.) has been reported.

From an extensive *in vitro* and *in vivo* evaluation of a series of hydroxyphosphonylmethoxypropyl- and phosphonylmethoxyethyl- side chain-containing nucleoside analogues (2, 3), HPMPC (Fig. 1a) emerged as the most selective antiherpetic agent of this series (2, 3). HPMPC has potent *in vitro* antiviral activity against herpesviruses (HSV-1 and -2, Vericella-Zoster virus, human CMV, and murine CMV), as well as vaccinia viruses and adenoviruses (2–4). It is more potent than ganciclovir against CMV and than acyclovir against Vericella-Zoster virus. *In vivo*, HPMPC was shown to be efficacious against HSV-1, HSV-2, and murine CMV infections in mice (3, 5, 6). Other models of animal infection, such as simian varicella virus infection of monkeys (7) and guinea pig CMV infection of guinea pig (8), have also been used to demonstrate the antiviral effect of HPMPC. As a nucleoside monophosphate analogue, HPMPC is effective against thymidine kinase-deficient herpesvirus (5, 9, 10). HPMPC inhibits human CMV replication selectively through inhibition of human CMV DNA synthesis (6).

This report describes cellular metabolism and enzymatic studies with HPMPC, which may assist in defining the biochemical basis of its antiviral effect.

Experimental Procedures

Materials. [2-^{14}C]HPMPC was synthesized by U. Haynes and J. Swigor, Bristol-Myers Squibb Company. The specific activity was 5.9 mCi/mmol. DNA polymerase α was affinity purified from HeLa cells by Dr. Alan Wahl, Bristol-Myers Squibb Company. HSV DNA polymerases were purified from HSV-1 (Z630)- or HSV-2 (MS)-infected HeLa cells (11). [5,5′-^{3}H]dCTP (specific activity, 47 Ci/mmol) was purchased from DuPont-NEN Research Products.

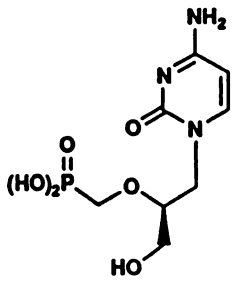
Cell metabolism studies. Monolayers of MRC-5 cells at 80% confluency were incubated with tissue culture medium (Eagle's minimum essential medium) containing radioactively labeled HPMPC, as shown below (usually 200 μM for 24 h). At the end of the incubation period, cells were processed in the following manner, to determine the intracellular level of HPMPC and its metabolites. The monolayer was washed with phosphate-buffered saline, and the cells were removed by

ABBREVIATIONS: HPMPA, (5)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]adenine; HPMPC, (*S*)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine; HSV, herpes simplex virus; HPLC, high performance liquid chromatography; TEA, triethylammonium; HPMPCpp, 1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine diphosphate; HPMPCp, 1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine phosphate; ara-C, cytosine arabinoside; CMV, cytomegalovirus.

trypsinization and counted. After centrifugation the cell pellet was extracted twice with 60% (v/v) methanol in water, at −20°. The combined extracts were evaporated under vacuum, redissolved in water, and analyzed by HPLC for soluble cellular metabolites. The HPLC system used a Whatman Partisil SAX-10 column. The mobile phase was pumped at 1 ml/min and was a linear gradient, from 0 to 55 min, of 15–700 mM potassium phosphate buffer at pH 3.5. Radioactivity was detected and quantitated on-line with a Radiomatic Flo-One Beta liquid scintillation detector. To determine the radioactive material not extractable with 60% methanol, cell pellets obtained after the extraction were digested in 500 μl of 5 N NaOH at 37° for 24 hr. After neutralization with 12 N HCl, scintillation fluid was added, and radioactivity was counted in a Beckman LS 6800 counter. For studies in the HSV-1-infected MRC-5 cells, the monolayers were exposed to HSV-1 (KOS), at a multiplicity of infection of 2 plaque-forming units/cell, for 1 hr before incubation with 200 μM HPMPC. After 24-hr drug exposure, cells were processed and cell extracts were analyzed as described above.

