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MECHANISM OF UPTAKE OF THE PHOSPHONATE ANALOG (*S*)-1-(3-HYDROXY-2-PHOSPHONYLMETHOXY-PROPYL)CYTOSINE (HPMPC) IN VERO CELLS

MICHELE C. CONNELLY,* BRIAN L. ROBBINS* and ARNOLD FRIDLAND*†‡
*Department of Infectious Diseases, St. Jude Children's Research Hospital, and
†Department of Pharmacology, University of Tennessee, Memphis, TN 38101, U.S.A.

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Abstract—The cellular uptake of phosphonylmethoxypropyl cytosine (HPMPC) was characterized to gain insight into the molecular properties that allow this anticytomegalovirus drug to permeate cell membranes. The time course of uptake of HPMPC into Vero cells was linear between 10 and 75 min and proportional to the concentration in the medium from 10^{-6} to 10^{-2} M. HPMPC uptake was temperature sensitive and the rate of uptake was considerably lower at 27° than at 37° and almost totally inhibited at 4°. In competition studies with naturally occurring nucleosides, nucleotides or the phosphonylmethoxyethyl derivatives, none affected the uptake of HPMPC at concentrations up to 2000-fold molar excess. The uptake of [^{3}H]HPMPC into Vero cells was compared with that of [^{14}C]sucrose, a probe for fluid-phase endocytosis. Kinetics for both compounds were very similar, as were the effects of the microtubule antagonist colchicine and the tumor promoting agent phorbol myristate acetate. Colchicine and the phorbol ester are known to, respectively, inhibit and stimulate endocytosis. It is concluded from these data that HPMPC enters Vero cells by fluid-phase endocytosis and that once internalized it may accumulate in the lysosome. Protonation of the negative charge on the phosphonyl group in HPMPC may allow its diffusion across the lysosome membrane and eventual activation to its putative active diphosphorylated form in the cell cytoplasm.

Derivatives of the acyclic nucleoside phosphonate analogs (phosphonylmethoxy)alkyl purine and pyrimidine have been shown to have potent anti-HIV and/or anti-HSV activity [1, 2]. Among the most promising of these acyclic nucleotide analogs is the cytosine derivative (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC§). This compound has demonstrated *in vitro* activity against a wide range of DNA viruses including herpes simplex virus type 1 (HSV-1) and -2, varicella zoster virus, human and murine cytomegalovirus (CMV) and simian varicella virus both *in vitro* and *in vivo* [2–4].

Biochemical studies have shown that HPMPC inhibits HSV DNA synthesis at concentrations that are significantly lower than that required for inhibition of cellular DNA synthesis and that HPMPC is converted to its mono- and diphosphorylated derivatives by cellular enzymes [5]. HPMPC acts as a stable monophosphate analog and retains the anionic charge characteristic of nucleoside monophosphates; therefore, its ability to enter cells may be limited. Nonetheless, it is clear that the acyclic nucleotide analogs such as HPMPC do enter cells in sufficient concentrations to produce impressive biological effects in different cell systems. The mechanism of cellular uptake of these analogs is still unclear. The reports on the purine nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)adenine (PMEA) suggest that cellular uptake in Vero cells is carried mediated [6], whereas PMEA uptake in H9 human lymphoid cells seems to be by endocytosis [7]. In this paper, an investigation of the mechanism of transport of HPMPC in Vero cells was initiated to determine if the uptake process for this drug is carrier mediated.

‡ Corresponding author: Dr. Arnold Fridland, Department of Infectious Diseases, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, TN 38101-0318. Tel (901) 522-0459; FAX (901) 527-6616.

§ Abbreviations: HPMPC, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; HSV-1, herpes simplex virus type 1; CMV, cytomegalovirus; NBMPR, 6-[(4-nitrobenzyl)thiol]-9-β-D-ribofuranosylpurine; EMEM, Eagle's minimum essential medium with Earle's salt.

