Aryl Ester Prodrugs of Cyclic HPMPC. I: Physicochemical Characterization and *In Vitro* Biological Stability

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Purpose. The chemical, enzymatic, and biological stabilities and physical properties of a series of salicylate and aryl ester prodrugs of the antiviral agent, cyclic HPMPC, were evaluated to support the selection of a lead compound for clinical development.

Methods. Chemical stabilities of the prodrugs in buffered solutions at 37°C were determined. Stability was also studied in the presence of porcine liver carboxyesterases (PLCE) at pH 7.4 and 25°C. Tissue stabilities were examined in both human and dog intestinal homogenates, plasmas and liver homogenates. Prodrug and product concentrations were determined by reverse phase HPLC.

Results. Chemical degradation of the prodrugs resulted in the formation of both cyclic HPMPC and the corresponding HPMPC monoester. Chemical stability was dependent on the orientation of the exo-cyclic ligand; the equatorial isomers were 5.4- to 9.4-fold more reactive than the axial isomers. In the presence of PLCE, the salicylate prodrugs cleaved exclusively to give cyclic HPMPC and not the HPMPC monoester. In plasma, but not intestinal or liver homogenates, the salicylate esters of cyclic HPMPC cleaved readily with a rate dependent on the chain length of the alkyl ester substituent.

Conclusions. The carboxylate function on the salicylate prodrugs of cyclic HPMPC provides an additional handle to chemically modify the lipophilicity, solubility and the biological reactivity of the prodrug. In tissue and enzymatic studies, the major degradation product is cyclic HPMPC. The salicylate ester prodrugs are attractive drug candidates for further in vivo evaluation.

KEY WORDS: prodrug; cyclic phosphonate; reactivity; salicylate esters; cidofovir; cyclic HPMPC; nucleotides.

INTRODUCTION

Nucleotide analogs containing a phosphonate moiety have received considerable attention as antiviral agents (1,2). These nucleotide analogs are converted readily to the active diphosphate species by cellular kinases and exhibit extended intracellular half-lives relative to nucleoside analogs. The ionic character of phosphonate analogs limits their permeability across mucosal and cellular membranes and thus, their bioavailability following oral administration is generally low in animals and humans (3,4). Preclinical and clinical studies have shown that the oral bioavailabilities of the phosphonates can be improved significantly by chemical modification to lipophilic prodrugs (5–7). Recently, the bis[(pivaloyloxy) methyl] ester prodrug of [2-(phosphonomethoxy) ethyl] adenine (PMEA) was shown to increase the oral bioavailability of the parent nucleotide analog in HIV infected patients from less than 5% to 42% (8).

Cidofovir ((S)-[[2-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1-(hydroxymethyl) ethoxy]methyl]phosphonic acid, dihydrate; HPMPC) is a phosphonate nucleotide analog approved for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS. Cyclic HPMPC ((S)-[[2-(4-amino-2oxo-1(2H)-pyrimidinyl)-1-(hydroxymethyl) ethoxy]methyl]phosphonic acid, dihydrate), is a cyclic analog of cidofovir which exhibits similar *in vitro* and *in vivo* antiviral activity to cidofovir but is 10–40 fold less nephrotoxic in rats, guinea pigs, and monkeys (9,10). *In vitro* metabolism studies have revealed that cyclic HPMPC is stable in plasma, liver and intestinal homogenates [3], however, inside intact cells it is rapidly converted to cidofovir by cCMP phosphodiesterase (11). Cyclic HPMPC can therefore be regarded as is an "intracellular prodrug" of cidofovir. The intracellular generation of cidofovir from cyclic HPMPC minimizes systemic exposure to cidofovir and reduces the potential for nephrotoxicity (9,10).

Like cidofovir, cyclic HPMPC is charged at physiological pH and has poor permeability across the human intestinal mucosa (3,12). Unlike cidofovir, which exists as a dianion at physiologic pH, cyclic HPMPC is a phosphonate mono ester and contains only a single ionizable oxygen on the phosphorous. This simplifies the synthesis of a suitable prodrug since only one negative charge needs to be masked. Cyclic HPMPC ester prodrugs are phosphonate diesters, with the original cyclic ester bond being "endo" and the additional ester bond from the promoeity being "exo." In addition to optimizing the physical properties and chemical stability of a potential cyclic HPMPC prodrug, it is important that the prodrug undergo exocyclic bond cleavage exclusively at the phosphonate ester yielding cyclic HPMPC. The endocyclic bond cleavage of the phosphonate ester is undesirable since it leads to the formation of acyclic HPMPC monoester (Scheme I). Unlike cyclic HPMPC, the activity and distribution of the acyclic HPMPC monoesters have not been defined and it is likely that the acyclic monoester could undergo further extracellular metabolism by phosphodiesterases (13,14), resulting in high relative systemic exposure of cidofovir.

