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Metabolic Diversity and Antiviral Activities of Acyclic Nucleoside Phosphonates

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SUMMARY

The acyclic nucleoside phosphonates (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC), (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA), and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) inhibited herpes simplex virus-1 replication in Vero cells, and the IC_{50} values ranged from 4 μM (for HPMPC and HPMPA) to 40 μM (for PMEA). Pretreatment of cells with HPMPC for 12–24 hr induced an effective antiviral state, and the cells maintained this antiviral state for >7 days. In contrast, much larger amounts (~2.5–5 × IC_{50} doses) of PMEA or HPMPA were required to establish an antiviral state, which lasted for only ~24 or 72 hr, respectively. A 12-hr treatment of the cells with the phosphonates was required for the establishment of optimal antiviral activity; surprisingly, longer durations of exposure to PMEA (but not HPMPA or HPMPC) resulted in diminished antiviral effect. We investigated the metabolism of PMEA and HPMPC to determine the cellular basis for these differences. The cellular uptake of HPMPC was ~8-fold greater than that of PMEA. The levels of the PMEA metabolites PMEA monophosphate and PMEA diphosphate increased for ~12 hr and plateaued thereafter. PMEA and its metabolites were cleared from the cells with a half-life of 4.9 hr. In contrast, the HPMPC metabolites HPMPC monophosphate (HPMPCp) and HPMPC diphosphate (HPMPCpp) accumulated throughout the 24-hr study period and, at equimolar drug concentrations (25 μM), reached intracellular levels ~2–3-fold greater than those of the PMEA metabolites. HPMPC also differed from PMEA in its capacity to generate a phosphodiester metabolite (HMPCp-choline), which was a predominant metabolite in HPMPC-treated cells. In addition, the rates of disappearance of intracellular metabolites of the two drugs were significantly different. Thus, the decay of HPMPCpp was quite slow and biphasic ($t_{½}$ = 24 and 65 hr) and that of HMPCp-choline was monophasic ($t_{½}$ = 87 hr). Together, these factors can explain the differing antiviral potencies seen with PMEA and HPMPC. The possible role of the choline adduct in the expression of antiviral activity of the drug remains to be elucidated, but the adduct may serve as an intracellular store for the long term maintenance of active HPMPCpp in cells. The results also highlight the extent of diversity in the cellular pharmacology and antiviral activities of the acyclic nucleoside phosphonates.

HSV is a common cause of frequent human infections and may be life-threatening in immunocompromised patients, such as those with acquired immunodeficiency syndrome or those receiving organ or tissue transplants (reviewed in Ref. 1). ACV is the drug of choice and functions as a selective inhibitor of herpesvirus replication because it requires viral thymidine kinase for its initial phosphorylation to ACV monophosphate (reviewed in Refs. 1 and 2). Other potentially useful compounds include foscarnet, a non-nucleoside inhibitor of HSV polymerase, and vidarabine (9-β-D-arabinofuranosyladenine), a nucleoside inhibitor, which are recommended for the management of infections due to ACV-resistant HSV. The poor oral bioavailability of the currently available agents, their toxicity, and the emergence of virus strains resistant to these compounds warrant a continued search for alternative antiviral agents (2, 3). Acyclic nucleoside phosphonates are a recently described class of antiviral agents with broad-spectrum activity against a number of DNA viruses, such as herpesviruses and retroviruses including HIV, the etiological agent of acquired immunodeficiency syndrome (4–10). A large number of acyclic nucleoside phosphonate analogs, which vary in the nucleoside base or in the

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ABBREVIATIONS: HSV, herpes simplex virus; HPLC, high pressure liquid chromatography; HPMPC, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; HPMPA, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEDAP, 9-(2-phosphonylmethoxyethyl)diaminopurine; ACV, acyclovir [9-(2-hydroxyethoxymethyl)guanine]; HIV, human immunodeficiency virus; ddCyd, dideoxycytidine; pfu, plaque-forming units; PMEAp, 9-(2-phosphonylmethoxyethyl)adenine monophosphate; PMEApp, 9-(2-phosphonylmethoxyethyl)adenine diphosphate; HPMPCp, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine monophosphate; HPMPCpp, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine diphosphate.