MATERIALS AND METHODS

Materials. Tritiated [5-^{3}H]HPMPC (sp. act. 21 Ci/mmol) was obtained from the Moravek Biochemical Co. (Brea, CA). The radiochemical was 97% pure (as analyzed by ion exchange HPLC) and was used without further purification. [^{14}C]Sucrose (sp. act. 475 mCi/mmol) was obtained from the New England Nuclear Corp. (Boston, MA). 6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR) and dipyridamole were obtained from the Sigma Chemical Co. (St. Louis, MO). All other nucleosides and chemicals were from Sigma.

Measurement of [^{3}H]*HPMPC uptake*. Cellular uptake studies were carried out using Vero cells. These cells were routinely maintained by serial culture in Eagle's minimum essential medium (EMEM) supplemented with 5% (v/v) heat-inactivated fetal bovine serum and 2 mM L-glutamine (BioWhittaker Inc., MD). For uptake experiments, 4.4×10^6 cells/dish (60×15 mm) were incubated

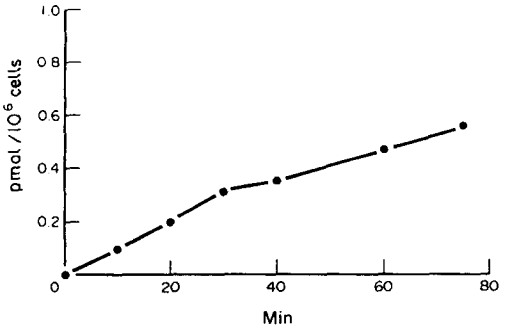

Fig. 1. Uptake of [^{3}H]HPMPC in Vero cells. Cells were incubated with 10 μM [^{3}H]HPMPC, and uptake was measured as described in Materials and Methods. Results are the averages of two separate experiments done in triplicate.

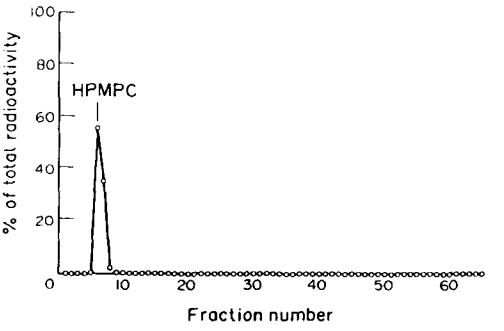

Fig. 2. High performance liquid chromatography of cell extracts after incubation of Vero cells for 60 min at 37° in medium containing 10 μM [^{3}H]HPMPC. HPLC was performed on ion exchange SAX column as described in Materials and Methods.

with 10 μM [^{3}H]HPMPC (5 μCi) in serum-free EMEM containing 25 mM HEPES for the indicated time periods. Control for accumulation of label at zero-time and its subsequent release was performed for each experiment to determine the efficiency of the washing procedure and the extent of tritium binding (adsorption) to the cell membrane and culture dish. From these controls it was calculated that the washing procedure left behind $<0.001\%$ of the extracellular radioactivity. All kinetic studies reported release after a zero-time loading was subtracted from reported values. After the incubation, the medium was removed and cells were washed by dipping the dish five times in separate beakers containing 1 L of ice-cold Dulbecco's phosphate-buffered saline supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Cells were then treated with 1.5 mL of trypsin-EDTA for 20 min at 37°, and cell-associated radioactivity was determined by liquid scintillation counting. Cells were incubated over a range of 4–37° for the temperature dependency studies; all other uptake studies were carried out at 37°.