In this report we describe the physiochemical characterization and *in vitro* biological stability of a series of aryl ester prodrugs of cyclic HPMPC. The rationale for the synthesis of cyclic HPMPC prodrugs containing salicylate ester promoeities is two-fold. Attachment of the salicylate ester to the phosphorus through the phenolic oxygen offers an additional functionality to modify the physical properties and reactivity of the prodrug at a site remote from the nucleotide. Secondly, cleavage at the salicylate ester site results in the formation of the salicylic acid prodrug which may be capable of internally catalyzing the decomposition of the phosphonate ester to yield cyclic HPMPC. A similar approach has recently been published for a phosphate dimer of 3′-azido-3′-deoxythymidine (AZT) (15).

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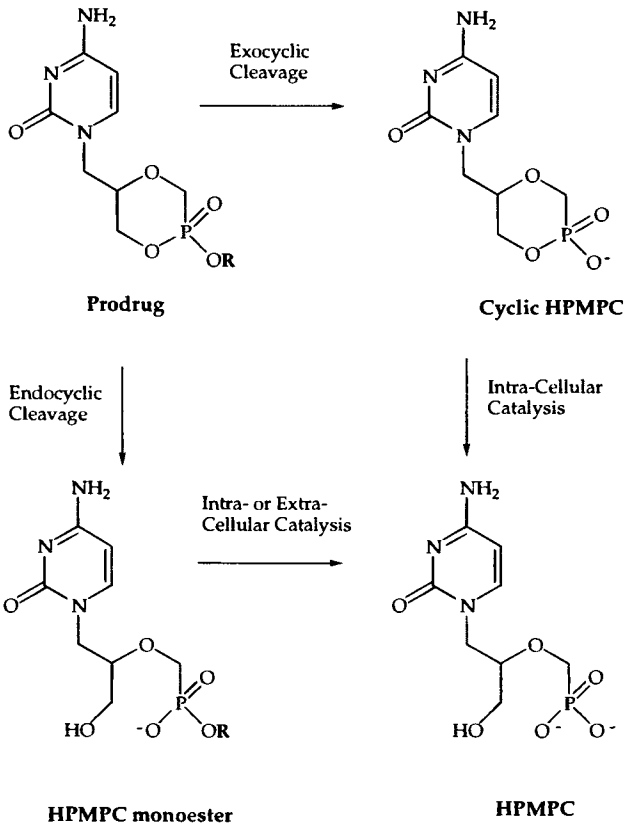


Scheme I. Metabolic and hydrolytic degradation pathways involving P-O bond cleavage for aryl ester prodrugs of cyclic HPMPC.

MATERIALS AND METHODS

Materials

Cidofovir, cyclic HPMPC, and the cyclic HPMPC prodrugs were synthesized at Gilead Sciences. Methanol and acetonitrile were HPLC grade. Water was de-ionized and glass distilled using a Corning Mega-pure system model mp-1, (Moleshiem, France). All other chemicals were of reagent grade and used without further purification. Porcine liver carboxyesterase (PLCE) was purchased from Sigma (St. Louis, Missouri). Measurements of pH were made on a Radiometer PHM84 Research pH meter equipped with a Sensorex SG900C combination electrode.

Pooled normal human plasma was obtained from George King Biomedical, Inc. (Overland Park, KS). Human intestine/liver homogenates were purchased from Keystone Skin Bank (Exton, PA). Pooled cynomolgus plasma was obtained from Sierra Biomedical (Sparks, NV). Dog, rat and cynomolgus intestine/liver homogenates were prepared using a modification of a published protocol (16). Small intestine segments or livers were rinsed and homogenized in 50 mM sodium/potassium phosphate buffer. The crude homogenates were centrifuged at 9000 × g at 4°C for 20 minutes. The supernatants were removed and designated as S9 fractions. These were stored at −70°C until used.

