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Enhanced chemical stability of the intracellular prodrug, 1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl] cytosine, relative to its parent compound, cidofovir

Reza Oliyai [a], William A. Lee [a], Gary C. Visor [b], Lung-Chi Yuan [a,*]

[a] *Department of Formulation and Process Development, Gilead Sciences, Inc., 353 Lakeside Dr., Foster City, CA 94404, USA*
[b] *Roche BioScience, 3401 Hillview Ave., Palo Alto, CA 94304, USA*

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Abstract

Degradation kinetics of cyclic HPMPC (cHPMPC), 1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine, and its parent compound cidofovir (also known as HPMPC) were conducted in the pH range of 2–11 at 70°C. cHPMPC manifested greater chemical stability than cidofovir, except under alkaline conditions (pH > 9). Three degradation products—cidofovir, cyclic HPMPU and HPMPU—were identified for cHPMPC, and the product distribution was characterized via a stability-indicating HPLC assay. Cyclic HPMPU and HPMPU are the uracil analogs of cHPMPC and cidofovir, respectively, formed through a hydrolytic deamination pathway. The deamination and hydrolysis rate constants for cHPMPC under acidic conditions were derived from the degradation product curves. The deamination rate constants for cHPMPC were about 8-fold slower compared to that for cidofovir. The enhanced chemical stability for cHPMPC relative to cidofovir is attributed to the absence of intramolecular catalysis with cHPMPC.

Keywords: Chemical stability; Cidofovir; Cyclic HPMPC; Cytosine; Deamination; Prodrug

1. Introduction

Cyclic HPMPC (cHPMPC; 1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine) is a cyclic analog of cidofovir. Cidofovir (also known as HPMPC) is a cytosine nucleotide analog with potent antiviral activity against herpes viruses in both preclinical (Bronson et al., 1989; Ho et al., 1992) and clinical (Polis et al., 1995) studies. cHPMPC is stable in plasma; however, it undergoes an enzymatic conversion to

* Corresponding author. Tel.: +1-650-522-5806; fax: +1-650-522-5660; e-mail: lung-chi_yuan@gilead.com.

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cidofovir inside cells (Bischotberger et al., 1994). Thus, cHPMPC is an 'intracellular' prodrug of cidofovir with equivalent in vitro and in vivo potencies to cidofovir. In addition, cHPMPC demonstrates a 10-fold reduction in nephrotoxicity as compared to cidofovir in various animal species (Bischotberger et al., 1994). The reduced nephrotoxicity results from the limited systemic exposure to cidofovir ($<10\%$) and the rapid transport of cHPMPC through the proximal tubular cells of the nephrons (Cundy et al., 1996).

Similar to other cytosine containing compounds, the primary degradation products for cidofovir and cHPMPC are the deaminated uracil analogs (Garrett and Tsau, 1972; Chen et al., 1991). Degradation kinetics of cHPMPC were conducted in support of the development of a stable liquid dosage form for the treatment of cytomegalovirus infection in patients with acquired immunodeficiency syndrome (AIDS). Interestingly, cHPMPC exhibits greater stability over a wide pH range relative to its parent compound, cidofovir. In this paper, the structural basis for the enhanced stability is explored using additional cidofovir analogs, and a mechanism to explain these observations is proposed.

2. Materials and methods

2.1. Materials

cHPMPC (Bischotberger et al., 1994), cidofovir (Bronson et al., 1989), the mono ethyl ester of cidofovir (HPMPC-POEt), and the benzyl ether of cidofovir (HPMPC-OBz) were synthesized at Gilead Sciences, Inc. Tetrabutylammonium dihydrogen phosphate was purchased from Fluka Chemika (purity $>97\%$). All salts and solvents were either reagent or high-performance liquid chromatography (HPLC) grade and used as received. Deionized water was used for buffer and HPLC mobile phase preparation.