Methods for detecting HPMPC metabolites. An HPLC procedure similar to that described by Ho *et al.* [5] was used to ascertain the extent to which intracellular HPMPC was metabolized during the uptake studies. Aliquots of cells incubated with [^{3}H]-HPMPC for periods of up to 75 min were washed with phosphate-buffered saline, scraped from the dishes, extracted with 70% ice-cold methanol, and centrifuged; then the supernatant was analyzed by HPLC. The HPLC conditions used were a Whatman Partisil 10 SAX column using a linear gradient from 10 to 700 mM ammonium phosphate buffer for 45 min, pH 3.5, pumped at 1.5 mL/min. Fractions were collected (1 mL) in a scintillation vial, and radioactivity was determined. Radioactive profiles were compared with that of authentic HPMPC nucleotide standards.

RESULTS

Kinetics of HPMPC uptake in Vero cells. Figure 1 shows a time course for the uptake of 10 μM [^{3}H]-HPMPC in Vero cells. Uptake of HPMPC increased almost linearly over a 75-min period of incubation, permitting the determination of an uptake rate. In several different experiments, the average rate of HPMPC uptake was 0.6 ± 0.04 pmol/10^6 cells/hr. Methanol extracts of the cell pellets were prepared and analyzed by ion exchange HPLC. This analysis revealed that $>99\%$ of the cell-associated radioactivity after 60 min of incubation coeluted with authentic HPMPC (Fig. 2). Thus, intracellular phosphorylation was not a significant factor within the time frame of our experiments. Ho *et al.* [5] have reported previously that HPMPC undergoes very slow conversion to the mono- and diphosphorylated derivatives in cells. Our experiments showed that several hours were required before phosphorylated products, $HPMPC_p$ and $HPMPC_{pp}$, could be detected (data not shown). Figure 3 summarizes results of uptake time courses performed at HPMPC concentrations between 10^{-6} and 10^{-2} M.

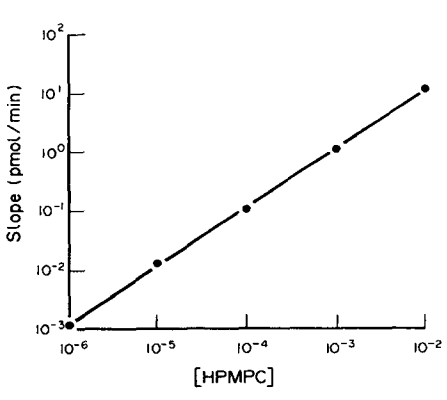

Fig. 3. Kinetics of [^{3}H]HPMPC uptake. The time course of [^{3}H]HPMPC uptake was observed for 10–75 min as in Fig. 1 in the presence of various concentrations of unlabeled HPMPC ranging from 10^{-6} to 10^{-2} M. The slopes of the lines fit to the data were determined by linear regression.

As can be seen from Fig. 3, the uptake of drug into the cells was directly proportional to the concentration of HPMPC in the medium.

In additional experiments, various nucleosides, nucleobases and nucleotides such as cytidine, cytosine, deoxycytidine, PMEA, and CMP were tested for their effects on the uptake of HPMPC in Vero cells. None of the compounds when tested at concentrations of up to 2000-fold excess had any effects on the cellular uptake of HPMPC in Vero cells (Table 1). Similarly, the nucleoside transport inhibitors dipyridamole and nitrobenzylthioinosine had no inhibitory effects on the uptake of HPMPC.

Effect of sodium on the uptake of HPMPC. Since the transport of PMEA in Vero cells has been shown to be sodium dependent [6], the effect of sodium on HPMPC uptake was also examined. No significant difference was found in the uptake of [^{3}H]-HPMPC in sodium-poor medium containing *n*-methylglucammonium chloride (MGA) compared with medium containing 0.15 mM sodium (data not shown).