Chemical and Enzymatic Stability

The stability of the prodrugs in solution were evaluated in 10 mM buffer concentration and total ionic strength was adjusted to 0.15 M with KCl. Concentrated stock solutions of the cyclic HPMPC prodrugs (~2–4 × 10^{-3} M) were prepared in DMSO. Chemical hydrolysis was studied by addition aliquots (100 μl) of these stock solutions to 10 ml of buffer solution pre-equilibrated at 37°C in a water bath. At appropriate time intervals, samples were withdrawn and analyzed using a stability indicating HPLC method. The concentration versus time data were fitted to a first-order exponential decay model (KaleidaGraph, version 2.1, Abelbeck software) to obtain the observed rate constants (k_{obs}). All reactions were followed for a minimum of two half-lives.

For the enzymatic studies, the stock solutions (100 μl) of the cyclic prodrugs were diluted in 10 ml of 10 mM phosphate buffer pH 7.4 at 25°C in a water bath. Enzymatic hydrolysis were initiated by addition of PLCE to achieve final enzyme concentration of 40 U/ml. At appropriate time intervals aliquots (400 μl) were removed and added to ice-cold acetonitrile (400 μl), mixed on a vortex mixer and stored at −20°C. Samples were filtered and analyzed by HPLC.

Partition Coefficient and Solubility

Prodrugs were dissolved in 3 ml of octanol, presaturated with phosphate buffer, pH 6.5 (~1 mg/ml). One ml of the octanol solution was mixed with 1 ml of phosphate buffer, pH 6.5, presaturated with octanol, in a 2 ml foil wrapped vial. Vials were rocked on a rocker for an hour at ambient temperature (21–23°C). The two layers were separated by centrifugation at 2000 rpm for 30 minutes and the octanol layer was carefully removed by pipette. The concentrations of prodrug in the octanol and aqueous layers were determined. The octanol phase (1:5) and the aqueous phase (1:3) were diluted with methanol prior to HPLC analysis. Due to limited solution stability of some of the prodrugs, solubilities were measured by adding excess quantity of the prodrug (~5 mg of prodrug to 5 ml of buffer), vortexing and sonicating for 10 min, followed by filtration, pH measurement and dilution for quantitation by HPLC analysis.

Analysis of Stability/Solubility Samples

Quantitation was performed using a HPLC system equipped with a Model P4000 solvent delivery system, a Model UV2000 detector, a Model AS3000 Autosampler (Thermo Separation, San Jose, CA), and Beckman PeakPro software for data acquisition with a Chromlink Module. The HPLC system employed a C_{18} reverse-phase analytical column (15 cm × 3.9 mm, Inertsil, ODS-2) (Alltech, Deerfield, IL) with a mean particle diameter of 5 μm. All analyses were performed with a linear step gradient at ambient temperature. Detection was by UV at 274 nm, and the injection volume was 20 μl. Mobile phase A contained 5 mM tetrabutyl ammonium hydrogen phosphate (TBAHP) and 20 mM phosphate buffer in water at pH 6.8 and mobile phase B contained 100% acetonitrile. Initial mobile phase condition of 95:5 (A:B) was run isocratically for 5 min followed by a linear gradient to 35:65 (A:B) over 18 min followed by 7 min of equilibration (30 min total run time). Flow rate was set at 1.0 ml/min and injection volume of 20 μl was used. The HPLC assay was linear within the range of 2–100 μg/mL with RSD value for repeatability of less than 2%. The retention times for some representative prodrugs such

as **1ax**, **2ax**, **4ax**, **9ax**, **10ax**, **11ax**, **12ax**, and **13ax** are 17.5, 18.2, 22.2, 20.8, 14. 5, 15.6, 17.5, and 20.2 minutes, respectively. The retention time for cyclic HPMPC is 2.3 minutes.

In Vitro *Tissue Stability*

Metabolic stability studies in plasma and intestineal homogenate were performed at a final drug concentration of 10 μg/ml (in DMSO) and in 90% biological fluid. Each compound was incubated with liver S9 fraction in the presence of an NADPH generating system to facilitate cytochrome P450 mediated metabolism (16). All tubes were preincubated without the drug in a shaker bath at 37°C (New Brunswick Scientific, Edison, NJ) and 100 oscillations/min for 5 minutes. The drug solution in DMSO was added to the test incubations, mixed and maintained at 37°C and 100 oscillations/min. Aliquots (50 μl) were withdrawn at 1, 30 and 60 minutes and quenched with 100 μl of 0.1% TFA in acetonitrile. Quenched samples were centrifuged for 5 minutes at 14,000 rpm in an Eppendorf Centrifuge 5402, and the supernatant was used directly for HPLC analysis. A control incubation was performed by heating the matrix at 100°C for 15 minutes to inactivate enzymes/proteins. The heat-treated matrices were centrifuged at 14,000 rpm for 5 minutes and the supernatant was used for the study.

