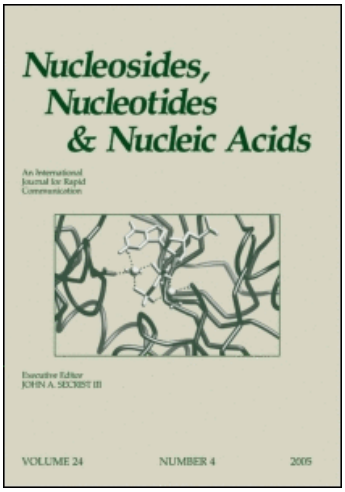

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Synthesis, Biological Activity and Decomposition Studies of Amino Acid Phosphomonoester Amidates of Acyclovir

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Abstract: Highly stable and water soluble amino acid phosphomonoester amidates of acyclovir (ACV) were synthesized and shown to function predominantly as prodrugs of ACV and not acyclovir monophosphate (ACV-MP) with activities within two fold of the amino acid prodrug of ACV, valaciclovir (VACV). Metabolism studies revealed that incubation of cell-free extracts of Vero cells with the L-leucine phosphomonoester amidate of ACV (**3c**), resulted in a burst of ACV-MP production followed by the rapid generation of ACV.

Acyclovir {9-[(2-hydroxyethoxy)methyl]guanine, Zovirax, ACV} **1**, an acyclic analog of the natural nucleoside 2'-deoxyguanosine and guanosine, is a clinically useful antiviral agent that exhibits potent and selective activity against herpesviruses.[1,2] ACV is currently the drug of choice for the treatment and prophylaxis of infections caused by the herpes group of viruses, however, it is only 15-21% bioavailable after oral administration. Although this is sufficient for therapy against herpes simplex virus infections, it is not adequate for treatment of infections with viruses less sensitive to ACV, such as varicella zoster virus.[3] The low bioavailability can be attributed to the poor aqueous solubility (1.4 mg/ml) and low lipophilicity of the drug, the latter being responsible for its poor percutaneous penetration.[4,5] A solution to this delivery problem maybe the development of prodrugs of ACV with more desirable physicochemical properties. Several prodrugs of

ACV have been synthesized with limited success.[6-23] Nevertheless, the L-valyl ester of ACV, valaciclovir (VACV), has been shown to greatly enhance the oral bioavailability of ACV and thus its clinical efficacy.[24-28]

Unfortunately, viral resistance to ACV can develop due to changes in the ability of the viral thymidine kinase to convert ACV to the corresponding monophosphate. These enzyme modifications arise as a result of mutations in the corresponding genes for this enzyme.[29-31] Therefore, several prodrugs have been synthesized with the hope of delivering ACV as a phosphorylated species.[8, 20-22, 32] These approaches would avoid the necessity for activation by the viral thymidine kinase at the expense of removing selective conversion of ACV to acyclovir monophosphate (ACV-MP) by virally infected tissues.