acyl side chain, have been synthesized and shown to display substantial differences in the spectra of their activities (4–12). Of these, two compounds, PMEA and HPMPC (Fig. 1), are currently being evaluated in clinical trials for their efficacy against HIV and cytomegalovirus infections, respectively (13, 14). PMEA is a broad-spectrum antiviral agent active against both retroviruses and DNA viruses, whereas HPMPC is highly effective against herpesviruses but not retroviruses (5, 10). These acyclic nucleoside phosphonate analogs are highly resistant to degradation by phosphatase or nucleotidase but are metabolized intracellularly to their corresponding biologically active diphosphoryl derivatives by cellular enzymes (15–17). The diphosphoryl derivatives presumably inhibit the viral polymerase function, by competition with their deoxynucleoside triphosphate counterparts for binding to reverse transcriptase and/or by their chain-terminating activity, in the case of the PMEApp, which lacks a 3′-OH group (15–17). An attractive feature of these compounds is their lack of requirement for initial phosphorylation by viral or cellular nucleoside kinases, a rate-limiting step that is required for most other antiviral nucleosides (5).

It has generally been demonstrated that the acyclic nucleoside phosphonates confer a long-lasting antiviral effect and thus require infrequent dosing *in vivo* (18–28). Several *in vitro* studies have demonstrated that a brief exposure to HPMPC confers long-lasting antiviral effects in cell culture (29, 30). However, in a recent report, the antiviral effect of PMEA was evident only when it was present throughout the infection (31). Here we have investigated the metabolism and antiviral activities of HPMPC, PMEA, and the related derivative HPMPA (Fig. 1), to determine the cellular basis for the long-lasting effects of HPMPC and to determine whether this is a common feature of all acyclic nucleoside phosphonates.

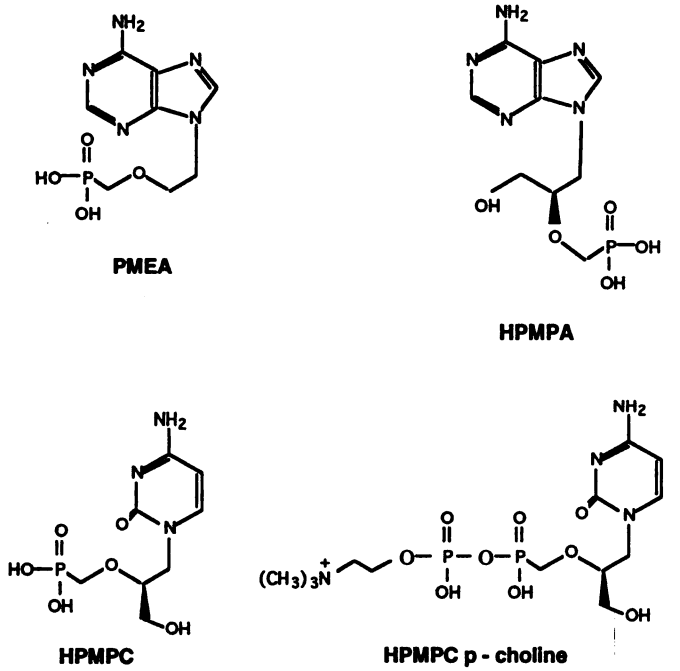

Fig. 1. Structures of the acyclic nucleoside phosphonates PMEA, HPMPA, HPMPC, and HPMPCp-choline.

Materials and Methods

Cells and virus. Vero cells (CCL 81), originally from the American Type Culture Collection (Rockville, MD), were maintained in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, nonessential amino acids, and 2 mM L-glutamine, in a 37° incubator with 5% CO_2. The virus used was HSV-1 strain KOS. Virus was grown and titered in Vero cell monolayers.

Compounds and enzymes. The acyclic nucleoside phosphonates PMEA, PMEDAP, HPMPC, and HPMPA (12, 32), as well as authentic phosphorylated standards for PMEA and HPMPC, were kindly supplied by Drs. Norbert Bischofberger and John Martin (Gilead Sciences, Forest City, CA). [^{3}H]PMEA (11 Ci/mmol) and [^{3}H]HPMPC (21 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). [^{3}H]Choline chloride (75 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Stock solutions of drugs were made in dimethylsulfoxide and stored at −20° until used. Snake venom phosphodiesterase and alkaline phosphatase were products of Sigma Chemical Co. (St. Louis, MO).