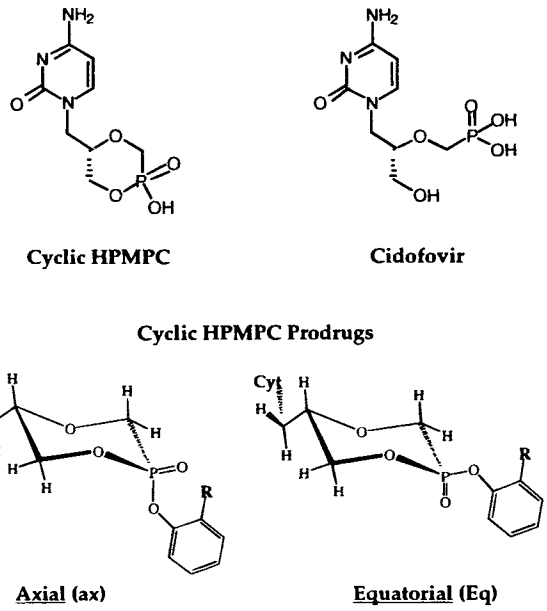
Analysis of Biological Samples

Aliquots were analyzed using an HPLC system comprised of a Model P4000 solvent delivery system with a Model AS3000 autoinjector and a Model UV1000 UV detector. The column was a Zorbax RX-C8 (5 μm, 250 × 4.6 mm) (MAC-MOD, Chadds Ford, NY) equipped with a Brownlee RP-8 Newguard guard column (7 μm, 15 × 3.2 mm) (Alltech, Deerfield, IL). The mobile phases used for reverse phase HPLC were: A, 20 mM potassium phosphate buffer, pH 7.0; B, 65% acetonitrile in 20 mM potassium phosphate, pH 7.0. The flow rate was 1.0 ml/min and the column temperature was maintained at 30°C by a column oven. The gradient profile was a linear gradient to 100% B by 15.0 minutes, returning immediately to 100% A. Detection was by UV at 274 nm, and the injection volume was 50 μl. Total cycle time between injections was 22 min. The bioanalytical method described above was designed specifically to follow the disappearance of each prodrug.

RESULTS AND DISCUSSION

Chemistry

Figure 1 lists the cyclic HPMPC prodrugs synthesized in this study. Esterification of (*S*)-cyclic HPMPC results in the formation of a chiral phosphorous and the potential for diastereomers. Scheme II depicts the general synthetic scheme used for the preparation of the axial and the equatorial prodrugs. The cyclic HPMPC chloridate was converted to the equatorial isomer by the addition of the sodium salt of the substituted phenol and the axial isomers were further enriched by the conditions described by Arimilli and co-workers (17). The chemical synthesis employed resulted predominantly in a single diastereomer (>95%). On the basis of ^{1}H, ^{13}C, and ^{31}P NMR spectra, the 6-membered phosphonate ring was assigned a chair



R	
$COOCH_2CH_3$	Ethylsalicylyl (1)
$COO(CH_2)_3CH_3$	Butylsalicylyl (2)
$COO(CH_2)_4CH_3$	Pentylsalicylyl (3)
$COO(CH_2)_5CH_3$	Hexylsalicylyl (4)
$COOCH_2CH(C_2H_5)(CH_2)_3CH_3$	Isoethylhexylsalicylyl (5)
$COO(CH_2)_9CH_3$	Dodecylsalicylyl (6)
$COOC_6H_{11}$	Cyclohexylsalicylyl (7)
$COOCH_2CH_2C_6H_5$	Phenethylsalicylyl (8)
$COOC_6H_5$	Phenylsalicylyl (9)
H	Phenyl (10)
OC_2H_5	*o*-Ethoxyphenyl (11)
$C(O)(CH_2)_3CH_3$	*o*-Valerylphenyl (12)
$(CH_2)_4CH_3$	*o*-Pentylphenyl (13)