2.2. Instrumentation

Measurement of pH was carried out using a Corning Ion Analyzer 250 pH meter, equipped with a Sensorex SG900C combination electrode. HPLC assays were performed using either a fully automated, computer-controlled Hewlett-Packard 1090 M HPLC system or a Spectra-Physics HPLC system equipped with a Model P4000 solvent delivery system, a Model UV2000 detector, a Model AS3000 Autosampler, and Beckman PeakPro software with a Chromlink module.

2.3. Dissociation constant (K_a)

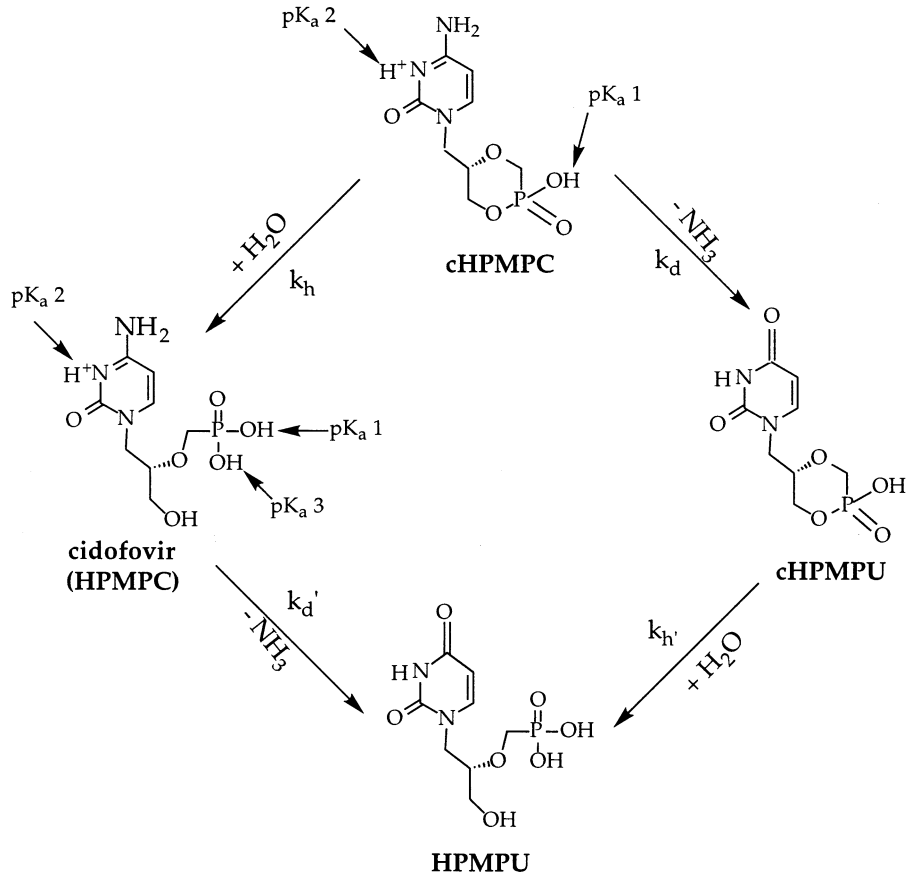
The dissociation constants for cHPMPC and cidofovir were measured by potentiometric titration at 25 and 70°C. cHPMPC and cidofovir (0.010 M) were prepared in CO_2 free deionized water and titrated stepwise with 0.20 N HCl. A Brinkmann RM6 Lauda water bath was used to maintain a constant temperature during the titration.

2.4. Stability

A series of buffers were prepared for solution stability studies. The pH 2–3 buffers were prepared from H_3PO_4 and NaH_2PO_4. The pH 4–5 buffers were prepared from CH_3COOH and CH_3COONa. The pH 6–8 buffers were prepared from NaH_2PO_4 and Na_2HPO_4. The pH 9–10 buffers were prepared from H_3BO_4, pH adjusted with NaOH. The pH 11 buffer was prepared from NaH_2PO_4 and Na_3PO_4. Total buffer concentration was 50 mM, and total ionic strength was adjusted to 0.15 M with NaCl except the 50 mM NaH_2PO_4–Na_3PO_4 buffer. The total ionic strength for pH 11 buffer was 0.16 M. All the pH values reported in tablets and figures are based on pH measurments at 70°C. Stock solutions of cHPMPC and cidofovir (0.2 mg/ml) were prepared in the above buffers. The stability was monitored at 70°C.