Antiviral assays. The effect of various compounds on HSV replication was monitored by plaque reduction or virus yield reduction assays. For plaque assays, Vero cells were seeded in 24-well tissue culture plates and grown to confluence in 18–24 hr. The cells were washed with phosphate-buffered saline, pH 7.4, and a 100-μl aliquot of a pretitered inoculum of HSV-1 (KOS strain), adjusted to contain 100 pfu of the virus, was added to each well. After 1 hr of adsorption at 37°, the cells were incubated with medium containing various drug concentrations. After 48 hr, the monolayers were stained with 0.1% crystal violet solution, the plaques were enumerated, and the IC_{50} values for the various drugs were determined. Appropriate controls for cell, virus, and drug cytotoxicity were included with each assay. For yield reduction assays, confluent monolayers of Vero cells grown on 24-well tissue culture plates were infected at a multiplicity of 1 pfu/cell and incubated in the absence or presence of various compounds. After 48 hr, the cells were lysed by three cycles of freezing and thawing. The cell debris was removed by centrifugation, and the total virus present in the supernatant was titered by plaque assays in Vero cells. In certain experiments, the cells were pretreated for varying periods of time and washed with buffer before virus infection, and the effects of drug treatments were monitored either by plaque reduction or by reduction in virus yield.

Metabolism of PMEA and HPMPC in Vero cells. The radiochemical purity of [^{3}H]PMEA and [^{3}H]HPMPC was first ascertained by analytical reverse phase HPLC and UV spectroscopy. As warranted, [^{3}H]PMEA was purified on an ion exchange Partisil 10 SAX column, using 0.003 M ammonium phosphate, pH 4.3, and 5% methanol buffer before use. Exponential cultures of Vero cells grown on 60- × 15-mm dishes were incubated with 2 ml of 25 μM [^{3}H]PMEA or 25 μM [^{3}H]HPMPC, at 37°. At various time points, the labeled cell extracts were prepared according to previously described procedures (33, 34). Briefly, the cells were washed extensively in ice-cold phosphate-buffered saline, pH 7.4, and extracted with 500 μl of ice-cold 70% methanol in 15 mM Tris, pH 7.4, for 30 min on ice. The methanol extracts were clarified by centrifugation at 14,000 rpm for 5 min in an Eppendorf microcentrifuge and the supernatant was stored at −20° until further analysis.

PMEA and HPMPC metabolites in the labeled cell extracts were analyzed by HPLC on a Whatman Partisil 10 SAX column, as described previously (33, 34). A linear gradient of 5–600 mM ammonium phosphate buffer, pH 4.0, at a flow rate of 1.5 ml/min, was used for the elution of PMEA and its metabolites. HPMPC metabolites were analyzed using a gradient of 15–700 mM ammonium phosphate buffer, pH 3.5, at a flow rate of 1.5 ml/min. The samples were spiked with unlabeled authentic PMEA or HPMPC standards before injection, and the radioactive peaks were identified by comparison with the UV profiles of the authentic standards.

Enzymatic degradation of drug metabolites. Portions of extracts prepared from about 10^6 Vero cells were treated for 3 hr

with 5 units of alkaline phosphatase in 0.1 M Tris·HCl buffer, pH 8.0, in the presence of 0.05 M $MgCl_2$. For cleavage with snake venom phosphodiesterase, cell extracts were treated with 1 mg of enzyme in Tris·HCl buffer, pH 8.9, in the presence of 0.015 M $MgCl_2$. The reaction products were then separated by HPLC as described above.