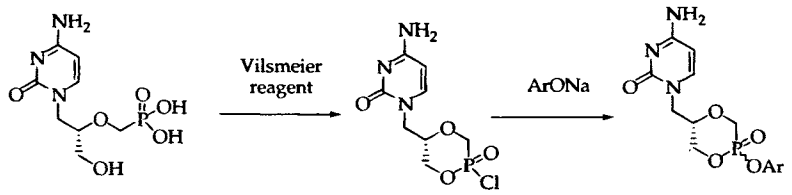
Fig. 1. Conformational isomers of aryl ester prodrugs of cyclic HPMPC.

conformation with cytosine preferentially occupying an equatorial position (17). The axial and equatorial isomers were distinguished unambiguously by additional 2-dimmentional NMR experiments (17). As a consequence of the conformational bias of the cytosine, the diastereomeric exocyclic esters were either axial or equatorial to the plane of the 6-membered ring (Fig. 1).

The carboxyl function on the salicylate provided an additional chemical handle to modify the lipophilicity and reactivity of the prodrug at a site remote from the phosphorus. As depicted in Scheme III, metabolism at the carboxylate will result in the formation of the salicylic acid prodrug, which potentially can degrade spontaneously to cyclic HPMPC and salicylic acid. If operable *in vivo*, this mechanism would minimize concerns regarding the irreversible inactivation of serine proteases resulting from catalysis at the phosphorus (18,19).

Chemical and Enzymatic Hydrolysis

The kinetics of degradation of the cyclic HPMPC prodrugs in buffered solution were described by a first-order exponential decay. The observed half-lives at pH 7.4 and 37°C are shown



Scheme II. General synthetic scheme for the preparation of axial and equatorial prodrugs.

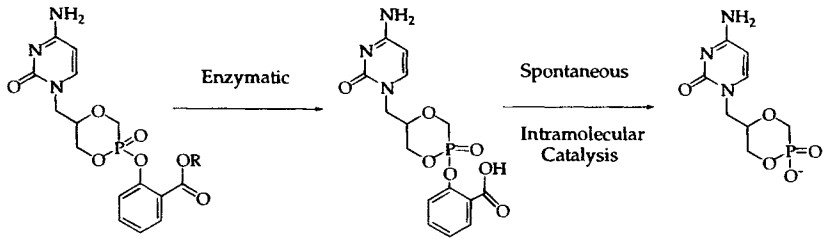
in Table 1. Chemical hydrolysis of the prodrugs resulted in the formation of two degradation products, cyclic HPMPC and the corresponding HPMPC monoesters with a retention time unique for each prodrug. The HPMPC monoester of butyl salicylate was isolated by HPLC and identified by NMR and mass spectrometry (data not shown). These results indicate that initial chemical hydrolysis occurs at the phosphorus and not the carboxylate ester. These findings are in contrast to those previously described for salicylate esters of AZT dimer (15) and suggest a unique hydrolysis pathway for the β-oxy phosphonates.

The chemical stabilities of the individual diastereomers were dependent on the orientation of the exo-cyclic ligand; the equatorial isomers were 5.4 to 9.4-fold more reactive than the corresponding axial isomer for all the prodrugs examined. The ratio of the hydrolysis rates for equatorial and axial isomers (ke/ka) are nearly identical to data previously reported for the alkaline hydrolysis of six-membered cyclic phosphonate diesters and phosphate triesters (20,21). The difference in reactivity was attributed to the greater ground state free energy of the axial isomer relative to the equatorial isomer. This enhanced stability in the ground state is due to the stereoelectronic anomeric effect of the lone pair electrons on the endocyclic oxygen ester. Due to limited chemical stability of the equatorial isomers, these compounds were considered less desirable from a formulation and manufacturing perspective and fewer compounds were characterized.

In buffered solution, the percent exocyclic P-O bond cleavage (Table 1) was dependent on the pH and the orientation of exocyclic ester. For example, hydrolysis of the axial isomer, **2ax**, yielded 59% exocyclic P-O cleavage at pH 7.4 and the equatorial isomer, **2eq**, resulted in 68% exocyclic cleavage. At pH 2, **2ax** yielded 74% exocyclic cleavage. In general, the chemical hydrolysis of the equatorial isomers showed a slightly greater preference for exo-cyclic bond cleavage as compared to the corresponding axial isomers. The rates of chemical degradation at pH 2 were studied for a limited number of the prodrugs (**1ax**, **2ax**, **10ax**, and **11ax**). The half-lives at pH 2 for **1ax**, **2ax**, **10ax**, and **11ax** were 41 hr, 45 hr, 142 hr, and 103 hr, respectively. These half-lives were significantly longer than at pH 7.4 indicating that the oral absorption will not be affected by chemical hydrolysis in the gastro intestinal tract.