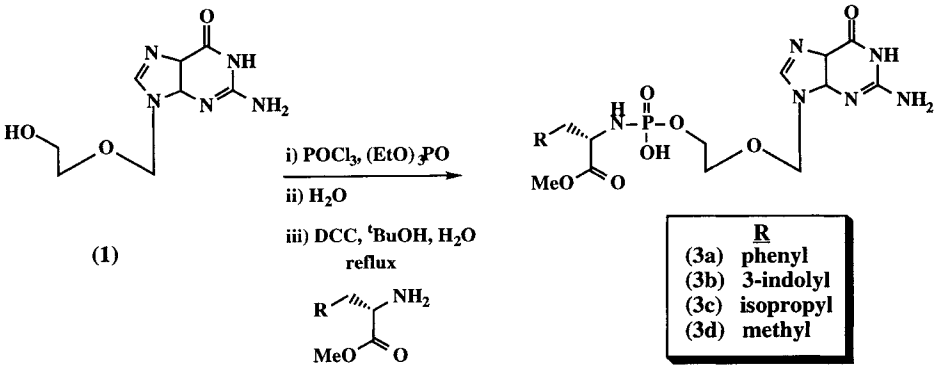
Recently, our laboratory has reported the chemical synthesis and biological activity of a series of aromatic amino acid phosphomoester amidates of zidovudine (AZT) and 5-fluoro-3'-deoxyuridine (FUdR).[33-35] One of these derivatives, 3'-azido-3'-deoxythymidine-5'-*N*-(1-carboxymethyl-2-indolyl-ethyl)phosphoramidate, was found to be 8-fold more active than the parent nucleoside and at least 10-fold less toxic. Surprisingly, when the diester derivatives were incubated in fetal calf serum or human serum at pH 7.2, 37°C, for two to six days, no degradation to the correponding 5'-monophosphate or nucleoside was observable. Preliminary mechanistic studies demonstrated that amino acid phosphoramidates of AZT and FUdR were internalized by lymphocytes and leukemia cells.[33, 35] In contrast to peripheral blood mononuclear cells (PBMCs) incubated with AZT, little or no free nucleoside and nearly four fold more phosphorylated AZT was observed in PBMCs incubated with these AZT derivatives.[33] However, studies with thymidine kinase deficient cells and a radiolabeled phosphoramidate monoester of FUdR revealed that the metabolism of these compounds likely proceeds through P-N bond cleavage, by an unknown phosphoramidate hydrolyzing enzyme, followed by P-O bond cleavage.

The behavior of AZT and FUdR phosphoramidates in cell culture lead us to investigate the utility of amino acid phosphomonoester amidates of ACV as prodrugs for the delivery of phosphorylated ACV. A series of amino acid phosphomonoester amidates of ACV were synthesized and their ability to inhibit the replication of HSV-1 and ACV resistant HSV strains in Vero cells was examined. In addition, the decomposition of the phosphoramidates in fetal calf serum and cell extracts was examined.

RESULTS AND DISCUSSION

Chemistry

The preparation of amino acid phosphoramidates of ACV (**3a-3d**) is shown in Scheme 1. Initially, phosphorylation of ACV (**1**) was attempted using cyanoethylphosphate



Scheme 1

and DCC,[36] leading to a mixture of products. Direct phosphorylation of ACV was accomplished using phosphorus oxychloride in triethylphosphate, yielding the acyclic monophosphate in 60%.[37, 38] ACV-MP (**2**) was then coupled with DCC in refluxing *tert*-BuOH/H_2O to the carbomethoxy esters of L-alanine, L-leucine, L-phenylalanine and L-tryptophan. The crude product mixtures were purified by column chromatography on silica gel followed by reverse phase medium pressure chromatography on a C-18 column, to give **3a-3d** in a yield of 28-89%. The low yield for the the alanine conjugate (**3d**) is probably a result of the enhanced propensity of alanine to form diketo-piperazine relative to the other amino acids.

Previously, we have demonstrated that phosphoramidate diesters of AZT, 5-fluoro-2'-deoxyuridine (FUdR) and 1-β-arabinofuranosylcytosine (ara-C), unlike the corresponding monophosphates, were stable indefinitely at 37°C in human and fetal calf serum. [34, 35] Before testing the biological activity of the ACV derivatives, the stability of the representative phosphomonoester amidate **3a** in growth media was determined. The degree of decomposition for the phosphoramidate in serum was determined by incubation in 20% fetal calf, pH 7.2 at 37°C followed by analysis of the remaining phosphoramidate by reverse-phase HPLC at 31 hr, 51 hr, 72 hr and 96 hr. These experiments revealed that >99% of the added phosphoramidate remained intact after incubation in serum for four days. Therefore, unlike 5'-phosphorylated nucleosides, the phosphoramidates are not rapidly degraded by nonspecific serum phosphohydrolases or phosphorylases and are chemically more stable than VACV ($t_{1/2}$ = 13 hr, pH 7.2)

Biological Activity

The compounds were shown to be non-toxic to Vero cells and tested for their ability to inhibit the cytotoxicity of HSV toward these cells. As can be seen in Table 1 the **3c, 3d**

Table 1. Biological Activity of Amino Acid Phosphomonester amidates of Acyclovir against Acyclovir Sensitive and Resistant HSV isolates.