Results

Antiviral activity of the various phosphonates. We compared the antiviral effects of the phosphonates PMEA, PMEDAP, HPMPA, HPMPC, and foscarnet in plaque reduction assays. All of the compounds were found to inhibit HSV-1, and the IC_{50} values ranged from 4 μM to 40 μM (Table 1). To determine whether the different compounds could confer an antiviral state when cells were pretreated with the drugs before virus infection, we also investigated the plaque-forming efficiency of HSV in Vero cells that had been pretreated with the various compounds for a period of 24 hr. Under these conditions, the IC_{50} values for HPMPC were comparable to the values obtained in plaque reduction assays, where the drug was maintained throughout the 48-hr infection period. In contrast, ~2–5-fold greater concentrations of foscarnet, PMEA, or HPMPA were required for 50% plaque reduction in pretreated cultures, compared with cultures where the drugs were maintained throughout the course of infection. These differences were statistically significant ($p < 0.01$).

We next examined the time course of appearance of the antiviral state in drug-pretreated cultures, to ensure that the reduced antiviral activity in cultures pretreated with drugs other than HPMPC was not due to insufficient exposure, which might limit the uptake or metabolism of these compounds, and the results are shown in Table 2. A maximal antiviral state (as indicated by the IC_{50} values) was achieved by 12 hr after the addition of the drugs for all of the compounds, i.e., HPMPC, HPMPA, and PMEA. Surprisingly, the antiviral activity in cells exposed to PMEA for longer periods was considerably lower than that in cells exposed for 12 hr. In contrast, prolonged incubation with HPMPA or HPMPC did not result in any increase, or decrease, in the antiviral states of the exposed cultures.

Vero cells were also pretreated with PMEA, HPMPA, or HPMPC for 12 hr and then incubated with drug-free medium for varying periods, in an attempt to determine the duration of persistence of the antiviral state. At different times (days 1, 3, 4, 7, and 8), the cells were monitored for their viability and morphology, infected with a standardized inoculum of HSV (~100 pfu), and examined for plaque formation after 48 hr. As shown in Fig. 2, cells exposed to PMEA or HPMPA rapidly lost their antiviral state, by ~24 hr or ~72 hr, respectively. In contrast, cells exposed to HPMPC resisted HSV infection for >7 days.

TABLE 2

Antiviral activity in Vero cells exposed to acyclic nucleoside phosphonates for varying periods of time

Vero cells grown to confluence on 24-well tissue culture plates were pretreated with increasing concentrations of the various drugs. At the indicated times, the drugs were removed and the cells were washed before infection with a standardized inoculum of HSV-1 (~100 pfu/well); the number of plaques formed in different cultures was enumerated after 48 hr of infection. IC_{50} values (mean ± standard deviation) were calculated from dose-response curves obtained for each exposure period.

Time of exposure	IC_{50}		
	PMEA	HPMPA	HPMPC
hr		*μM*	
6	>200	28 ± 4	7 ± 3
12	43 ± 6	17 ± 3	3 ± 0.1
24	87 ± 21	16 ± 2	3 ± 0.6
48	>200	16 ± 3	3 ± 1.0
72	>200	22 ± 7	3 ± 1.7

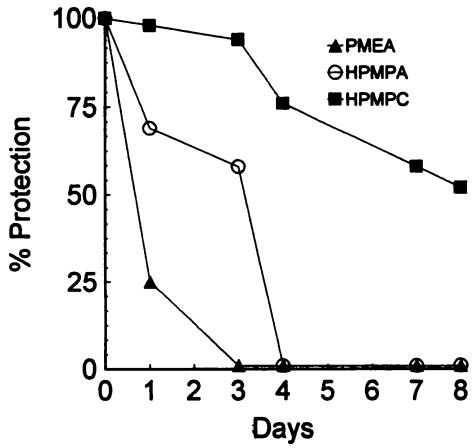

Fig. 2. Persistence of antiviral activity in Vero cells pretreated with acyclic nucleoside phosphonates. Exponential cultures of Vero cells grown in 24-well microtiter plates were incubated with PMEA (90 μM), HPMPA (30 μM), or HPMPC (10 μM). After 24 hr, the drugs were removed and cells were incubated in drug-free medium. At the indicated times after pretreatment, the cells were infected with HSV-1 (KOS) (~1 pfu/cell). After 48 hr, the total intracellular and extracellular virus was harvested by repeated freezing and thawing and was assayed by plaque formation on Vero monolayers. The results are expressed as percentage protection, which corresponds to the percentage reduction in the yield of infectious virus in drug-teated cultures, compared with untreated controls.