The extent of endo-cyclic bond cleavage was unexpectedly high considering the relative leaving group abilities of the phenoxide and the alkoxide ligands and the positive entropic effect resulting from exocyclic cleavage. In the reference cited above, no endocyclic cleavage of the cyclic phosphonate aryl ester was reported (20). Unlike cyclic five-membered phosphates or phosphonates, there is little or no ring strain associated with the six-membered ring analogs. In the case of the cyclic HPMPC prodrugs, the presence of an additional oxygen at the 4 position in the ring possibly adds ring strain and thus increases the relative rate of ring opening (22). Alternatively, if attack at phosphorus to form the pentacovalent phosphorane intermediate is rate-limiting, there will be very little leaving group selectivity. The product distribution can be explained by hydroxide attack at phosphorus opposite to the exo-cyclic P-O bond, resulting in the placement of the exo-cyclic ester in the axial position of the trigonal bipyramid intermediate and subsequent breakdown to cyclic HPMPC. Hydroxide attack opposite to the endocyclic P-O bond results in the analogus placement for the endocyclic ester; with pseudorotation of this intermediate energetically prohibited, and thus breakdown of the intermediate to the acyclic monoester (23,24).

Porcine liver carboxyesterases (PLCE), an esterase with broad substrate specificity, was used to model the enzymatic



Scheme III. Conversion scheme involving C-O bond cleavage for salicylate ester prodrugs of cyclic HPMPC.

Table 1. Physicochemical Properties of Cyclic HPMPC Prodrugs

Prodrug[a]	log P[b]	Solubility (mg/mL)	$t_{1/2}$ (min) pH 7.4, 37°C	% EXO	$t_{1/2}$, PLCE (min) pH 7.4, 25°C
1ax	0.2	>1.0	352	63	>25
1eq	0.0	>1.0	40	75	5
2ax	1.1	0.2	271	59	14
2eq	N.D.	N.D.	35	68	N.D.
3ax	1.6	0.085	282	60	8
4ax	2.1	0.027	220	60	7
4eq	N.D.	N.D.	30	71	N.D.
5ax	2.8	N.D.	362	63	30
7ax	1.6	N.D.	264	64	22
8ax	1.7	0.07	174	64	7
9ax	1.2	0.055	150	60	7
9eq	N.D.	N.D.	28	70	N.D.
10ax	−0.2	>1	375	29	30
10eq	−0.1	>1	40	45	N.D.
11ax	0.1	>1	560	52	>100
11eq	0.3	>1	73	52	N.D.
12ax	0.8	N.D.	260	57	>100
13ax	2.1	N.D.	822	18	>100

Note: N.D. = not determined.
[a] Designations ax and eq refer to axial or equatorial orientation of the aryl ester.
[b] Octanol-pH 6.5 phosphate buffer.

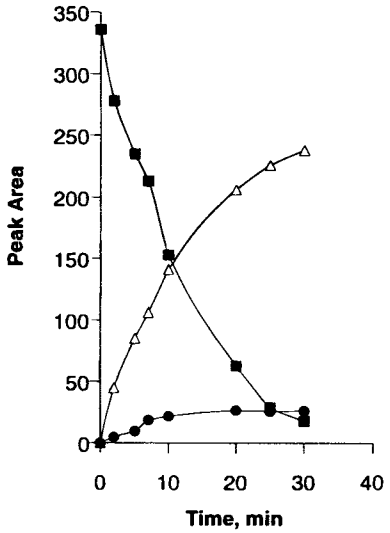


Fig. 2. Product distribution curves of hexylsalicylate cyclic HPMPC (**4ax**) in the presence of 40 U/ml of PLCE at 25°C in a pH 7.4 phosphate buffer: (■ Prodrug **4ax**; △ cyclic HPMPC; ● Intermediate).

conversion of the prodrugs to the parent compound. The half-lives of cyclic HPMPC prodrugs in the presence of PLCE are shown in Table 1. The rate of hydrolysis of compound **6ax** could not be accurately determined due to its limited aqueous solubility. All the salicylate prodrugs (**1–9**) and the phenyl prodrug (**10**) were substrates for PLCE and resulted in >98% exocyclic cleavage to yield cyclic HPMPC. In the presence of 40 U/ml PLCE the $t_{1/2}$ values for the axial isomers at 25°C ranged from 7–30 minutes. In contrast, the alkyl and ethoxy substituted phenyl esters of cyclic HPMPC (**11–13**) were poor substrates for PLCE (>98% remaining after 10 min incubation). For prodrugs **10–13** the PCLE-catalyzed hydrolysis can occur only at the phosphorous atom (P-O bond) and only phenyl cyclic HPMPC (**10ax**) was observed to degrade under the conditions of the PLCE experiment. Apparently, there is a strong inhibitory effect of the non-salicylate ortho substituents in the PLCE catalyzed P-O bond hydrolysis.