Compound	EC_{50} (μM)[a] HSV-1 (F) ACV-r	EC_{50} (μM)[a] HSV-2 (D5-4) Vero cells	CC_{50} (μM)[a] Toxicity
3a	2.0	>100	>100
3b	7.7	>100	>100
3c	1.5	>100	>100
3d	1.8	>100	>100
ACV	0.02	31.5	>100
VACV	0.84[b]	-	>500[b]

[a] The variance for the EC_{50} and CC_{50} values was less than ±10%. [b] Adapted from Beauchamp et al.[15]

and **3a** were 75, 90 and 100-fold less active than ACV, respectively, while the tryptophan derivative, **3b**, was nearly 400-fold less active than ACV. Consequently, with the exception that the EC_{50} value for **3b** is 4 to 5 times larger than the values for **3a**, **3c** and **3d**, little preference for either a methyl, isopropyl or phenyl side chain was observed. These results are in sharp contrast to the 8-fold increase, relative to AZT, in the ability of the tryptophan phosphoramidate of AZT to inhibit HIV-1 replication in human peripheral blood mononuclear cells.[33] Nevertheless, the IC_{50} values for **3a**, **3c**, and **3d** were within two fold of the IC_{50} for VACV.[15]

In addition, the phosphoramidates did not exhibit any appreciable activity toward either a strain of HSV with a resistant thymidine kinase (HSV-2 (D5-4) ACV-r). Therefore, because these compounds are stable in growth media, the catabolism of these agents by Vero cells probably occurs intracellularly, resulting in their ultimate conversion to ACV and not to significant amounts of the monophosphate over the couse of the experiment.

Internalization by Vero cells

To determine the extent of cellular uptake of ACV phosphoramidates, Vero cells were incubated with 100 μM of compound **3c** and the amount of intracellular intact phosphoramidate determined at 1 hr, 4 hr and 8 hr time points via a RP-HPLC assay. As can be seen from Figure 1., the intracellular amounts of **3c** increased with time from 10

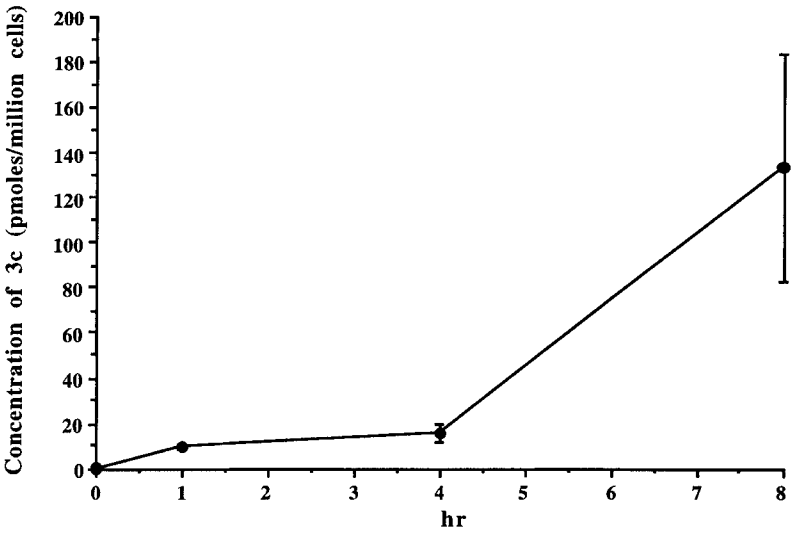


Figure 1.

Intracellular concentration (pmoles/million cells) of compound **3c** from Vero cells incubated with 100 μM **3c**. Error bars represent standard deviations for three separate experiments.

pmoles/million cells at the end of the first hour to 132 pmoles/million cells at the end of 8 hours. Although saturation was not observed over the course of this experiment, the uptake proceeded non-linearly, increasing 1.5-fold from 1 hr to 4 hr and 13-fold from 4 hr to 8 hr.