Similarity of HPMPC uptake and fluid-phase endocytosis. Fluid-phase endocytosis is known to be temperature dependent and sensitive to various metabolic inhibitors [8–10]. Figure 4 shows that reducing the temperature from 37° to 27° slowed the rate of [^{3}H]HPMPC uptake by about half, and at 4° net uptake of HPMPC was almost totally inhibited. The effect of colchicine on the uptake of HPMPC was also examined and was compared with that of sucrose, a marker of fluid-phase endocytosis. To allow comparison between the two substrates, the results were expressed as microliters of medium cleared of drug per milliliter of cell H_2O. As shown in Fig. 5, there was very little difference between the uptake of HPMPC (0.36 μL/min/mL) and sucrose (0.34 μL/min/mL) in Vero cells. Addition of 10 μM colchicine to the incubation medium caused a partial inhibition of the uptake of HPMPC and sucrose in Vero cells.

Phorbol myristate acetate stimulates endocytosis in macrophages [11]. Figure 5 shows that the rate of uptake of both HPMPC and sucrose in Vero cells was enhanced at least 2-fold in the presence of 50 ng/mL of this compound. This suggests that modulation of the rate of endocytosis may alter response of target cells infected with virus to HPMPC or its derivatives.

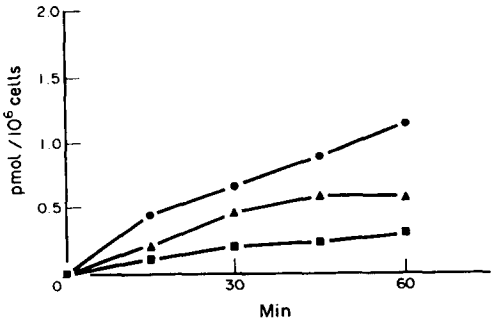

Fig. 4. Time course of the uptake of radioactivity in Vero cells incubated with 10 μM [^{3}H]HPMPC at different temperatures. Cells were preincubated for 30 min at different temperatures in control medium at 37° (●), 27° (▲), or 4° (■) prior to the addition of [^{3}H]HPMPC. Uptake of radioactivity in Vero cells was determined as described in Materials and Methods. Each point represents the average of two determinations that varied by < 15%.

DISCUSSION

It is understood from experimental data that in order to exert its antiviral action HPMPC and its acyclic nucleotide analogs must be converted to the pharmacologically active antimetabolite $HPMPC_{pp}$. Drug uptake, the initial step in the intracellular accumulation of HPMPC, plays an essential role in achieving sufficient high levels of drug to maintain

Table 1. Compounds tested for their effects on [^{3}H]HPMPC uptake in Vero cells

Compound		Concentration of compound	Control (%)
Bases and nucleosides	Cytosine	1 mM	100
	Thymine	1 mM	108
	2′-Deoxycytidine	1 mM	103
	Cytidine	1 mM	97
Nucleotides	PMEA	2 mM	107
	Cytidine 5′-phosphate	2 mM	97
	Thymidine 5′-phosphate	2 mM	101
	Deoxycytidine 5′-phosphate	2 mM	93
Nucleoside transport inhibitors	Dipyridamole	10 μM	102
	Nitrobenzylthioinosine	5 μM	100

The uptake of 10 μM [^{3}H]HPMPC was determined in the presence or absence of unlabeled compounds in the incubation medium for 60 min as described in Materials and Methods. The data are the means of two separate experiments performed in duplicate. The amount of [^{3}H]HPMPC uptake in the control Vero cells during this period was 0.76 pmol/10^5 cells.

