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Synthesis, transport and antiviral activity of Ala-Ser and Val-Ser prodrugs of cidofovir

Larryn W. Peterson^a, Jae-Seung Kim^b, Paul Kijek^b, Stefanie Mitchell^b, John Hilfinger^b, Julie Breitenbach^c, Kathy Borysko^c, John C. Drach^{c,d}, Boris A. Kashemirov^a, and Charles E. McKenna^{a,*}

^a Department of Chemistry, University of Southern California, Los Angeles, California 90089-0744 USA

^b TSRL, Inc., Ann Arbor, Michigan 48108 USA

^c College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109-1065 USA

^d School of Dentistry, University of Michigan, Ann Arbor, Michigan 48109-1078 USA

Abstract

We report the synthesis and biological evaluation of Ala-(Val)-L-Ser-CO₂R prodrugs of **1**, where a dipeptide promoiety is conjugated to the P(OH)₂ group of cidofovir (**1**) via esterification by the Ser side chain hydroxyl group and an ethyl group (**4** and **5**) or alone (**6** and **7**). In a murine model, oral administration of **4** or **5** did not significantly increase total cidofovir species in the plasma compared to **1** or **2**, but **7** resulted in a 15-fold increase in a rat model and had *in vitro* EC₅₀ values against human cytomegalovirus comparable to **1**. Neither **6** nor **7** exhibited toxicity up to 100 μM in KB or HFF cells.

Keywords

Prodrug; Cidofovir; Antiviral; Human cytomegalovirus; Oral bioavailability

Cidofovir (HPMPC, **1**) is a broad-spectrum antiviral agent (Vistide®) used to treat AIDS-related human cytomegalovirus (HCMV) retinitis.^{1, 2} Currently, cidofovir is of particular interest as a potential therapy for orthopox virus infections, including smallpox.³ An important limitation of cidofovir as a therapeutic agent is its low oral bioavailability (less than 5 %)^{4, 5} and poor transport into cells, which is attributed to its ionizable phosphonic diacid group. This problem necessitates intravenous administration, and therefore, the development of an orally available form of cidofovir would be highly advantageous. As a result, there are efforts to increase the bioavailability of **1** by attachment of auxiliaries to the parent drug to mask the anionic phosphonate group.

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*Corresponding author. Tel.: +1-213-740-7007; fax: +1-213-740-0930; mckenna@usc.edu.

Supplementary Material

Supplementary data (experimental details, including synthesis of prodrugs, oral bioavailability assay and antiviral assays, and ¹H and ³¹P NMR spectra for intermediates and compounds **4**, **5**, **6** and **7**) can be found in the online version.

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Several prodrug strategies have been considered to circumvent this problem.⁶⁻⁹ We previously reported the synthesis and biological evaluation of a series of peptidomimetic cyclic cidofovir prodrugs containing an amino acid or dipeptide (**3**) promoiety linked through an amino acid side chain hydroxyl group,¹⁰⁻¹⁵ but application of this prodrug approach to acyclic nucleoside phosphonates (ANP), such as **1** itself, was not explored.

Alternative prodrug strategies^{6, 8, 16} involving esterification of the drug phosphonic acid groups with an ether lipid alcohol have been applied to cidofovir and analogous prodrugs have been described.^{17, 18}

We report here the synthesis and biological evaluation of two novel classes of cidofovir prodrugs in which a peptidomimetic promoiety is attached to the phosphonate via a serine side chain hydroxyl group. In one version, both charges on the phosphonate are masked: the dipeptide promoiety (X-L-Ser-CO₂R) esterifies one P-OH, while the other is esterified by a simple alkyl (ethyl) group (X = L-Ala, R = Me (**4**) or X = L-Val, R = Me (**5**) (Fig. 1)). In the other, one P-OH is left unmodified, where X = L-Val, R = iPr (**6**) or X = D-Val, R = iPr (**7**) (Fig. 1).

Mono-esterification of **1** using PyBOP coupling conditions proved difficult as intramolecular cyclization intervened. However, **4** and **5** could be synthesized starting from the trityl-protected monoethyl ester salts **10** or **11**, which in turn can be prepared from a readily available intermediate (**8**) from the synthesis of **1**.¹³ Because **8** is a diethyl ester, it is soluble in organic solvents, which facilitates purification. Synthesis of **8** was carried out as previously described,¹⁹ and the crude product was purified by centrifugal chromatography on silica gel using a gradient of dichloromethane and acetone.