Decomposition in Cell Extracts

Recently, we have shown that the tryptophan 5'-phosphomonoester amidate of FUdR is converted by CEM cell extracts to the corresponding FUdR 5'-monophosphate followed by rapid conversion to FUdR.[35] Consequently, although the biological activity of the amino acid phosphoramidates of ACV provide little evidence of the presence of significant amounts of ACV-MP in Vero cells treated with these compounds, we examined the metabolism of **3c** by Vero cell extracts for the generation of ACV-MP and ACV.

Typically, **3c** was incubated in Vero cell lysates, pH 7.5, 37°C for a specified period of time and analyzed by anion exchange and reversed phase HPLC analysis. As can be seen in Figure 2, within the first 30 min, a burst of ACV-MP was readily observed followed by a time dependent decrease. The decrease in the ACV-MP concentration corresponded to a time dependent increase in the concentration of ACV. When the

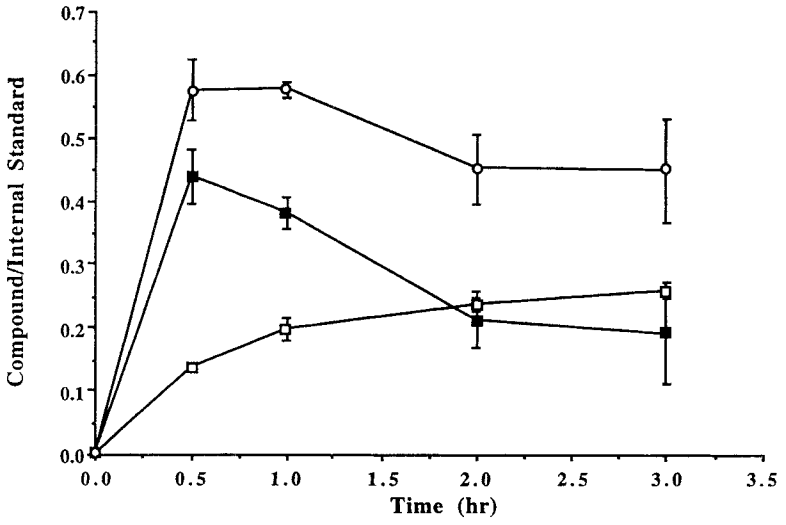


Figure 2.

Production of ACV-MP , —■—, ACV , —□—, and (ACV-MP + ACV) —○— from cell-free extracts of Vero cells incubated with **3c**. The units of the abscissa are given as the ratio of either ACV-MP, ACV or (ACV-MP + ACV) and the internal standard, (-)-Carbovir. Error bars represent standard deviations for three separate experiments.

concentrations of ACV-MP and ACV are summed, the overall concentration of ACV generated from **3c** reached a maximum within the first 30 min of the incubation and slowly decreased over the next 2 hr.

The burst kinetics observed for the production of ACV-MP, lag in generation of ACV and plateau in the overall concentration of ACV are consistent with the conclusion that the major pathway for metabolism of **3c** by cell free extracts consists of P-N bond cleavage followed by conversion to ACV. The subsequent decrease in the overall concentration of ACV probably reflects the slow decomposition of ACV or conversion of ACV-MP to the corresponding diphosphate and triphosphate.

In summary, these results imply that amino acid phosphoramidates of ACV function primarily as prodrugs of ACV and not ACV-MP. The mechanism of activation appears to proceed by first conversion to ACV-MP, followed by rapid P-O bond cleavage. The high stability and rapid internalization of **3c** suggests that the conversion process occurs intracellularly. Recently, we have shown that amino acid phosphoramidates of FUdR and 5'-fluoro-deoxythymidine (FLT) are substrates for a punitive phosphoramidate hydrolase.[35, 39] The relationship of this observation to ACV phosphoramidate hydrolysis by primate and human cells is currently under investigation and will be reported in due

course. Furthermore, because the *in vitro* biological activity of these compounds is comparable to VACV and their stability is considerably greater than observed for VACV, we are currently evaluating the effectiveness of amino acid phosphomonoester amidates to enhance the bioavailability of ACV.