The solution stability studies of HPMPC-POEt and HPMPC-OBz were studied similarly at 0.2 mg/ml, pH 3.9, 70°C.

The rates of compound disappearance were fit to a first-order exponential decay to obtain an observed rate constant (k_{obs}). The pH–rate profiles and product–time curves were fit to the general non-linear curve fitting model using the



Scheme 1. Degradation pathways for cHPMPC.

program KaleidaGraph™ (Version 2.1.3; Abelbeck Software).

2.5. HPLC methodologies

Four stability-indicating HPLC methods were used for the potency assays of cidofovir, cHPMPC, HPMPC-POEt and HPMPC-OBz, respectively. All methods employed a Hypersil ODS C_{18} column (5 μm, 4.6 × 150 mm; Alltech Associates, Inc.) at ambient temperature with a flow rate of 2.0 ml/min and UV detection at 274 nm. Mobile phases were adjusted to pH 6.0 with concentrated phosphoric acid. The mobile phase for cidofovir consisted of 5.0 mM of tetrabutylammonium dihydrogen phosphate (TADP) and 3.5 mM of Na_2HPO_4; for cHPMPC, the mobile phase was 1.5 mM of TADP and 3.5 mM of Na_2HPO_4; for HPMPC-POEt, the mobile phase was 3.0 mM of TADP and 3.5 mM of Na_2HPO_4; for HPMPC-OBz, the mobile phase consisted of 18% of acetonitrile and 82% of 5.0 mM of TADP and 3.5 mM of Na_2HPO_4.

3. Results

3.1. Dissociation constants

The pK_a values of cHPMPC were 2.03 ± 0.11 for the phosphonic acid group and 4.55 ± 0.02 for the N3 site on the cytosine ring determined at 25°C. The corresponding pK_a values of cHPMPC determined at 70°C were 2.08 ± 0.08 and $4.17 \pm$

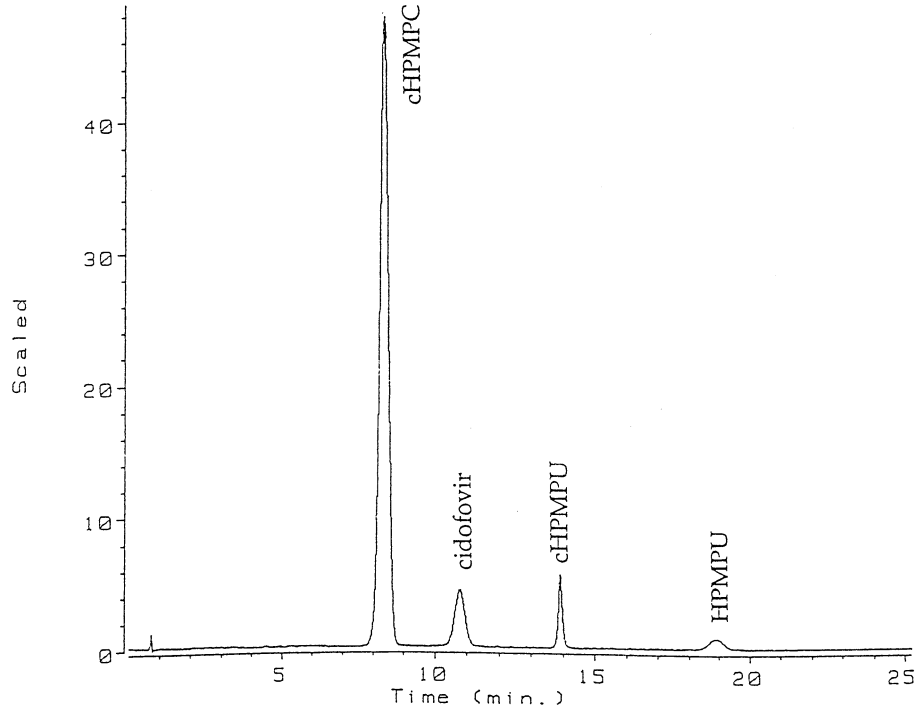


Fig. 1. A degradation chromatogram of cHPMPC (0.2 mg/ml) at pH 2.2 and 50°C for 54 days.