The salicylate ester prodrugs may either undergo catalysis by PLCE at the phosphorus (P-O bond) or carboxyl group of the salicylate. Cleavage at the latter site would result in the formation of the corresponding salicylic acid prodrug of cyclic HPMPC which could participate in a intramolecular catalysis to form cyclic HPMPC or the mono-salicylic acid ester of HPMPC (Scheme III). Except for **4ax**, **6ax** and **9ax**, no significant peaks other than the prodrug, cyclic HPMPC and salicyclic acid were observed in the HPLC chromatograms. In the case of **4ax**, **6ax** and **9ax**, PLCE catalyzed hydrolysis resulted in the formation of a common intermediate (identical HPLC retention times) which subsequently degraded to cyclic HPMPC. Figure 2 shows the time-course for the degradation of prodrug **4ax**, the formation of cyclic HPMPC, and the formation of the intermediate in the presence of PLCE. Attempts to characterize the mass of this intermediate using an LC-MS were unsuccessful. The intermediate decomposed under all experimental conditions tried to yield a M/C peak at 261, consistent with the mass of cyclic HPMPC. Low levels (<2%) of this intermediate were observed during PLCE hydrolysis of the other salicylate ester prodrugs.

Small quantities of the intermediate in solution were isolated for further kinetic study by filtering a suspension of **6ax** and PLCE. At pH 7.4 and 25°C, the intermediate degraded to cyclic HPMPC and salicyclic acid with a half-life of 18 minutes, independent of PLCE concentration. The proposed structure for the intermediate is the salicylic acid prodrug of cyclic HPMPC resulting from PLCE catalyzed hydrolysis at the salicylate ester. Since the intermediate is only observed to a significant extent during the degradation of **4ax**, **6ax** and **9ax**, the PLCE catalyzed hydrolysis of the other salicylate prodrugs is believed to occur predominantly by direct cleavage of the P-O bond. This mechanism is unique for the salicylate esters and phenol; the hydrolysis of non-ester ortho substituted prodrugs are not catalyzed by PLCE. Additionally, in the case of salicylate ester prodrugs of AZT dimers, PLCE catalysis resulted in cleaving the carboxylate ester and not the phosphate ester (15). From these data, we hypothesize that the intermediate is the salicylic acid prodrug of cyclic HPMPC (Scheme III) and PLCE catalyzed hydrolysis of the salicylate prodrugs of this cyclic phosphonate occurs at both phosphorous and the carboxylate ester.

Physicochemical Properties

The relative hydrophobicities of cyclic HPMPC prodrugs (Table 1) were evaluated by measuring the octanol-buffer (pH 6.5) partition coefficient (log P). For the salicylate ester series, the log P values were proportional to the molecular weight of the alcohol, with an increase of 0.4 to 0.5 log units for each additional methylene group. The increase in apparent lipophilicity was accompanied by a decrease in the aqueous solubility at pH 6.5 (Table 1).

In Vitro *Biological Stability*

Since the equatorial isomers displayed poor chemical stability and are not ideal clinical candidates only the biological stability of the axial isomers were studied. The stability of the salicylate prodrugs, **1ax–9ax**, were evaluated in plasma and intestinal and liver homogenates from dogs and humans (Table 2). Previously, we have found the dog to be a predictive model for the oral bioavailability of adefovir dipivoxil and bis POC PMPA in human (6,25). Due to the complexity of these tissues it was only possible to monitor the loss of prodrug by RP-HPLC. Preliminary experiments in these fluids using ^{14}C-labeled **2ax** suggests that the major product observed (>90%) was cyclic HPMPC. This observation is consistent with the PLCE-catalyzed hydrolysis of salicylate prodrugs in which cyclic HPMPC was also the predominate product.