MATERIALS AND METHODS

Materials: NMR (^1H and ^{31}P) spectra were recorded on a Varian VXR-300 spectrometer. An external standard of 85% H_3PO_4 was used for all ^{31}P-NMR spectra. FAB mass spectra were obtained on a VG 7070E-HF mass spectrometer. Analytical TLC was performed on Analtech Silica Gel GHLF (0.25 mm) plates. Column chromatography of water soluble compounds on silica gel was performed using the following solvent gradient : $CHCl_3$:MeOH:H_2O (5:2:0.25), then $CHCl_3$:MeOH:H_2O (5:3:0.5) and finally $CHCl_3$:MeOH:H_2O (5:4:1). Reverse phase MPLC was performed at a pressure of 10-12 psi using an FMI lab pump equipped with a flow meter and pulse dampener. A C-18 column (3/4" x 18", 230-400 mesh) was used with a solvent flow rate of ~1.5 mL/min. A solvent gradient of H_2O:CH_3CN (95:5) to H_2O:CH_3CN (80:20) was used. Concentration under reduced pressure refers to solvent removal on a Buchi rotary evaporator. High vacuum refers to < 10^{-2} psi attained with a DuoSeal mechanical pump. Acyclovir was obtained as a gift from Glaxo-Wellcome, Inc. (USA) and PFA trisodium hexahydrate was obtained as a gift from Astra, Inc. (Sweden).

9-[(2-Hydroxyethoxy)methyl]guanine 2-monophosphate (2)

Triethylphosphate (10 mL) was placed in a dry flask and cooled to 0°C in an ice bath under nitrogen. Distilled phosphorus oxychloride (0.82 mL, 8.78 mmol) was added and then ACV, **1**, was added in one portion. The reaction mixture was stirred at 0°C for 22 hr and then poured into cold water (15 mL). The resulting suspension was extracted with $CHCl_3$ (2 x 20 mL) and diethyl ether (2 x 20 mL) and the aqueous phase adsorbed onto deactivated charcoal (5 g) in a sintered funnel. The charcoal was washed with water (50 mL) and the product isolated by eluting with 1.5 *N* NH_4OH in EtOH:H_2O (1:1, 100 mL). The latter eluate was concentrated to dryness and then redissolved in a small amount of water and introduced onto an ion exchange column (BioRad AG 50W-X8, 100-200 mesh, H^+, 3/4" x 6"). The column was eluted with water and 10 mL fractions were collected. The fractions containing the product were combined and lyophilized. (170 mg, 63% yield). ^1H-NMR (D_2O) : δ 8.78 (1H, s, H8), 5.63 (2H, s, OCH_2N), 3.95 (2H, m, $POCH_2$), 3.81 (2H, m, $POCH_2C\underline{H}_2$). ^{31}P-NMR (D_2O) : δ 1.33.

9-[(2-Hydroxyethoxy)methyl]guanine 2-*N*-(1-carboxymethyl-2-phenyl-ethyl)phosphoramidate (3a)

To a flask containing ACV-MP **2** (80 mg, 0.262 mmol) was added phenylalanine methyl ester (392 mg, 1.84 mmol), DCC (270 mg, 1.31 mmol), ^{t}BuOH (5 mL) and water (1mL). A reflux condensor was attached to the flask and the reaction mixture heated in a boiling water bath for 4 hr. After cooling to room temperature the solvents were removed under reduced pressure. The residue was resuspended in water (15 mL) and extracted with diethyl ether (4 x 15 mL). The aqueous phase was then lyophilized. Column chromatography on silica gel using a $CHCl_3$:MeOH:H_2O solvent gradient followed by reverse-phase medium pressure liquid chromatography (MPLC) on a C-18 column with a H_2O:CH_3CN solvent gradient gave the product as a colorless solid. The product was isolated as a colorless solid (109 mg, 89% yield). ^{1}H-NMR (D_2O) : δ 7.77 (1H, s, H8), 7.09-6.92 (5H, m, Ph), 5.28 (2H, s, OCH_2N), 3.80 (1H, dd, C<u>H</u>CO_2Me), 3.64 (1H, m, $POCH_2$), 3.56-3.40 (3H, m, $POCH_2$, $POCH_2C\underline{H}_2$), 3.51 (3H, s, CO_2CH_3), 2.70 (2H, d, Ph$C\underline{H}_2$). ^{31}P-NMR (D_2O) : δ 7.17. FAB-MS : [M+1] 466.99, [M+1+gly] 559.13; HPLC t_R 8.02 min.[43]