Phosphate triesters or phosphonate diesters can be mono-dealkylated by treatment with sodium iodide (NaI)²⁰ or lithium bromide (LiBr)^{21, 22} depending on the alkyl group. Since the reaction proceeds via an S_N2 attack of the halide on the alkyl group, NaI is commonly used for mono-dealkylation of methyl esters, whereas LiBr is preferred for more sterically-hindered esters, such as ethyl esters.²³ The reaction does not proceed beyond the formation of the phosphate diester (or the phosphonate monoester) because attack by the anion on the alkyl group is prevented by the presence of the negative charge on the monosodium or monolithium salt.²⁰

The monoester **10** was therefore prepared as shown in Scheme 1. Purified **8** and LiBr were dissolved in dry acetonitrile, and the solution was refluxed for 30 h. The reaction was monitored by ³¹P NMR. Additional equivalents of LiBr were added, and the reaction was continued until completed.

The ³¹P NMR spectrum of the reaction product obtained by precipitation with diethyl ether revealed four peaks. The major peak at δ 15.5 ppm corresponded to the desired product **9**. Additional signals at δ 16.0, 21.8, and 22.2 ppm were assigned to the monoester without the trityl group, the diethyl ester (**8**), and the diethyl ester without the trityl group, respectively. When the reaction was repeated with varied equivalents of LiBr, reaction times and solvents, partial loss of the trityl group always persisted.

In view of this problem and the resultant low yields, a better procedure was sought. Racha *et al.* described the use of NaOH to selectively remove one of the ethyl esters from a diethyl phosphonate.²⁴ Although mono-dealkylation might be accomplished by treatment of the diester with BTMS,²⁵ it was found that treatment with NaOH resulted directly in pure product.²⁴ Treatment of **8** was attempted with 1.7 M NaOH in 1:1 ethanol:water (Scheme 1) mono-dealkylated the phosphonate diester, and also cleanly removed the benzoyl group in a one pot reaction. After stirring at room temperature overnight, the benzoic acid side product

was removed by passing the reaction mixture through a column containing Dowex 50Wx8 resin, providing pure **11** in 70 % yield. Besides much improved yields, this method provides a shorter reaction time and a less complicated product mixture, facilitating purification.

For conjugation of the trityl-protected cidofovir monoethyl esters **10** or **11** with a dipeptide X-Ser promoiety, L-valine and L-alanine were chosen as the N-terminal amino acids ("X") by analogy with the cyclic cidofovir Val-Ser-CO₂R prodrugs (**3**) previously synthesized and studied in our laboratory.^{14, 19} The Boc-protected dipeptide promoieties (**12**) were prepared using a standard EDC coupling method.²⁶ Conjugates **4** and **5** were synthesized as outlined in Scheme 2. The Boc-protected intermediate **13** was prepared by treatment of **10** or **11** with the N-Boc-protected dipeptide promoiety (**12**) using PyBOP and DIPEA in DMF at 40 °C. The reaction was easily monitored by ³¹P NMR, and when no cidofovir monoethyl ester (**10** or **11**) remained, the reaction was stopped. The PyBOP byproduct and excess promoiety were removed with a diethyl ether wash before aqueous workup and purification of crude **13** by preparative silica gel TLC. Following deprotection of the Boc and trityl groups with TFA in CH₂Cl₂, the target compounds **4** and **5** were purified by preparative TLC and isolated by precipitation with diethyl ether from a saturated methanolic solution (23 and 7 %, respectively). Their structures were confirmed by ¹H and ³¹P NMR and by HR-MS.²⁷ For conjugates **4** and **5**, a chiral center at phosphorus is formed when the promoiety is attached. Consequently, **4** and **5** were isolated as the resulting diastereomeric mixtures, the composition of which was determined by ³¹P NMR.

A synthetic route previously reported for preparation of alkoxyalkyl esters of cidofovir involved coupling **2** with an alkoxyalkyl alcohol using Mitsunobu chemistry.²⁰ The phostonate ring was then opened by heating the product in 2 M NaOH to 80 °C, producing an acyclic cidofovir derivative in yields of 32–85 %.²⁸ These relatively harsh conditions, if applied to synthesis of a peptidomimetic cyclic cidofovir conjugate, such as **13** (Scheme 2) would likely result in decomposition due to attack of OH[−] on the methyl ester.