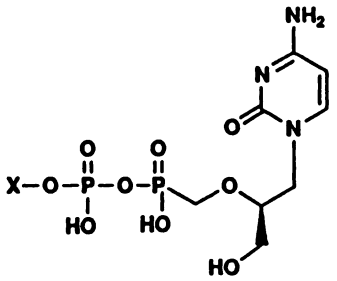
Synthesis of HPMPCpp. A solution of (*S*)-1-[3-benzyloxy-2-(phosphonomethoxy)propyl]cytosine (0.74 g, 2.0 mmol) in 1:1 *t*-butanol/water (40 ml) was treated with morpholine (0.70 g, 8.0 mmol) and heated to reflux (bath temperature of 100°). A solution of 1,3-dicyclohexylcarbodiimide (1.65 g, 8.0 mmol) in 1:1 *t*-butanol/water (20 ml) was added dropwise to the solution over 1.5 hr. The reaction mixture was stirred at reflux for 14 hr, and the resulting slurry was treated with morpholine (0.35 g, 4.0 mmol) and 1,3-dicyclohexylcarbodiimide (0.83 g, 4.0 mmol) and heated for an additional 6 hr. The mixture was allowed to cool to room temperature and concentrated *in vacuo*. Water (50 ml) was added to the residue, insoluble material was removed by filtration, and the filtrate was washed with diethyl ether (2 × 75 ml). The aqueous layer was concentrated *in vacuo* and coevaporated with ethanol (2 × 75 ml). The residue was placed under high vacuum (0.1 mm Hg) for 1 hr and then dissolved in anhydrous dimethyl sulfoxide (15 ml). Tri-*n*-butylammonium pyrophosphate (2.74 g, 6.0 mmol, Sigma) was added in one portion, and the reaction mixture was stirred at room temperature for 48 hr. Diethyl ether (100 ml) was added to the solution, causing precipitation of a gummy solid onto the sides of the flask. The ethereal solution was decanted and the remaining residue was washed with additional diethyl ether (50 ml). The ethereal solution was again decanted, and the final residue was placed under high vacuum for 18 hr.

a.



HPMPC

b.



HPMPCp-adduct: X = choline or ethanolamine

Fig. 1. a, Structural formula of HPMPC. b, Proposed structural formula of HPMPCp adduct.

The unpurified diphosphate was dissolved in a mixture of methanol (40 ml) and water (10 ml) and treated with ammonium formate (1.0 g) and 10% palladium on carbon (1.0 g). The reaction mixture was heated at 70° for 1.5 hr and then filtered, while hot, through a 1-inch pad of Celite; the collected solid was rinsed with hot water. The filtrate was concentrated *in vacuo* to afford a yellow viscous residue.

Synthesis of cyclic HPMPC. A solution of 1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (2.23 g, 8.00 mmol) in water (10 ml) and dimethylformamide (30 ml) was treated with 4-morpholino-*N,N'*-dicyclohexylcarboxamide (2.35 g, 8.00 mmol). The mixture was stirred at room temperature for 14 hr, the resulting clear solution was concentrated *in vacuo*, and the residue was evaporated from dimethylformamide (2 × 30 ml). The residue was redissolved in dimethylformamide (50 ml) and added dropwise over 2.5 hr to a solution of dicyclohexylcarbodiimide (14.9 g, 72.0 mmol) in anhydrous pyridine (800 ml), heated at reflux. The reaction mixture was heated at reflux for an additional 12 hr and then concentrated *in vacuo*. The residue was dissolved in water (200 ml) and treated with diethyl ether (200 ml). Insoluble material was removed by filtration, and the aqueous layer was separated, washed with diethyl ether (2 × 200 ml), and then concentrated *in vacuo*. The residue was dissolved in water (200 ml), and the acidity of the solution was adjusted to pH 1 by addition of 1 N aqueous hydrochloric acid. The solution was purified on a reverse phase column, using water as the eluent. Fractions containing UV-active material were pooled and concentrated *in vacuo* to provide 1.21 g (58%) of a white powder.

Purification and characterization of HPMPCp and HPMPCpp. HPMPCp and HPMPCpp in the reaction mixture were resolved from each other by fractionation on a Trisacryl-DEAE ion exchange column, using a gradient from 20 to 500 mM TEA-HCO_3, pH 7.3. The eluent was monitored with an on-line ISCO UA-5 absorbance detector at 280 nm. However, as judged by NMR analysis of the fractions obtained, both preparations were contaminated by significant amounts of PP_i.