9-[(2-Hydroxyethoxy)methyl]guanine 2-*N*-(1-carboxymethyl-2-indolyl-ethyl)phosphoramidate (3b)

As described above, ACV-MP (90 mg, 0.295 mmol) was coupled with tryptophan methyl ester (450 mg, 2.07 mmol) in the presence of DCC (304 mg, 1.48 mmol). Column chromatography on silica gel followed by reverse phase MPLC gave the product as a colorless solid (112 mg, 75% yield). ^{1}H-NMR (D_2O) : δ 7.75 (1H, s, H8), 7.41 (1H, d, indole H4), 7.31 (1H, d, indole H7), 7.06 (1H, t, indole H6), 7.02 (1H, s, indole H2), 6.95 (1H, t, indole H5), 5.25 (2H, s, OCH_2N), 3.97 (1H, dd, C<u>H</u>CO_2Me), 3.74 (1H, m, $POCH_2$), 3.59 (1H, m, $POCH_2$), 3.54 (3H, s, CO_2CH_3), 3.46 (1H, m $POCH_2C\underline{H}_2$), 3.37 (1H, m, $POCH_2C\underline{H}_2$), 2.97 (2H, broad d, Trp$C\underline{H}_2$). ^{31}P-NMR (D_2O) : δ 7.57. FABMS : [M+1] 505.95; HPLC t_R 9.18 min.[43]

9-[(2-Hydroxyethoxy)methyl]guanine 2-*N*-(1-carboxymethyl-2-methyl-propyl)phosphoramidate (3c)

As described above, ACV-MP (80 mg, 0.262 mmol) was coupled with leucine methyl ester (320 mg, 2.21 mmol) in the presence of DCC (270 mg, 1.31 mmol). Column chromatography on silica gel followed by reverse phase MPLC gave the product as a colorless solid (98 mg, 87% yield). ^{1}H-NMR (D_2O) : δ 7.93 (1H, broad s, H8), 5.48 (2H, s, OCH_2N), 3.86 (2H, broad t, $POCH_2$), 3.69 (3H, broad m, $POCH_2C\underline{H}_2$, C<u>H</u>CO_2Me), 3.64 (3H, s, CO_2CH_3), 1.55 (1H, m, C<u>H</u>$_2$CHCO_2Me), 1.40 (2H, m,

$C\underline{H}_2CHCO_2Me$, $Me_2C\underline{H}$), 0.78 (6H, t, $(CH_3)_2$). ^{31}P-NMR (D_2O) : δ 7.69. FABMS : [M+1] 432.98, [M+1+gly] 523.11; HPLC t_R 7.19 min.[43]

9-[(2-Hydroxyethoxy)methyl]guanine 2-*N*-(1-carboxymethyl-ethyl)phosphoramidate (3d)

As described above, ACV-MP (86 mg, 0.282 mmol) was coupled with alanine methyl ester (300 mg, 2.91 mmol) in the presence of DCC (290 mg, 1.31 mmol). Column chromatography on silica gel followed by reverse phase MPLC gave the product as a colorless solid (31 mg, 28% yield). ^{1}H-NMR (D_2O) : δ 7.93 (1H, broad s, H8), 5.48 (2H, s, OCH_2N), 3.86 (2H, dd, $POCH_2$), 3.70 (3H, broad m, $POCH_2C\underline{H}_2$, $C\underline{H}CO_2Me$), 3.63 (3H, s, CO_2CH_3), 1.22 (3H, d, $C\underline{H}_3CH$). ^{31}P-NMR (D_2O) : δ 7.63. FABMS : [M+1] 390.89, [M+gly] 482.91; HPLC t_R 9.69 min.[43]