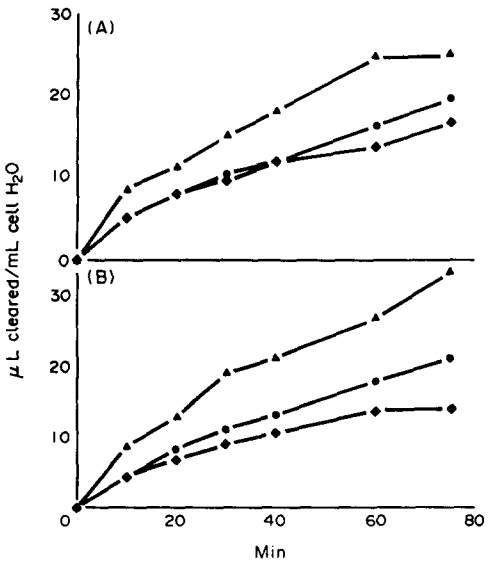

Fig. 5. Comparison of the rate of uptake of [^{3}H]HPMPC and [^{14}C]sucrose. Vero cells were incubated with (A) 10 μM [^{14}C]sucrose (●) or (B) 10 μM [^{3}H]HPMPC (●) in the presence of 10 μM colchicine (◆) or 50 μM phorbol myristate acetate (▲). To permit comparison, the data were normalized as the volume of extracellular medium taken up.

production of phosphorylated drug in target cells. There are only two reports on the uptake of the purine acyclic phosphonyl derivative PMEA, and these have given conflicting results. Prus *et al.* [6] reported that uptake of PMEA in Vero cells appears to be carrier mediated since it was sodium dependent, concentrative and inhibited by other nucleotides. In contrast, Palu *et al.* [7] found no evidence for a carrier-mediated process of PMEA uptake in human lymphoblastoid H9 cells and suggested that the mechanism of PMEA uptake was by endocytosis.

In this study we have investigated the uptake of the acyclic nucleoside phosphonate analog HPMPC in greater detail in order to further understand the mechanisms for its antiviral activity. HPMPC is a stable monophosphate analog because the carbon-phosphorous bond is not susceptible to phosphorylytic cleavage [1, 3]. In the present study, we show that Vero cells incubated with radiolabeled HPMPC and sucrose accumulated the drugs at a similar rate which approached the extracellular concentration by approximately 1.8%/hr. The rate of sucrose uptake is an accepted measure of fluid-phase endocytosis in mammalian cells, and the similarity between these rates is compelling evidence that the same mechanism, namely fluid-phase endocytosis (i.e. not adsorptive), is operative for the uptake of HPMPC. Additional evidence for this mechanism is the fact that the uptake of HPMPC, like that of sucrose, is temperature sensitive, inhibited by colchicine, and stimulated by phorbol myristate acetate [10, 12].

The permeation of HPMPC (10 μM) in Vero cells was slow and contrasted with the 500-fold faster uptake reported by Pruss *et al.* [6] for PMEA, but is similar with data recently reported by Palu *et al.* [7] for PMEA uptake in H9 lymphoid cells. The reason for the discrepancy between the different results is not clear; however, it seems possible that some of the uptake kinetics demonstrated by Prus *et al.* for PMEA may represent cellular uptake of radioactive contaminants present in the sample.

The intracellular fate of radiolabeled HPMPC following entry of the drug is not directly indicated by our experiments, but there is every reason to suppose that it reaches the lysosomes. At the lysosomal pH of 4.5, HPMPC would be partially protonated. A decrease in its negative charge should allow it to partially partition into membrane lipids and diffuse across lysosomal membranes at a slow but increased rate for further metabolism and eventual inhibition of viral replication in infected cells.

Appreciation of the mechanism of uptake of HPMPC may help to explain the numerous *in vitro* and *in vivo* observations of the efficacy of HPMPC and its acyclic phosphonate derivatives acting as inhibitors of viral replication [1–4]. For example, Bronson *et al.* [3] and Yang and Datema [13] showed that a single dose of HPMPC 4 days prior to infection by HSV type 2 protects mice against an otherwise fatal infection. The apparent linearity of uptake of HPMPC with time (Fig. 1) provides evidence that the endocytosed HPMPC probably remains within cells for a considerable time. Ho *et al.* [5] showed that HPMPC and its metabolites have intracellular half-lives in the range of 6–48 hr. It should also be noted that fluid-phase endocytosis is cell cycle dependent and greatest during the G_1 phase [13]. Thus, it might be expected that the effectiveness of HPMPC and its derivatives may be variable among different cell types.

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