The HPMPCpp preparation described above was again fractionated through an ion exchange column, using a gradient from 10 to 500 mM TEA-HCO_3, pH 7.3. Aliquots of the fractions eluted with a 0.32–0.41 M TEA-HCO_3 buffer gradient were analyzed by HPLC and shown to contain intact HPMPCpp. These fractions were pooled and lyophilized. ^{31}P NMR analysis of the pooled material showed that HPMPCpp was free of PP_i contamination.

Removal of PP_i from the HPMPCp preparation by pyrophosphatase treatment was also feasible. Thus, the pooled fractions, containing 5 mg of HPMPCp (as measured by absorbance at 269 nm), were treated with pyrophosphatase (50 units) at 25° for 15 min. By using Centricon-30 microconcentrators, an enzyme-free HPMPCp filtrate was obtained. This material was fractionated on a Trisacryl DEAE ion exchange column, using a gradient from 10 to 350 mM TEA-HCO_3, pH 7.3. Fractions corresponding to the HPMPCp peak, as verified by HPLC analysis, were pooled; the material was lyophilized and used for ^{31}P NMR analysis.

DNA polymerase assays. Inhibition of viral DNA polymerase activity by HPMPCpp was measured in 100-μl assay mixtures containing 50 mM Tris buffer, pH 8.0, 4 mM $MgCl_2$, 200 mM KCl, 2 μg of bovine serum albumin, 0.2 μg/μl activated salmon sperm DNA, 0.5 mM dithiothreitol, 50 μM dATP, dGTP, and TTP, fixed varying concentrations of [^{3}H]dCTP, and varying concentrations of HPMPCpp. For DNA polymerase α inhibition by HPMPCpp, the assay mixtures consisted of 50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothreitol,

2 μg of bovine serum albumin, 0.2 μg/μl activated salmon sperm DNA, 50 μM each of dATP, dGTP, and TTP, and varying concentrations of [^{3}H]dCTP and HPMPCpp. Ten microliters of a predetermined amount of cellular or viral DNA polymerase were added to start the reaction. At specified time intervals up to 20 min, aliquots of assay solution were dispensed onto cellulose paper discs and dipped into 5% trichloroacetic acid/1% pyrophosphate to quench the reaction. The discs were washed, dried, and counted, in vials with Insta-gel XF scintillant added, using a Beckman LS 6800 counter. Incorporation of radioactivity in the activated salmon sperm DNA increased linearly during the incubation period. Kinetic constants were obtained by nonlinear iterative curve-fitting, based on competitive inhibition.

Results

Identification of HPMPCpp and HPMPCp by ^{31}P NMR. The ^{31}P NMR spectrum for HPMPCpp (Fig. 2A) showed three main resonance peaks, one at +9 ppm, corresponding to the phosphonate phosphorous, one at −22 ppm, corresponding to the β-phosphorous, and one at −6 ppm, corresponding to the γ (terminal)-phosphorous. A small peak at +10 ppm was due to some breakdown to HPMPC. This was confirmed by HPLC analysis; the HPMPCpp preparation was 95% pure, with contaminating HPMPC representing 5% of the material.

The ^{31}P NMR spectrum for the HPMPCp preparation (Fig. 2B) showed two resonance peaks, at +7 ppm and at −6 ppm, corresponding to the phosphonate and β-phosphorous of HPMPCp, respectively. Around 10% of the PP_i originally present in the pooled fractions was left (resonance peak at −6.7 ppm), indicating that the enzyme treatment was effective but incomplete. No resonance peak for P_i was seen in the ^{31}P NMR spectrum, which demonstrated the complete removal of P_i generated from pyrophosphatase treatment.