Compound Purity

The purity of **3a**, **3b**, **3c** and **3d** were evaluated by RP-HPLC prior to biological testing. In brief, separation and quantitation of ACV and ACV 2-monophoshate, **2**, present in the compounds to be tested was performed on a 4.6 x 250 mm 5-μm Spherisorb® reverse phase C8 column. The HPLC system consisted of a Spectra-Physics SP8800 ternary HPLC pump and SP4600 integrator; a Kratos Spectraflow 757 absorbance detector; and a Rheodyne manual injector. A standard curve (2 μM - 200 μM) based on peak area was constructed for ACV with 2',3'-didehydro-3'-deoxythymidine (d4T) (50 μM) used as the internal standard. The compounds were eluted by using a gradient of 50 mM ammonium acetate (solvent A) and acetonitrile (solvent B) and monitored at 260 nm. For **3d**, the gradient ran at 1.5 mL/min and changed linearly from 98% A to 92% A over the first 10 min. From 10 to 15 min there was a linear gradient change from 92% A to 85% A. From 15 to 20 min there was a linear gradient change back to 98% A. For all other compounds tested, the gradient ran at 1.5 mL/min and changed linearly from 90% A to 75% A over the first 15 min. From 15 to 20 min there was a linear gradient change back to 90% A. The peak area corresponding to retention times of ACV and ACV-MP in an 800 μM sample of the compound containing 50 μM d4T were compared to the peak area of d4T. The concentration of ACV and ACV-MP were determined from the standard curve and expressed as a percent of 800 μM.

HSV Bioassay

Prototype and clinical viruses were evaluated for drug susceptibility by plaque reduction assays in Vero cells, as described previously. [40-42] The compounds were tested for activity against ACV and ACV/PFA-resistant viruses by a plaque reduction assay in

Vero cells. ACV was used as the positive control. The median effective concentration (EC_{50}) values for the viruses was estimated by regression analysis as described previously.[42] The median inhibitory concentration (IC_{50}) values for the compounds was also assessed in Vero cells as described previously.[40] For these studies the F strain HSV-1 originally obtained from Dr. B. Roizman (University of Chicago) was used. In addition, inhibition studies were conducted with two well characterized HSV-2 clinical isolates D5A and CD. These viruses are resistant to ACV and ACV/PFA, respectively.

Determination of the Rate of Decomposition in Serum

A representaitive compound, **3b**, was chosen to determine the relative stability of the phosphoramidates in fetal bovine serum. In brief, 100 μM **3b** was incubated in a solution of 20% heat inactivated fetal bovine serum in PBS (pH 7.4) at 37°C for 31 hrs, 51 hrs, 72 hrs and 94 hrs in triplicate. Cold HPLC grade methanol (326.3 μL) was added to precipitate proteins and 3.75 μL of 10 mM d4T in MeOH was added as an internal standard. Samples were then centrifuged (13,200 g, 10 min., 4°C) and a 20 μL aliquot from the methanolic solution was subjected to HPLC analysis. Analysis of the remaining phosphoramidate was performed on a 4.6 x 250 mm 5-μm Spherisorb® reverse phase C8 column. The HPLC system and conditions were as described above. Concentration of the remaining phosphoramidate was determined by similar methods as described above. Decomposition rates were determined by linear plots of the remaining concentration of phosphoramidate vs. time.

Metabolism of (3c) in Vero Cell Lysates

Vero cells were trypsinized and separated from their culture medium by centrifugation (430 g, 5 min., room temp.). The residue (about 100 μL) was resuspended in 2 mL of buffer (Tris·HCl 20 mM, NaCl 500 mM, pH 7.5) and sonicated with a VirSonic 300 cell disrupter with microtip adapter (4 x 4 second bursts on ice). Lysate from ~4 x 10^6 cells (488 μg total protein by Bradford assay) was incubated with **3c** (1 mM) for indicated lengths of time at 37°C in triplicate. Enzymatic reactions were halted by quickly freezing the samples in dry ice and storing at -20°C until assayed. MeOH (180 μL) was added to precipitate proteins and to ensure termination of enzymatic reactions. 20 μL of 0.5 mM (-)-cis-2-amino-1,9-dihydro-9-[4-(hydroxymethyl)-2-cyclopenten-1-yl]-6H-purin-6-one (i.e., (-)-carbovir) in 25% methanol solution was added as an internal standard. Samples were then centrifuged (13,200 g, 15 min., 4°C) and a 20 μL aliquot from the methanolic lysate was subjected to HPLC analysis. Separation and quantitation of the nucleoside ACV was performed on a 4.6 x 250 mm 5-μm Spherisorb® reverse phase C8 column that was protected by a 4.6 x 7.5 mm All-Guard™ Econosphere™ RP-C8