Replacement of the carboxylate methyl ester with an isopropyl ester (**14** and **15**; Scheme 3) was also desirable to increase the stability of the dipeptide moiety against enzymatic degradation. Incorporation of the isopropyl ester presumably stabilized the promoiety against removal by beta-elimination¹⁰ and allowed the isolation of the first acyclic analogue of this class. To further suppress decomposition of the prodrug by cleavage of the external rather than the internal P-O bond, milder conditions for opening the phostonate ring were explored. When **14** and **15** were placed in bicarbonate buffer at pH 7.5, the phostonate ring was opened in good yield to produce the acyclic monoester, Val-Ser-CO₂iPr HPMPC (**6** and **7**; Scheme 3). Because the formation of some cyclic cidofovir (**2**) could not be avoided, **6** and **7** were purified by preparative HPLC on a Varian C₁₈ column, and then characterized by ¹H and ³¹P NMR and by HR-MS. Their purity was verified by combustion analysis or HPLC.²⁹

The X-L-Ser-CO₂Me HPMPC(OEt) prodrugs **4** (X = L-Ala) and **5** (X = L-Val) were evaluated for oral availability by direct injection of the drugs into the gastrointestinal tract of a rat at a level of 10 mg/kg (Fig. 2). Oral uptake of **4** was only modestly enhanced, and **5** showed no significant enhancement compared to **1** and **2**. Furthermore, the only species detected in the plasma was the monoethyl ester of cidofovir (**16**; Fig. 3).

When the dipeptide HPMPC(OEt) conjugates **4** and **5** were evaluated for oral transport in a murine model, the major species observed in the plasma after oral administration of the prodrug was again HPMPC(OEt) (**16**; Fig. 3). Cidofovir monoethyl ester (**16**) was therefore evaluated in an *in vitro* antiviral assay to determine if a) it was processed by cellular enzymes and phosphorylated to an active triphosphate form; or b) its ethyl group was

removed, resulting in **1**. The *in vitro* antiviral activity of **16** against vaccinia virus (VV) was examined in a plaque reduction assay using Ara-A and **1** as positive controls (Fig. 4). No effect on the virus was detected at any concentration of **16** tested (1 to 100 μ M), showing that **16** was inactive as such and was not significantly converted to **1** under the assay conditions.

D-Val-L-Ser-CO₂iPr cHPMPC and D-Val-L-Ser-CO₂iPr HPMPC (**15** and **7**) were also evaluated for oral bioavailability by direct injection of the drug into the gastrointestinal tract of a rat at a level of 10 mg/kg (Fig. 5). Both prodrugs exhibited enhanced oral bioavailability relative to **1** and **2**. Intriguingly, despite the presence of an unmodified P-OH group, the acyclic cidofovir analogue **7** showed the largest AUC and C_{max} among all the prodrugs tested. The enhanced bioavailability of these compounds may be partially due to their enhanced stabilities.

Prodrugs **6** and **7** were evaluated for *in vitro* antiviral activity against VV, cowpox virus (CPX), and human cytomegalovirus (HCMV). Their EC₅₀ values (25 to > 100 μ M) were higher than **1** and **2** (20 to 40 μ M; Table 1). A similar pattern was observed for CPX with **1** and **2** showing greater activity against this virus than the prodrugs tested. However, **6** had submicromolar antiviral activity against HCMV (IC₅₀ = 0.32 μ M), 10-fold lower than ganciclovir, the positive control. When the prodrugs were evaluated for cytotoxicity with KB and HFF cells, no toxicity (CC₅₀) at concentrations up to 100 μ M was observed (Table 2).

The parent compound **1** and its cyclic analogue **2** had *in vitro* antiviral activity against the orthopox viruses consistent with published values³⁰ and were more active against the poxviruses and HSV-1 than the prodrugs (Table 1). The reduced antiviral activity may be due to incomplete biotransformation in the *in vitro* assays, reflecting differences in the assays themselves. In particular, the HCMV assay requires incubation for 10 days, but for the VV and CPX assays, the incubation time is ~ 3 days. It is possible that the prodrugs are less potent in the poxvirus assays because the shorter incubation time did not allow sufficient time for the prodrug to exert a therapeutic effect by conversion to **1**.

Another reason for the observed differences in antiviral activity might be the expression of different viral-encoded enzymes that activate the prodrugs.^{31–35} Recent reports describe the presence of a serine/threonine phosphatase encoded by HCMV,^{31, 32} while a dual specificity tyrosine/serine phosphatase that is encoded by VV^{33–35} is expressed late in the virus life cycle.^{33, 35} It is unknown whether these enzymes would accept the phosphonate-amino acid conjugates, and additional studies will be necessary to elucidate the factors contributing to the observed differences in prodrug antiviral activity.