TABLE 1

Inhibition of HSV-1 plaque formation in Vero cells by various phosphonates

Plaque reduction assays were carried out on as described in the text. For continuous exposure, Vero cells grown to confluence in 24-well tissue culture plates were infected with an inoculum of HSV-1 (KOS) adjusted to contain ~100 pfu and, after a 1-hr adsorption period, the cells were washed and incubated with varying concentrations of the indicated drugs. For pretreatment studies, uninfected Vero cells were incubated with varying concentrations of the drugs for 24 hr and washed before infection with HSV; the drugs were not included during the postadsorption period. Each value represents the mean ± standard deviation of three independent assays. *p* values were calculated by Student's *t* test.

Treatment	ED_{50}				
	PMEA	PMEDAP	HPMPA	HPMPC	Foscarnet
			μM		
Continuous exposure	40 ± 4	10 ± 2	4 ± 0.4	4.0 ± 0.8	25 ± 5
24-hr pretreatment	90 ± 13 ($p < 0.01$)	35 ± 5 ($p < 0.01$)	20 ± 2.0 ($p < 0.01$)	4.2 ± 0.6 (NS[a])	57 ± 4 ($p < 0.01$)

[a] NS, not significant.

Metabolism of PMEA and HPMPC in Vero cells. The uptake of HPMPC into Vero cells was substantially greater than that of PMEA. After incubation of the Vero cells with 25 μM drug, the intracellular concentrations of PMEA and its metabolites PMEAp and PMEApp reached maximal levels of 1.3, 0.08, and 0.34 pmol/10^6 cells, respectively, and declined thereafter (Fig. 3). In comparison, with 25 μM HPMPC the intracellular concentrations of HPMPC reached a level of 8.3 pmol/10^6 cells, and HPMPCp and HPMPCpp accumulated steadily throughout the 24-hr period of incubation, reaching levels of 0.3 and 0.7 pmol/10^6 cells, respectively (Fig. 4). Furthermore, a fourth prominent metabolite was formed from HPMPC in the Vero cells and reached concentrations greater than those of HPMPCpp (~2.5 pmol/10^6 cells) after the 24-hr time period (Fig. 4). Unlike the aforementioned HPMPC nucleotides, this metabolite resisted degradation by alkaline phosphatase but was quantitatively susceptible to degradation by venom phosphodiesterase, yielding HPMPC as the product (Table 3). Other workers have observed the presence of such a metabolite in human embryonic fibroblasts and have identified it as HPMPC diphosphocholine (35, 36). To confirm the nature of this liponucleotide, we labeled Vero cells for 12 hr in choline-free medium containing HPMPC (25 μM) and [*methyl*-^{3}H]choline (0.05 μM). Chromatography of the cellular extract (Table 4) did reveal incorporation of radioactivity into the HPMPC liponucleotide fraction.

Catabolism of PMEA and HPMPC and their metabolites in Vero cells. To determine the clearance of the anabolites accumulated from these two agents, Vero cells were labeled for 24 hr with radioactive PMEA or HPMPC, washed, resuspended in fresh drug-free medium, and sampled periodically. Cell extracts were prepared and analyzed by HPLC to determine the kinetics of clearance of the phosphonates and their metabolites. As shown in Fig. 5, marked differences were seen in the catabolism of PMEA and HPMPC. PMEA and its metabolites PMEAp and PMEApp were rapidly cleared from the cells, with a half-maximal clearance time ($t_{1/2}$) of <5 hr. In contrast, the clearance of various HPMPC metabolites was extremely slow, as reported by others (35), and appeared to follow biphasic kinetics. The half-lives for HPMPCp and HPMPCpp were ~24 hr in the initial phase and ~65 hr in the later phase. For HMPCp-choline a monophasic pattern, with a half-life of ~87 hr, was clearly noted.