precolumn. The HPLC system was identical to the one described above. Metabolites were eluted by using a gradient of 50 mM ammonium acetate (solvent A) and acetonitrile (solvent B). The gradient ran at 1.5 mL/min and changed linearly from 100% A to 99% A over the first 8 min. From 8 to 20 min there was a linear gradient change from 99% A to 89% A. At this point, the system was held constant at 89% A for two min then from 23 to 25 minutes there was a linear gradient change back to 100% A. Metabolite identification was based on retention times of coinjected synthesized standards of ACV and **3c**. The corresponding retention times for ACV, (-)-carbovir and **3c** when monitored at 260 nm were 8.0, 19.7, and 22.6 min, respectively. Separation and quantitation of the nucleotide ACV-MP was performed on a 4.6 x 150 μm 5-mm Spherisorb® SAX column that was protected by a 4.6 x 7.5 mm All-Guard™ PARTSIL 10-μm SAX precolumn. Metabolites were eluted using an isocratic solvent system of 97% 0.01 M KH_2PO_4 (pH 4.5) and 3% 0.5 M KH_2PO_4 (pH 4.5) at a flow rate of 1.0 mL/min. Metabolite identification was based on the retention time of coinjected synthesized standard of ACV-MP. The corresponding retention times for ACV-MP and (-)-carbovir when monitored at 260 nm were 8.3 and 3.34 minutes, respectively. Relative amounts of ACV and ACV-MP were expressed as peak area ratios compared to the peak area of (-)-carbovir.

Internalization of (3c) by Vero Cells

Vero cell cultures were grown at 37°C in a 10% CO_2-90 % air environment and counted by the trypan blue exclusion method. Seventeen culture flasks were each seeded with approximately, 1.2 x 10^6 cells and incubated for 48 hr. At the end of 48 hr, fresh media was added and the cells cultured with 100 μM **3c**. After incubation of the cells for 1 hr, 4 hr and 8 hr, the media was removed and the cells washed three times with PBS. Cells were then lysed by the addition of 60% methanol in water, overnight at -20°C. Cellular debris was then pelleted by centrifugation and the cellular extracts were then removed by pipette, dried and stored at -20°C until assayed. The dried lysates were reconstituted in 35 μL of a 25 % methanol solution containing (-)-carbovir (50 μM). Twenty microliters of the lysate solutions were typically used for RP-HPLC analysis with a 4.6 x 250 mm 5-μM Spherisorb® reverse phase C8 column. The injected aliquot was eluted with a gradient of acetonitrile (solvent A) and 50 mM ammonium acetate (solvent B). The gradient was run at a flow rate of 1.5 mL/min from 100% B to 99% B for 5 min, from 99% B to 80% B over the next 11 min, and 100 % B over the last 4 min. (-)-Carbovir and **3c** were observed spectrophotometrically at 260 nm with retention times of 14.5 min and 15.7 min, respectively. For each experiment, the ratio of the peak area of **3c** to the peak area of (-)-carbovir was converted to a concentration with a standard curve. The standard curve was established by mixing 5 μL of varying concentrations of aqueous solutions of

3c with 30 μL of a 25% methanolic solution of carbovir. Dried lysates of Vero cells were reconstituted with the carbovir and **3c** mixtures and the corresponding peak areas determined and graphed as a log-log plot.

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43. Although the purity of **3a-3d** was verified by NMR, HPLC and Mass Spectrum analysis, their hydroscopic nature prevented the obtaining of satisfactory (<0.5% of theoretical) elemental analysis.