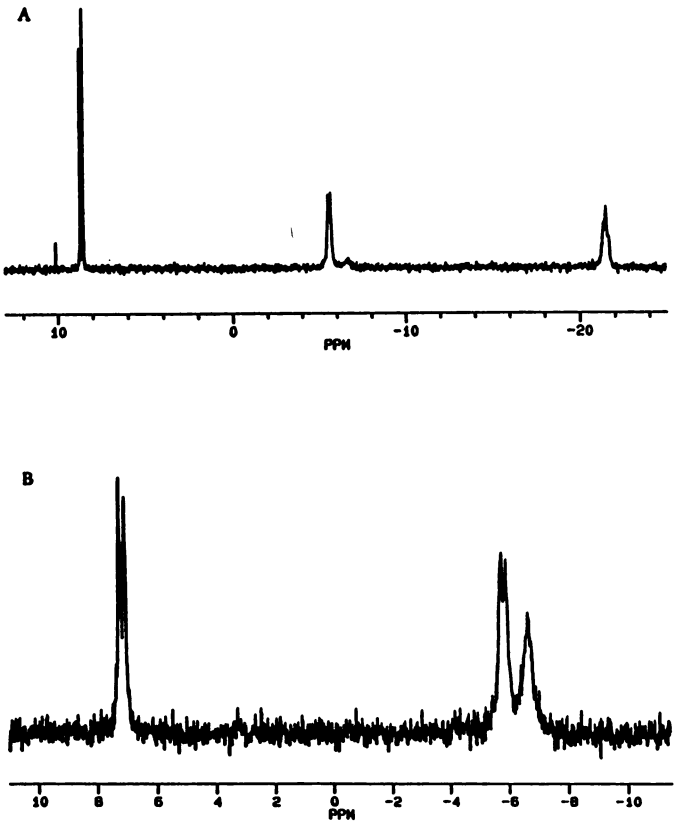


Fig. 2. A, ^{31}P NMR spectrum of HPMPCpp at pH 7, showing phosphonate phosphorus signal at +8.6 ppm, β-phosphorus at −21.5 ppm, and γ-phosphorus at −5.6 ppm. B, ^{31}P NMR spectrum of HPMPCp, showing phosphonate phosphorus signal at +7.1 ppm and β-phosphorus at −5.5 ppm (with some PP_i in the sample; signal at −6.7 ppm), after pyrophosphatase treatment.

Metabolism of [^{14}C]HPMPC by MRC-5 cells. To investigate the intracellular metabolism of HPMPC, 200 μM radiolabeled HPMPC was incubated with MRC-5 cells. This concentration of HPMPC does not cause visible toxicity to the monolayer. Ion exchange HPLC of a 60% methanol extract of the drug-treated cells, taken at 24 hr, displayed three peaks of radioactivity, in addition to HPMPC, at retention times of 15, 28, and 53 min (Fig. 3A). Also, extracts from cells incubated with labeled HPMPC were spiked with authentic, chemically synthesized HPMPCp and HPMPCpp. HPLC analysis of these mixtures at pH 5.5, to resolve HPMPCpp from endogenous ATP (Fig. 3B), showed that the UV absorbance due to the standards coincided with the peaks of radioactivity. To further identify the two peaks eluting at 28 and 53 min as HPMPCp and HPMPCpp, corresponding fractions that contained radioactivity were collected separately, acidified to pH 1 with HCl, and incubated at 37° for 48 hr, to hydrolyze the phosphoester linkage. The solutions were then neutralized and analyzed by HPLC. All the radioactivity was associated with HPMPC (data not shown). Thus, the identity of the metabolites as HPMPCp and HPMPCpp was confirmed.

Identification of the material eluting at 15 min at pH 3.5 has been more problematic. It is neither cyclic HPMPC (produced by activation and internal cyclization) nor (*S*)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]uridine (produced by deamination), because authentic samples had different retention times. Incubation of the material from this labeled peak with alkaline phosphatase did not degrade the compound, whereas incubation with phosphodiesterase caused about 30% of the material to be converted to HPMPC. Under similar condition, 95% of CDP-choline was digested by phosphodiesterase treatment and no digestion was observed when CDP-choline was treated with alkaline phosphatase. This would indicate the presence of a blocked phosphate ester. Attempts to double-label the compound by treating cells simultaneously with both [^{14}C]HPMPC