0.05, respectively. The pK_a values of cidofovir were 2.15 ± 0.13 (phosphonic acid), 4.57 ± 0.07 (cytosine) and 7.00 ± 0.06 (phosphonic acid) determined at 25°C. The corresponding pK_a values of cidofovir determined at 70°C were 2.15 ± 0.08, 4.21 ± 0.06 and 7.16 ± 0.02, respectively.

3.2. Degradation pathways

Scheme 1 depicts the proposed degradation pathways for cHPMPC and cidofovir. The primary degradation products of cHPMPC in solution are cHPMPU and cidofovir. Both cidofovir and cHPMPU degraded further to the terminal product, HPMPU. Degradation products were identified by comparison of the UV spectra and HPLC retention times to those of authentic samples. cHPMPU resulted from deamination of cHPMPC, and cidofovir is formed by hydrolysis of cyclic phosphonate ester. A representative chromatogram of cHPMPC and its degradation products, cidofovir, cHPMPU and HPMPU, is shown in Fig. 1.

The distribution between three degradation products of cHPMPC at 70°C for 6 weeks as a function of pH is illustrated in Fig. 2. cHPMPU was the major degradation product at pH 3–9, and cidofovir was the predominant product at

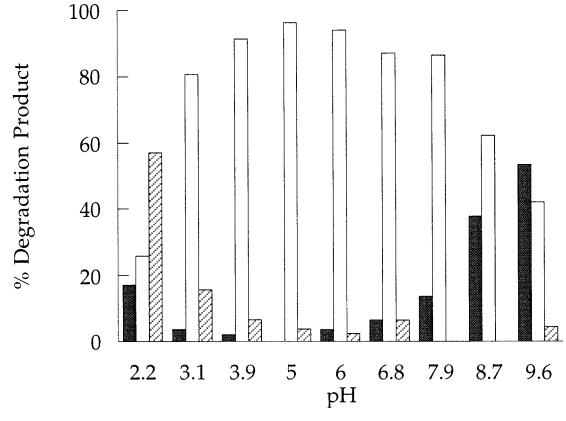


Fig. 2. Plots of the normalized degradation product distribution of cHPMPC as a function of pH at 70°C: cidofovir (solid), cHPMPU (open), and HPMPU (shade).

Table 1
Pseudo first-order rate constants of cHPMPC and cidofovir as a function of pH at 70°C

cHPMPC		Cidofovir	
pH	k_{obs} ($s^{-1} \times 10^8$)	pH	k_{obs} ($s^{-1} \times 10^8$)
2.2	31.1	1.9	58.7
3.1	13.7	3.0	61.8
3.9	7.44	3.9	40.5
5.0	3.01	4.9	14.3
6.0	1.86	5.9	7.91
6.8	1.61	6.4	6.47
7.9	1.60	7.0	4.54
8.7	2.85	7.4	3.18
9.6	9.24	8.1	2.16
		8.7	1.54
		9.6	1.37
		10.6	2.18

basic pH values (pH $\geq$ 9). HPMPU was observed at all pH values except pH 8–9. At basic pH values (pH 8.7 and 9.6), an approximate 2–3% loss in mass balance was observed after exposure at 70°C for 6 weeks. Cytosine ring opening by hydroxide ions to produce degradation products with no chromophore at 274 nm are thought to be responsible for the loss of mass balance (Lonnberg et al., 1986). Mass balance was obtained at all other pH values.