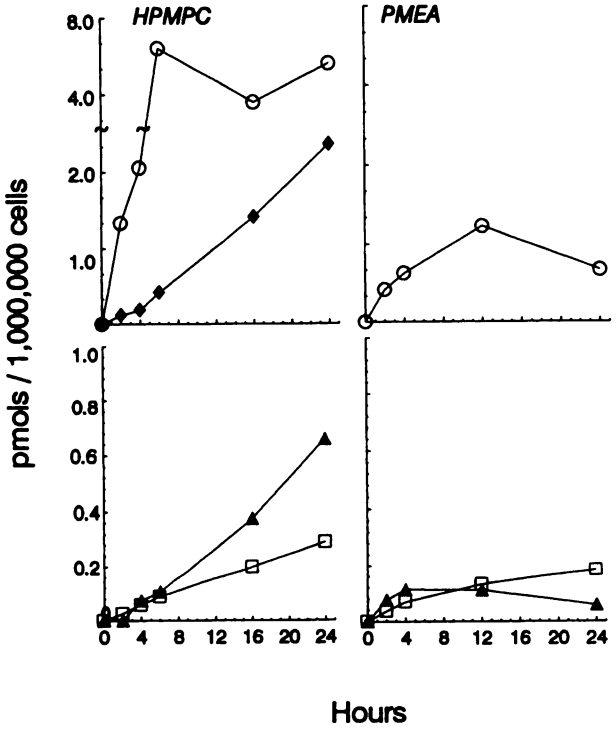

Fig. 3. Uptake and metabolism of PMEA and HPMPC in Vero cells. Exponential cultures of Vero cells were radiolabeled with a 25 μM solution of [^{3}H]PMEA or [^{3}H]HPMPC. At the indicated times, cells extracts were analyzed by anion exchange HPLC, as described in the text, for the levels of the phosphonates (○), their monophosphorylated (□) and diphosphorylated (▲) derivates, and the choline adduct of HPMPCp (♦). The values are shown as pmol/10^6 cells.

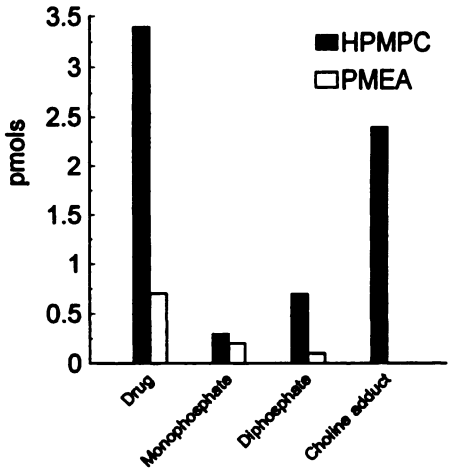

Fig. 4. Intracellular levels of drug and drug metabolites in Vero cells treated with 25 μM [^{3}H]PMEA or [^{3}H]HPMPC for 24 hr. The values are shown as pmol/10^6 cells.

TABLE 3

Identification of HPMPC metabolites by anion exchange HPLC

Vero cells were incubated with 25 μM [^{3}H]HPMPC for 24 hr, washed free of drug, and extracted with 70% ice-cold methanol as described in Materials and Methods. The supernatant fraction was analyzed for HPMPC metabolites either before or after enzyme treatment, as indicated. The results of a representative experiment are shown.

Treatment	Composition			
	HPMPC	Metabolite X	HPMPCp	HPMPCpp
		%		
None	70.0	18.0	3.7	7.7
Alkaline phosphatase	83.4	16.6	0	0
Phosphodiesterase	89.0	1.5	3.0	6.3

TABLE 4

Effect of choline on the formation of HPMPCp-choline in Vero cells

Vero cells were incubated for 12 hr in choline-free medium with 5% dialyzed fetal calf serum, in the presence of 0.05 μM [^{3}H]choline and 25 μM HPMPC. Cells were washed and extracted with 60% ice-cold methanol, and the extracts were analyzed by ion exchange HPLC as described in Materials and Methods. The results of a representative experiment are shown.