With few exceptions, the stabilities of the salicylate prodrugs in dog and human tissues were similar. The stabilities were surprisingly high in liver and intestinal homogenates of both species (no degradation at 60 min). Except for **1ax**, all the salicylates showed significant breakdown in human and dog plasmas. Interestingly the acyclic salicylate esters (**1ax–5ax**) showed a linear dependence (r^2 = 0.997) between the log P and the stability of the prodrug in human plasma (Fig. 3). This linear relationship observed in human plasma suggests that the ester group may play a role in the plasma degradation pathway.

The obvious pathway would be the intitial metabolism of the salicylate ester, resulting in the salicylic acid prodrug intermediate which then spontaneously degrade to cyclic HPMPC. The measured half-life at pH 7.4 for the degradation of this putative intermediate (18 min) is consistent with this proposal. The non-salicylate aryl esters, **10ax**, **11ax** and **12ax** showed no degradation at 60 min in human plasma, consistent with the hypothesis.

The rationale for the synthesis of the salicylate prodrugs was in part guided by a desire to initiate the enzymatic metabolism of the prodrug at a site remote from the phosphorus. As previously stated, phosphonate esters can be irreversible inhibitors of serine proteases. The results obtained in human plasma may suggest that the site of the initial hydrolysis is the salicylate ester, however, regardless of the detailed mechanism, no obvious irreversible inhibition of the enzyme(s) responsible for the metabolism of the salicylate prodrugs is observed.

Table 2. % Prodrug Remaining at 60 Minutes in Biological Fluids at 37°C

	Plasma		Intestinal homogenate		Liver homogenate	
Prodrug	Dog	Human	Dog	Human	Dog	Human
1ax	84	Stable	Stable	Stable	Stable	Stable
2ax	49	66	Stable	78	Stable	Stable
3ax	67	52	Stable	Stable	Stable	Stable
4ax	59	28	Stable	Stable	Stable	84
5ax	41	9	Stable	Stable	Stable	49
7ax	53	78	Stable	Stable	Stable	68
8ax	44	60	Stable	Stable	Stable	72
9ax	46	24	Stable	Stable	75	64

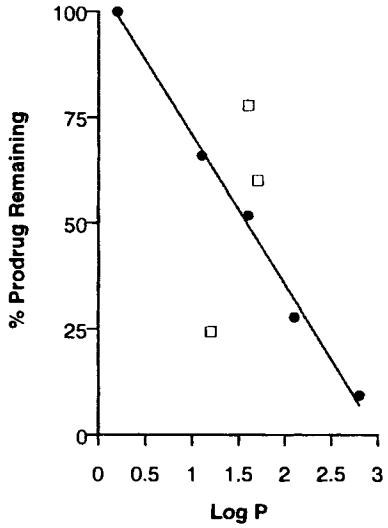


Fig. 3. Relationship between human plasma stability and the octanol-buffer partition coefficient for acyclic salicylate prodrugs (■) [**1ax, 2ax, 3ax, 4ax, 5ax**] and cyclic salicylate prodrugs (□) [**7ax, 8ax,** and **9ax**].

CONCLUSIONS

Aryl esters of cyclic HPMPC were synthesized in a stereospecific manner to give either an axial or equatorial orientation of the exo-cyclic ester with respect to the 6-membered phosphonate ester. Chemical stability was dependent on the orientation of the exo-cyclic ligand; the equatorial isomers were 5.4 to 9.4 more reactive than the axial isomers. Variation of the salicylate ester resulted in predictable changes in solubility and lipophilicity. Chemical stability was only slightly effected by the nature of salicylate ester, however, *in vitro* stability in the presence of PLCE and plasma did show a greater dependence on the salicylate ester moiety. Using PLCE as a model enzyme, the axial isomers of the salicylate prodrugs hydrolyze exclusively to give cyclic HPMPC and not the HPMPC monoester. In plasma, but not intestinal or liver homogenates, the salicylate esters of cyclic HPMPC also cleave readily to give cyclic HPMPC with a rate dependent on the chain length of the alkyl ester substituent. The plasma half-lives for the axial isomers were significantly shorter than the chemical hydroysis at pH 7.4 and 37°C suggesting that metabolism will govern the conversion of the prodrug to the parent drug *in vivo*. The lack of reactivity in intestinal and liver homogenate suggest that *in vivo* there will be circulating levels of intact prodrug. This leaves open the possibility for enhanced activity due to enhanced cellular permeability or distribution of the prodrug relative to cyclic HPMPC. The *in vivo* metabolism and bioavailability of the salicylate prodrugs of cyclic HPMPC will be published separately.

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