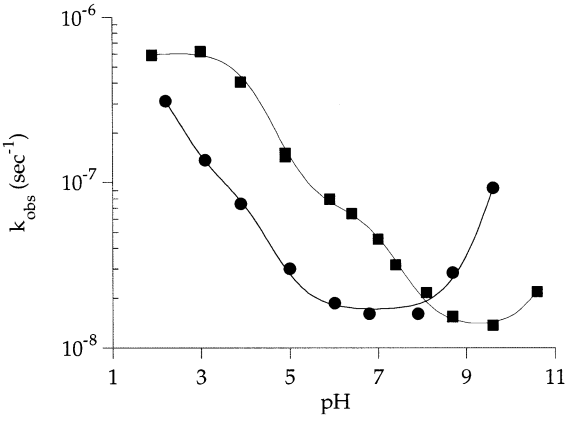


Fig. 3. A comparison of pH–rate profiles for cidofovir (square) and cHPMPC (circle) at 70°C.

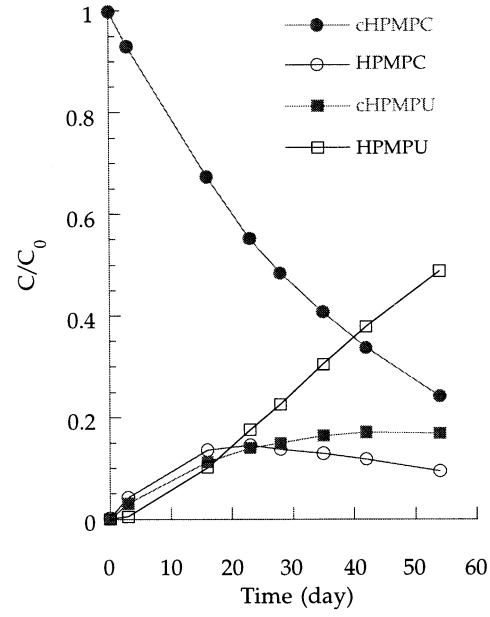


Fig. 4. Product distribution curves of cHPMPC degraded at 70°C in a pH 2.2 phosphate buffer: cHPMPC, cidofovir (HPMPC), cHPMPU, and HPMPU. Computed curves according to the mechanism shown in Scheme 1.

3.3. Degradation kinetics

The rates of disappearance of cHPMPC and cidofovir were fit to a first-order exponential decay model to obtain the observed rate constant, k_{obs} (Table 1). The role of buffer catalysis has not been determined in these studies, and thus the reported rate constants have not been corrected for buffer concentration. Fig. 3 depicts the pH–rate profiles for cidofovir and cHPMPC at 70°C. The shapes of pH–rate profile for cHPMPC and cidofovir are similar, with that of cHPMPC shifted by two pH units to the acidic region. Additionally, the magnitude of the degradation rate below pH 7 for cHPMPC is less than that for cidofovir.

The k_{obs} values for cHPMPC are composed of two individual rate constants as shown in Scheme 1: k_d (the deamination rate constant) and k_h (the hydrolysis rate constant). The k_{obs} values for cidofovir only describe the deamination rate constant ($k_{d'}$). Concentrations of cHPMPC, cidofovir (HPMPC), cHPMPU and HPMPU at time t can be described by the following equations:

$$[\text{cHPMPC}]_t = [\text{cHPMPC}]_0\, e^{-(k_h + k_d)t} \quad (1)$$

$$[\text{HPMPC}]_t = [\text{cHPMPC}]_0[k_h/(k_{d'} - k_h)] \times (e^{-k_h t} - e^{-k_{d'} t}) \quad (2)$$

$$[\text{cHPMPU}]_t = [\text{cHPMPC}]_0[k_d/(k_{h'} - k_d)](e^{-k_d t} - e^{-k_{h'} t}) \quad (3)$$

$$[\text{HPMPU}]_t = [\text{cHPMPC}]_0 - [\text{cHPMPC}]_t - [\text{HPMPC}]_t - [\text{cHPMPU}]_t \quad (4)$$

where $[\text{cHPMPC}]_0$ is the concentration of cHPMPC at time zero, k_d and $k_{d'}$ are the deamination rate constants of cHPMPC and cidofovir, respectively, and k_h and $k_{h'}$ are the hydrolysis rate constants of cHPMPC and cHPMPU, respectively (Scheme 1). Fig. 4 depicts the product formation–time curves of cHPMPC at 70°C at pH 2.2 by fitting the experimental points to Eqs. (1)–(4). The calculated k_d and $k_{d'}$ allow for direct comparison of the deamination rate of cHPMPC and cidofovir obtained under identical conditions.