In summary, two classes of peptidomimetic prodrugs of **1** in which the P(OH)₂ was esterified by a Ala-(Val-)L-Ser-CO₂R dipeptide promoiety, with the second P-OH either esterified by an ethyl group or left unmodified, were synthesized and evaluated for their transport properties and for their *in vitro* antiviral activity. The synthesis of the peptidomimetic conjugates of **1** were accomplished using a one pot method to prepare the drug scaffold of **4** and **5** followed by conjugation to the dipeptide promoiety using PyBOP. After unsatisfactory attempts to mono-esterify the phosphonate group of **1**, prodrugs **6** and **7** were prepared from their corresponding cyclic forms using basic conditions to open the phosphonate ring. Administration of **7** to the rat by oral injection resulted in a 15-fold increase in total cidofovir species in the plasma relative to **1** or **2**, despite the presence of one ionizable P-OH group in this prodrug. This enhanced oral bioavailability may be due to higher chemical and enzymatic stability compared to its cyclic analogue **15**. Improved stability might explain why the IC₅₀ value of **7** (1.3 μ M) against HCMV *in vitro* was 4-fold

less than that of **6**. We are currently investigating the relationship between transport and activation, in an attempt to determine an optimal balance between the two processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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27. Compound **4**: ^1H NMR (400 MHz, CD_3OD): δ 1.36 (t, $J = 6.2$ Hz, 3H), 1.58 (d, $J = 7.0$ Hz, 3H), 3.62–4.46 (m, 16H), 6.00 (d, $J = 7.4$ Hz, 1H), 7.72 (d, $J = 7.4$ Hz, 0.3H), 7.77 (d, $J = 7.4$ Hz, 0.7H). ^{31}P NMR (202 MHz, CD_3OD): δ 22.61 (s, 0.74P), 23.09 (s, 0.26P). HRMS-FAB m/z [$\text{M} + \text{H}$] $^+$ calcd for $\text{C}_{17}\text{H}_{30}\text{N}_5\text{O}_9\text{P}$: 480.1859. Found: 480.1863. Anal. calcd for $\text{C}_{17}\text{H}_{30}\text{N}_5\text{O}_9\text{P} \cdot 3(\text{C}_2\text{HO}_2\text{F}_3) \cdot 0.5(\text{C}_4\text{H}_{10}\text{O})$ C, 34.97 %; H, 4.46 %; N, 8.16 %. Found: C, 34.68 %; H, 4.22 %; N, 8.09 %. Compound **5**: ^1H NMR (400 MHz, CD_3OD): δ 1.12 and 1.14 (d, $J = 6.7$ Hz, 6H), 1.33–1.37 (m, 3H), 2.29 (m, 1H), 3.60–4.46 (m, 16H), 5.93 (d, $J = 7.3$ Hz, 1H), 7.61 (d, $J = 7.3$ Hz, 0.3H), 7.65 (d, $J = 7.3$ Hz, 0.7H). ^{31}P NMR (162 MHz, CD_3OD): δ 22.60 (s, 0.65P), 23.17 (s, 0.35P). HRMS-FAB m/z [$\text{M} + \text{H}$] $^+$ calcd for $\text{C}_{19}\text{H}_{34}\text{N}_5\text{O}_9\text{P}$: 508.2172. Found: 508.2180.
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29. Compound **6**: ^1H NMR (400 MHz, D_2O): δ 0.95 (d, $J = 6.9$ Hz, 6H), 1.11 and 1.13 (2d, $J = 6.2$ Hz, 6H), 2.12 (m, 1H), 3.43–3.49 (m, 2H), 3.59–3.77 (m, 5H), 3.90–4.01 (m, 2H), 4.05–4.11 (m, 1H), 4.57 (m, 1H), 4.91 (sept, 1H), 5.90 (d, $J = 6.9$ Hz, 1H), 7.53 (d, $J = 7.4$ Hz, 1H). ^{31}P NMR (162 MHz, CD_3OD): δ 17.32 (s, 1P). HRMS-FAB m/z [$\text{M} + \text{H}$] $^+$ calcd for $\text{C}_{19}\text{H}_{34}\text{N}_5\text{O}_9\text{P}$: 508.2167. Found: 508.2168. LC-MS: $R_t = 14.92$ min; [$\text{M} + \text{H}$] $^+$ 508.1. Compound **7**: ^1H NMR (400 MHz, D_2O): δ 0.91 and 0.92 (2d, $J = 6.8$ Hz, 6H), 1.12 and 1.14 (2d, $J = 6.2$ Hz, 6H), 2.05–2.17 (m, 1H), 3.46–3.52 (m, 2H), 3.60–3.79 (m, 5H), 3.99–4.05 (m, 2H), 4.10–4.16 (m, 1H), 4.51–4.53 (m, 1H), 4.93 (sept, 1H), 6.04 (d, $J = 7.8$ Hz, 1H), 7.73 (d, $J = 7.8$ Hz, 1H). ^{31}P NMR (162 MHz, D_2O): δ 16.90. HRMS-FAB m/z [$\text{M} + \text{H}$] $^+$ calcd for $\text{C}_{19}\text{H}_{34}\text{N}_5\text{O}_9\text{P}$: 508.2167. Found: 508.2172.
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Figure 1.