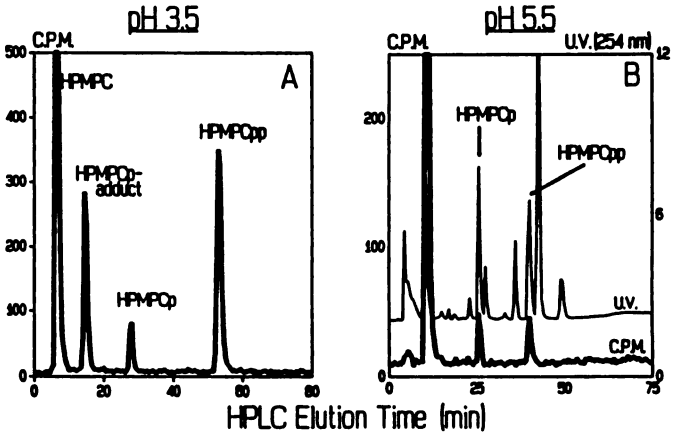


Fig. 3. Strong anion exchange HPLC separation of [^{14}C]HPMPC and metabolites in the 60% methanol extract from [^{14}C]HPMPC-treated MRC-5 cells. A, At pH 3.5, four radioactive peaks were eluted: HPMPC, 5 min; HPMPCp adduct, 15 min; HPMPCp, 28 min; and HPMPCpp, 53 min. B, At pH 5.5, the retention times were: HPMPCp, 26 min; HPMPCpp, 40 min; and ATP, 42.4 min. HPMPCp and HPMPCpp standards were spiked in this sample.

and [^{3}H]choline, however, were not successful. Only ^{14}C activity was detected in the 15-min peak, with no ^{3}H activity. Based on these observations and by analogy with the metabolism described for dideoxycytidine and ara-C (12, 13), this metabolite could be a phosphate ester adduct of HPMPC, such as HPMPCp-choline or HPMPCp-ethanolamine (Fig. 1b). Formation of the three HPMPC metabolites was also observed when [^{14}C]HPMPC was incubated with either Vero or Madin-Darby canine kidney cells.

To investigate the kinetics of entry of HPMPC into the cell and its rate of metabolism, extracts were prepared from cells incubated with labeled HPMPC for varying lengths of time (Fig. 4), and the concentrations of HPMPC and metabolites were determined. The concentration of cell-associated HPMPC increased with time over the 4-day period. However, even after a 4-day incubation, the highest HPMPC concentration reached inside the cells was only 6% of the extracellular drug concentration. This is unlike what has been observed for nucleoside analogues, which equilibrate across the cell membrane within seconds to minutes. The concentration of HPMPC metabolites, on the other hand, increased only in the first 24 hr and subsequently remained constant. The levels of the three metabolites thus reached a steady state after 24-hr incubation with HPMPC.

The effect of varying the concentration of HPMPC on production of metabolites was measured (Fig. 5) after a 24-hr incubation. The results show that the concentrations of metabolites present at 24 hr increased, relative to the extracellular concentration of HPMPC, in a linear dose-dependent manner. This result is important because it allows intracellular concentrations of metabolites (which presumably include the antiviral species) to be controlled by changes in the dose of HPMPC.

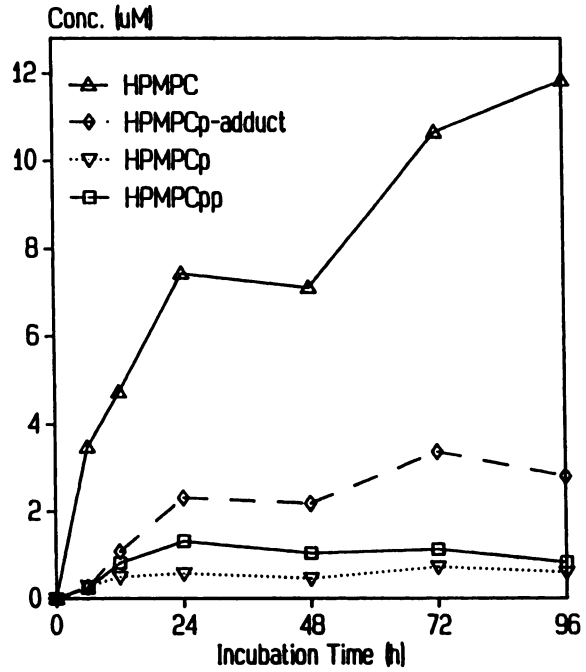


Fig. 4. Cellular uptake and phosphorylation of HPMPC by MRC-5 cells. Cells were treated with 200 μM [^{14}C]HPMPC. At the designated time intervals, fractions of cells were processed and the 60% methanol cell extracts were analyzed by strong anion exchange HPLC. Concentrations of HPMPC (△), HPMPCp adduct (◇), HPMPCpp (□), and HPMPCp (▽) at various times were as shown.