Table 2 summarizes the calculated rate constants for the deamination and hydrolysis of cHPMPC and cidofovir in the pH range of 2.2–3.9 at 70°C. The relative values of k_d and $k_{d'}$ ($k_d/k_{d'} = 0.11–0.12$) indicate that the hydrolytic deamination rate of cidofovir is approximately 8-fold larger than that of cHPMPC. At pH 3.9, the deamination reaction is the predominant degradation pathway for cHPMPC with minor degree of ring opening ($k_d/k_h = 35.5$). This analysis suggests that the greater stability of cHPMPC as compared to cidofovir at pH 3.9 is primarily the result of the decreased in the rate of deamination.

The relative rates of deamination of cidofovir, cHPMPC, mono ethyl ester of cidofovir (HPMPC-POEt) and *O*-benzyl ether of cidofovir (HPMPC-OBz) are presented in Table 3 at pH 3.9, 70°C. For HPMPC-POEt and HPMPC-OBz, the corresponding deaminated uracil analogs (Table 3) were the degradation products detected under the studied conditions. Modification of the free hydroxyl (HPMPC-OBz) and the phosphonate (HPMPC-POEt) groups of cidofovir reduces the observed rate of deamination of the cytosine moiety by about factor of two. These observations collectively suggest that the acyclic side chain of cidofovir is involved in catalysis of cytosine ring deamination.

4. Discussion

Detailed mechanistic studies of the hydrolytic deamination of cytosine nucleosides under acidic and neutral conditions have been extensively reported (Shapiro and Klein, 1966; Garrett and Tsau, 1972; Kusmierek et al., 1989). The rate-limiting step in the hydrolytic deamination of cytosine is the nucleophilic attack by water at C6 position on the cytosine ring (Wechter and Kelly, 1970). The rate of deamination of 1-β-D-arabinosylcytosine (ara-C) was reported to be 40 times faster than cytidine (Notari et al., 1969, 1970; Dudycz et al., 1977). The enhanced rate of deamination of ara-C was elucidated based on a mechanism involving an intramolecular nucleophilic participation of the 'β' 2′-hydroxyl group on the C5–C6 double bond of cytosine, leading to the formation of a five-membered ring intermediate (Notari et al., 1969, 1970).

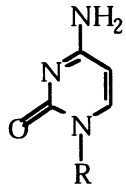
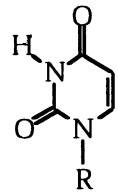
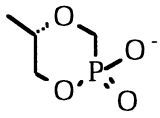
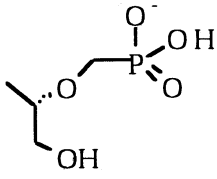
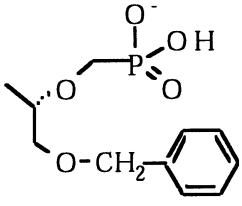
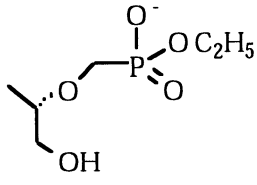
A similar type of mechanism may be invoked to describe the relative reactivity of cHPMPC and cidofovir. Cyclization of cidofovir to form cH-