Composition		
Choline	Choline phosphate	HPMPCp-choline
	pmol/10^6 cells	
0.08	1.24	0.32

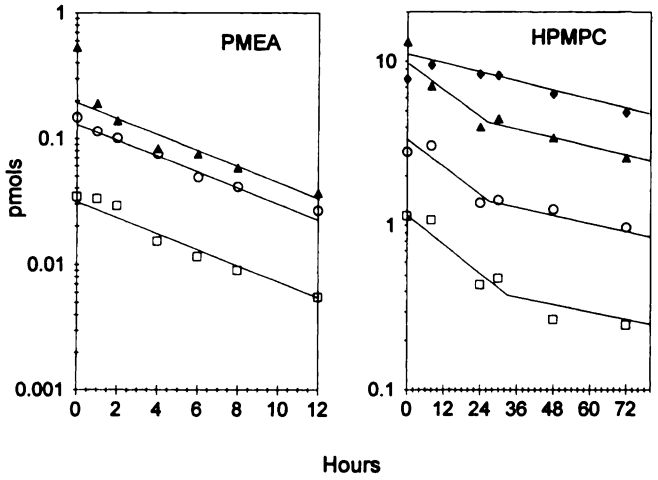

Fig. 5. Catabolism of PMEA and HPMPC by Vero cells. Exponential cultures of Vero cells were radiolabeled with a 25 μM solution of [^{3}H]PMEA or [^{3}H]HPMPC for 24 hr. The cells were washed and incubated in drug-free medium, and cells extracts were prepared at the indicated times. The samples were analyzed for the levels of the phosphonates (▲), their monophosphorylated (□) and diphosphorylated (○) derivates, and the choline adduct (◆) by anion exchange HPLC, as described in the text. The values are shown as pmol/10^6 cells.

Discussion

The acyclic nucleoside phosphonates tested in this study all inhibited HSV replication to varying degrees, and the rank order of potency was HPMPC > HPMPA > PMEDAP > PMEA. These results are in general agreement with previous reports (5). The factors that influence the antiviral efficacy are likely to include the cellular pharmacology of the various compounds, in addition to their intrinsic physicochemical properties. Available evidence indeed suggests that cellular uptake is a critical factor that affects PMEA efficacy, and bispivaloyloxymethyl-PMEA, a lipophilic prodrug of PMEA that is readily taken up by the cells, shows a significant increase in its antiviral efficacy against both HSV (data not shown) (32) and HIV (32, 34) *in vitro*, compared with PMEA.

The spectrum of antiviral activity of the nucleoside phosphonates is markedly affected by the nature of the acyclic side chains. Thus, the 9-(2-phosphonylmethoxyethyl) series of nucleoside phosphonates, e.g., PMEA, exhibit broad-spectrum activity against both RNA and DNA viruses, whereas 3-hydroxy-2-phosphonylmethoxypropyl derivatives exhibit potent antiviral activity against DNA viruses but not RNA viruses, although the diphosphorylated derivatives of all of these phosphonates can inhibit retroviral reverse transcriptase *in vitro*. In contrast, the phosphonate analogs 9-(2-phosphonylmethoxypropyl)adenine and 9-(2-phosphonylmethoxypropyl)diaminopurine selectively inhibit retroviruses but not herpesviruses. This suggests that the differential uptake and metabolism of these compounds in the target cells may be important determinants of their selectivity.

The acyclic nucleoside phosphonates are generally thought to confer a long-lasting antiviral effect and require infrequent dosing *in vivo*; for example, exposure of cytomegalovirus-infected cells to HPMPC for a single 6-hr period inhibited viral DNA synthesis over a period of at least 7 days (5). A similar long-lasting antiviral effect of HPMPC has been observed *in vivo* in various animal models, e.g., in the control of topical or systemic HSV-1 infections in mice and rabbits, cytomegalovirus infections in mice or rats, simian varicella virus infections in monkeys, or vaccinia virus infections in mice with severe combined immunodeficiency (20–28). Likewise, a single administration or infrequent administrations of PMEA were found to be effective for the prophylaxis or treatment of Moloney sarcoma virus infections in mice (18, 19). It has been suggested that the prolonged effectiveness may be related to long retention (~16–18 hr) of the active intracellular diphosphorylated metabolites of these compounds (15, 35–36). Our results are consistent with the idea that HPMPC can confer a long-lasting antiviral activity, which correlates well with the prolonged retention of HPMPC metabolites. However, we were unable to demonstrate a similar long-lasting antiviral effect with the acyclic nucleoside phosphonates PMEA and HPMPA. Moreover, the half-lives of PMEA metabolites were relatively short (i.e., $t_{1/2}$ ~ 5 hr) both in Vero cells and in CEM cells (34) and were considerably shorter than the value of ~18 hr reported for MT-4 cells (15). In a recent report, the anti-HSV-1 activity in HeLa cells also required the continuous presence of PMEA throughout the course of infection (31). We therefore believe that, unlike HPMPC, PMEA (and possibly other phosphonates) is unable to confer a long-lasting antiviral effect *in vitro* and the intracellular half-life of PMEA (and its metabolites) is relatively short, consistent with its biological activity. The *in vivo* efficacy observed with infrequent dosing of PMEA in animal models may therefore be, at least in part, related to the immunomodulatory effects of PMEA (37).