Structures of cidofovir (**1**), cyclic cidofovir (**2**), cyclic cidofovir prodrugs (**3**), and the prodrugs of **1** synthesized: X-L-Ser-CO₂Me HPMPC(OEt), where X = L-Ala (**4**) and L-Val (**5**), and X-L-Ser-CO₂iPr HPMPC, where X = L-Val (**6**) and D-Val (**7**).

Figure 2.

Dose-corrected total cidofovir found in the plasma after oral dosing of rats with **4** and **5** by direct injection of 3 mg (~10 mg/kg) of drug into the gastrointestinal tract. Plasma was sampled over a time course of 4 h.

Figure 3.
Conversion of the dipeptide HPMPC(OEt) conjugates **4** and **5** to **16**.

Figure 4. Vaccinia virus plaque reduction assay examining the antiviral activity of **16**. Ara-A and cidofovir (**1**), the controls, have EC₅₀ values of 2 μM and 20 μM, respectively.

Figure 5. Dose-corrected total cidofovir found in plasma after intestinal dosing of **1**, **2**, **3b**, **15** and **7**. Rats were dosed by direct injection of 3 mg (~10 mg/kg) of drug into the gastrointestinal tract, and plasma was sampled over a time course of 4 h.

Scheme 1.

Preparation of **10** and **11**. Reagents and conditions: a) LiBr, CH₃CN, reflux, 40 h, 50 %; b) NH₄OH, CH₃OH, RT, 16 h, 62 %; c) 1.7 M NaOH, H₂O/EtOH, RT, 16 h, 71 %.

Synthesis of dipeptide HPMPC(OEt) conjugates **4** and **5**. Reagents and conditions: a) **11**, PyBOP, DIPEA, DMF, 40 °C, 5 h; b) TFA, CH₂Cl₂, RT, 16 h. Overall yields: **4**, 23 %; **5**, 7%.

Scheme 3.

Synthesis of the acyclic cidofovir prodrugs **6** and **7**. Reagents and conditions: a) H₂O, pH 7.5, RT, 18 h. Yields: **6**, 45 %; **7**, 33 %.

Table 1

Antiviral activity of cidofovir, cyclic cidofovir, and select peptidomimetic prodrugs

	IC ₅₀ (μM)		
	VV	CPX	HCMV
HPMPC (1)	20	30	0.28, 0.11
cHPMPC (2)	40	30	0.25
L, L-CO ₂ Me cHPMPC (3a)	100 ^a	> 30 ^a	0.30 ^a
L, L-CO ₂ iPr HPMPC (6)	> 100	> 100	0.32
D, L-CO ₂ iPr HPMPC (7)	> 100	> 100	1.3
Ganciclovir	-	-	3 ^a

^aIC₅₀ values for **3a** and ganciclovir from Eriksson, *et al.*¹⁹

Table 2

Cytotoxicity in KB and HFF cells

	CC ₅₀ Values (μM)	
	KB	HFF
HPMPC (1)	> 100	> 100
cHPMPC (2)	> 100	100
L, L-CO ₂ Me cHPMPC (3a)	> 100 ^a	> 100 ^a
L, L-CO ₂ iPr HPMPC (6)	> 100	> 100
D, L-CO ₂ iPr HPMPC (7)	> 100	> 100

^aCC₅₀ values for **3a** from Eriksson, *et al.*¹⁹