Table 2
Summary of deamination and hydrolysis rate constants, k_d, k_h, $k_{d'}$, $k_{h'}$, and their ratios calculated from the degradation product curves of cHPMPC and cidofovir at 70°C

pH	k_d (s^{-1})	k_h (s^{-1})	$k_{d'}$ (s^{-1})	$k_{h'}$ (s^{-1})	$k_d/k_{d'}$	$k_h/k_{h'}$
2.2	1.09×10^{-7}	1.94×10^{-7}	9.35×10^{-7}	3.82×10^{-7}	0.12	0.51
3.1	1.07×10^{-7}	1.97×10^{-8}	9.26×10^{-7}	5.09×10^{-8}	0.12	0.39
3.9	6.99×10^{-8}	1.97×10^{-9}	6.37×10^{-7}	–[a]	0.11	–

[a] $k_{h'}$ at pH 3.9 was too small to be determined accurately.

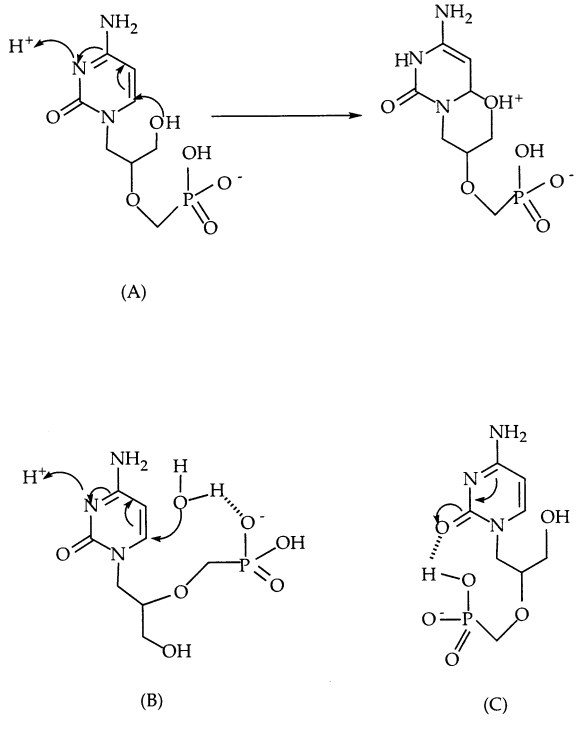
Table 3
Comparison of deamination rate constants for cHPMPC and its derivatives at pH 3.9, 70°C

R	Starting material	Deamination product	Deamination rate constant (k_d, $s^{-1} \times 10^8$)	Relative rate
				
	Cyclic HPMPC (cHPMPC)	Cyclic HPMPU (cHPMPU)	5.4	1.0
	Cidofovir (HPMPC)	HPMPU	44	8.1
	HPMPC-OBz	HPMPU-OBz	22	4.1
	HPMPC-POEt	HPMPU-POEt	26	4.8

PMPC results in the removal of side chain hydroxyl group and modification of a free phosphonate group by formation of a cyclic six-membered phosphonate mono ester. Structural differences between cidofovir and cHPMPC suggests that the free hydroxyl group and/or the free

phosphonate group in cidofovir can also accelerate the rate of deamination via anchimeric assistance or/and nucleophilic participation, similar to the mechanism proposed for ara-C. The free hydroxyl group on the cidofovir side chain may potentially catalyze the deamination through the formation of a six-membered ring intermediate (Scheme 2, A). The decrease in the rate of deamination of HPMPC-OBz is consistent with involvement of the free hydroxyl group in the deamination of cidofovir. Similarly, the deamination rate of the HPMPC-POEt was also reduced as compared to cidofovir, thus indicating that phosphonate moiety may also play a role in the deamination reaction.

Two potential mechanisms involving the phosphonate group of cidofovir are shown in Scheme 2 (B and C). The first involves a general-base catalysis and the second involves a general-acid catalysis. Both cytosine and cytidine have been shown to be subject to general-base and general-acid catalysis by phosphate (Notari et al., 1969).



Scheme 2. Proposed mechanisms for the deamination of cidofovir.

The differences in the conformational flexibility of the side chains may explain the degree and the magnitude of rate acceleration in the case of ara-C as compared to cidofovir.

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