The differences in the antiviral efficacy of HPMPC and PMEA can be explained, at least partly, by differences in their metabolism. First, the cellular uptake and accumulation of HPMPC in Vero cells were about 7 times greater than those of PMEA. Second, a greater level of active metabolites accumulated in Vero cells from HPMPC than from PMEA at equimolar drug concentrations. Indeed, after 24 hr of incubation, the intracellular HPMPCpp formation exceeded the PMEApp concentration by ~8-fold. In addition, HPMPC was metabolized in Vero cells to a form that was previously identified as HMPCp-choline (36). This metabolite was the most abundant HPMPC metabolite in Vero cells, exceeding the concentration of HPMPCpp by 3-fold. Thus, in the studies shown in Fig. 2, Vero cells incubated with HPMPC (25 μM) generated HPMPCpp levels of ~0.27 μM (based on a cell volume of 3 pl), compared with a HMPCp-choline concentration of ~1 μM. The formation of phosphodiesters of HPMPC is not unique to Vero cells and has also been reported in MRC-5 and LEP human embryonic lung fibroblasts (35, 36). Cihlar *et al.* (36) also demonstrated the incorporation of radioactivity into HMPCp-choline fractions in LEP cells incubated with HPMPC and radioactive choline, indicating that human cells can utilize HPMPC for phospholipid synthesis, presumably via phosphatidylcholine cytidylyltransferase. The substrate specificity of the enzyme for this analog, and particularly the question of whether HPMPC can be metabolized to more than one phospholipid, needs to be determined.

It is of interest that the slow disappearance of HPMPCpp does not extend to the corresponding purine phosphonate PMEApp, which appears to be eliminated from the cells at a rate that is normal for nucleotides, although similar prolonged retention of nucleotide analog has been observed for

dideoxyadenosine triphosphate, the active metabolite of the antiviral agent dideoxyinosine (38). For HPMPC, this behavior may reflect either a very low capacity of cellular nucleotide-degrading enzymes to act on HPMPCpp or the ability of the phosphodiester to act as a depot for replenishment of HPMPCpp, despite its intracellular decay. Other cytosine analogs, such as arabinosylcytosine (an anticancer drug) and ddCyd, can also be metabolized to phosphodiesters (39–41). For ddCyd, both the choline and ethanolamine derivatives have been detected, with the latter constituting the major ddCyd metabolite in human lymphoid cells (40). Although the possible role of ddCyd phosphodiesters in the antiviral activity of the drug is unknown, it should be noted that ddCyd is the most potent of all dideoxynucleosides in clinical trials and is administered at a dose one tenth of that used for 3′-azido-2′,3′-dideoxythymidine. Thus, it seems likely that ddCyd phosphodiesters, and by analogy HMPCp-choline, are biologically active and exert their effects either directly or through conversion to the nucleoside triphosphate analog. It is of interest to note that, at least in one study, ddCyd triphosphate was reported to have a long half-life in two different human lymphoid cells lines (42). In terms of clinical significance, the present study supports the rather infrequent (once or twice weekly) administration that has been recommended for HPMPC (14). However, it should also be apparent from the present studies that the unusual metabolic behavior of HPMPC is not similar to that of other acyclic phosphonate analogs currently being developed. Finally, it is possible that a prolonged persistence of unusual metabolites may contribute to drug toxicity over time, and more detailed studies to elucidate the mode of action of such compounds are warranted.

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