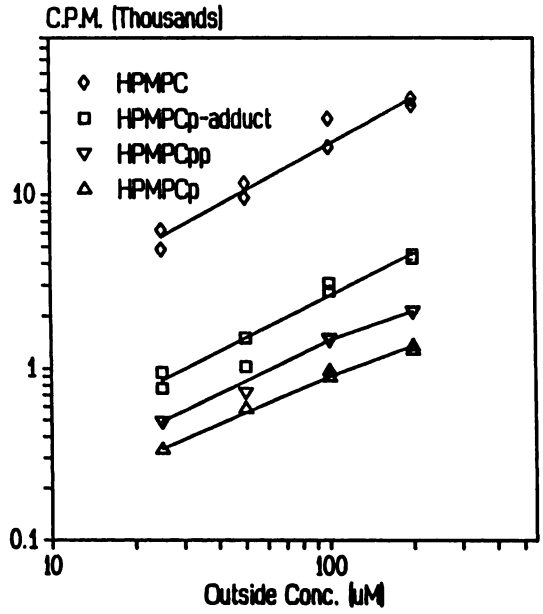


Fig. 5. Effect of HPMPC concentration in the medium on the concentrations of metabolites in MRC-5 cells. Cells were incubated with 25 μM, 50 μM, 100 μM, or 200 μM [^{14}C]HPMPC for 24 hr. The 60% methanol cell extracts were analyzed by strong anion exchange HPLC. Radioactivity associated with HPMPC and metabolites was determined. ◇, HPMPC; □, HPMPC adduct; ▽, HPMPCpp; △, HPMPCp.

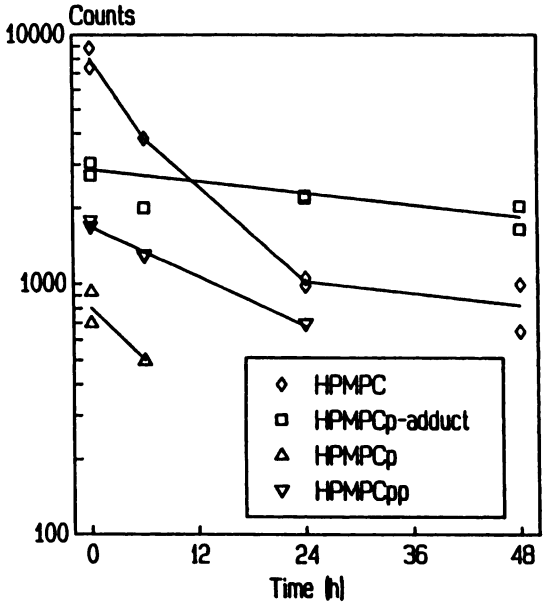


Fig. 6. Intracellular persistence of HPMPC and its metabolites after the removal of HPMPC from the medium. MRC-5 cells were exposed to [^{14}C]HPMPC in the medium for 24 hr and then incubated further in drug-free medium. The amount of cell-associated HPMPC and metabolites at 0, 6, 12, and 24 hr after drug removal was measured. ◇, HPMPC; □, HPMPC-adduct; ▽, HPMPCpp; △, HPMPCp.

To determine the persistence of intracellular pools of HPMPC metabolites, MRC-5 cells were exposed to 200 μM labeled HPMPC for 24 hr, washed, and resuspended in drug-free medium. At various times, cells were extracted and processed for metabolite quantitation. The results (Fig. 6) show that HPMPC efflux from the cell is slow, with about 50% being gone by 6 hr. Subsequently, the rate of exit becomes slower. The metabolite pools decay with half-lives of about 6 hr for

HPMPCp, 17 hr for HPMPCpp, and >48 hr for the HPMPCp adduct.

To investigate the role of virus infection on HPMPC metabolism, labeled HPMPC (200 μM) was added to HSV-1-infected or mock-infected cells, and the metabolites in extracts were quantitated 24 and 48 hr after infection (Fig. 7). The results show that production of HPMPCp, HPMPCpp, and HPMPCp adduct is unchanged by infection with the virus. Furthermore, in both virus-infected and mock-infected cells, 95–98% of the cell-associated radioactivity appeared in the 60% methanol extract. Incorporation of HPMPC into cellular DNA or RNA should, therefore, be minimal.

To evaluate how HPMPC affects cellular or viral DNA synthesis, the inhibitory effect of HPMPCpp on human DNA polymerase α and HSV-1 and HSV-2 DNA polymerase activity was measured, using activated salmon sperm DNA as template-primer. In all cases, HPMPCpp inhibited the polymerase activity in a competitive manner (Fig. 8) with respect to the natural substrate dCTP. Both HSV-1 and HSV-2 DNA polymerases demonstrated significantly greater affinity towards HPMPCpp (K_i = 0.8 and 1.4 μM, respectively) (Table 1) than did cellular DNA polymerase α (K_i = 51 μM).

The formation and the persistance of HPMPCpp in the uninfected cells suggested that preincubation of cells with HPMPC could prime them to an antiviral state, even when

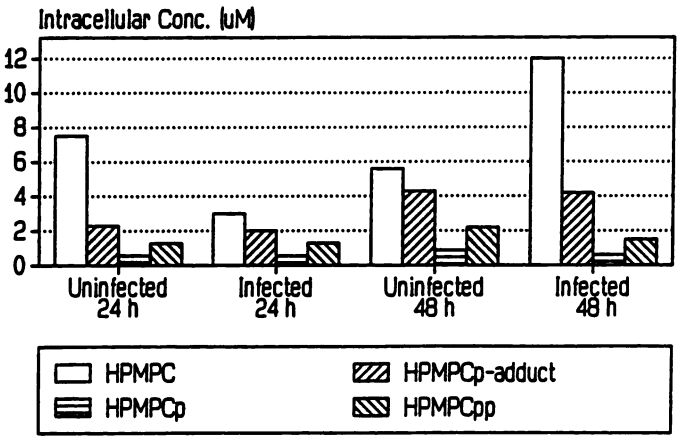


Fig. 7. Effect of HSV-1 infection on HPMPC metabolism in MRC-5 cells. Both mock-infected and HSV-1-infected (multiplicity of infection of 2) MRC-5 cells were treated with 200 μM [^{14}C]HPMPC for 24 or 48 hr. HPMPC and its metabolites were measured as described in Experimental Procedures.

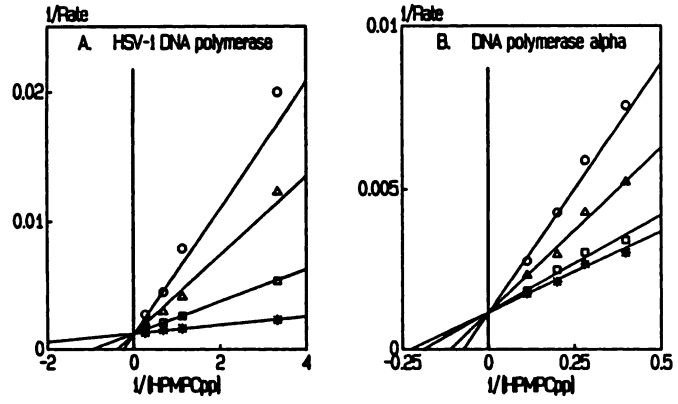


Fig. 8. Lineweaver-Burk plot of inhibition of HSV-1 (A) and human DNA polymerase α (B) by HPMPCpp.

TABLE 1

Inhibition constants of HPMPCpp with respect to DNA polymerases from HSV-1 and HSV-2 and human DNA polymerase α

Polymerases were assayed using activated salmon sperm DNA as template-primer.

	K_m ([^{3}H]dCTP)	K_i (HPMPCpp)	K_i/K_m
	μM	μM	
HSV-1 DNA polymerase	0.31	0.86	2.8
HSV-2 DNA polymerase	0.37	1.4	3.8
DNA polymerase α	4.7	51.0	10.8

excess extracellular compound was removed. Therefore, cells were incubated with various concentrations of either HPMPC, acyclovir, or ganciclovir for 24 hr, and the compounds were removed before infection. For comparison, a standard assay was also performed using preinfected monolayers with compound continuously present in the medium. The antiviral activity of HPMPC was similar with both assays (ID_{50} of ~12–25 μg/ml), whereas acyclovir and ganciclovir showed antiviral activity when they were present continuously (ID_{50} values of 2–4 and 4–8 μg/ml, respectively) but not after their removal (ID_{50} of >100 μg/ml).

Discussion

We have demonstrated the cellular uptake of HPMPC and its intracellular conversion to HPMPCp and HPMPCpp. These findings are consistent with previous studies on the metabolism of HPMPA (14). The antiviral activity of HPMPA has been proposed to result from its structural similarity to dAMP, after phosphorylation to a triphosphate analogue. HPMPA is a stable monophosphate analogue, because the carbon-phosphorous bond is not readily susceptible to enzymatic cleavage. In cells, HPMPA is phosphorylated by host enzymes to HPMPA phosphate and HPMPA diphosphate (14). The latter metabolite has been shown to inhibit herpesvirus-encoded enzymes, DNA polymerase, and ribonucleotide reductase (15, 16). Based on this analogy and the structural similarity of HPMPCpp to dCTP, it seems likely that HPMPCpp is the form that is active at the enzyme level. Our results from the enzyme kinetic study demonstrate selective inhibition of HSV-1 and -2 DNA polymerases by HPMPCpp. In addition to the differential binding affinity of HPMPCpp toward the viral and host DNA polymerases shown here, the incorporation of HPMPC into and/or the excision/repair of HPMPC from viral or host DNA could also contribute to the selective inhibition of viral DNA synthesis by HPMPC.

The increase in HPMPCpp concentration inside the cell in response to an increased concentration of extracellular HPMPC indicates that antiviral activity should also show dose responsiveness. Results obtained from the treatment of HSV infection with HPMPC in a murine model support (9, 17) this theory. Also, the long intracellular half-life of HPMPCpp (17 hr) after extracellular drug is removed would be expected to lead to a persistence of antiviral activity. This suggestion is supported both by *in vitro* data showing antiviral activity after removal of HPMPC (see Results) and *in vivo* data where antiviral activity is seen with infrequent (7, 9, 10, 17) and prophylactic (17) dosing schedules.

The intracellular production of the third metabolite is different from HPMPA metabolism. However, the formation of a choline or ethanolamine adduct would be expected from the

known metabolic pathways for deoxycytidine and its analogues dideoxycytidine (12) and ara-C (13). The long intracellular half-life of this metabolite (>2 days) may produce a reservoir of drug that is slowly released. This may contribute to the long duration of action of HPMPC and may have an impact on long term toxicity.

The enzymes involved in the proposed phosphorylations of HPMPC have not been identified. However, preincubation of cells with 20 μM cytidine or deoxycytidine was without effect on the pattern of labeled metabolites (result not shown). When cells were treated with 200 μM HPMPC, the intracellular concentration of HPMPC reached ≈12 μM at most, and 20 μM cytidine or deoxycytidine should compete favorably with HPMPC for phosphorylation under these conditions. Possibly the enzyme that phosphorylates dCMP and CMP does not phosphorylate HPMPC. Higher concentrations of either cytidine or deoxycytidine (410–440 μM) decrease the anti-CMV activity by 7–10-fold (4). Although this may be explained by less HPMPCpp being formed, it might also be the result of elevated dCTP produced competing against HPMPCpp at the polymerase level. Further work with cell extracts will be needed to address this issue. Recently, the one-step phosphorylation of HPMPA to HPMPA diphosphate, catalyzed by 5-phosphoribosyl-1-pyrophosphate synthetase, was reported (18). Thus, there is precedent for the involvement of enzymes other than kinases in the phosphorylation of phosphonates.

With acyclovir (19, 20) and ganciclovir (21, 22), the intracellular conversion to monophosphate is dependent to a large extent on viral infection. A virally encoded enzyme (thymidine kinase) for herpes simplex or a putative viral-induced host enzyme for CMV mediates this initial step. Subsequent conversions to diphosphate and triphosphate are then catalyzed by host enzymes. In this study we show that HPMPC activation is neither dependent on nor facilitated by virus-encoded or -induced enzymes. Thus, HPMPCpp is produced by uninfected cells, which primes them to resist virus replication before infection. This feature of the mechanism of action may also be responsible for the ability to use HPMPC in a prophylactic mode (17).

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