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Synthesis and biological activation of an ethylene glycol-linked amino acid conjugate of cyclic cidofovir

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Abstract—Cidofovir (HPMPC) is a broad-spectrum anti-viral agent whose potential, particularly in biodefense scenarios, is limited by its low oral bioavailability. Two prodrugs (**3** and **4**) created by conjugating ethylene glycol-linked amino acids (L-Val, L-Phe) with the cyclic form of cidofovir (cHPMPC) via a P–O ester bond were synthesized and their pH-dependent stability (**3** and **4**), potential for in vivo reconversion to drug (**3**), and oral bioavailability (**3**) were evaluated. The prodrugs were stable in buffer between pH 3 and 5, but underwent rapid hydrolysis in liver ($t_{1/2}$ = 3.7 min), intestinal ($t_{1/2}$ = 12.5 min), and Caco-2 cell homogenates ($t_{1/2}$ = 20.2 min). In vivo (rat), prodrug **3** was >90% reconverted to cHPMPC. The prodrug was 4× more active than ganciclovir (IC_{50} value, 0.68 μM vs 3.0 μM) in a HCMV plaque reduction assay. However, its oral bioavailability in a rat model was similar to the parent drug. The contrast between the promising activation properties and unenhanced transport of the prodrug is briefly discussed.

Cidofovir (Vistide®, HPMPC, **1**, Fig. 1) is a broad-spectrum anti-viral agent that is used in the treatment of cytomegalovirus (CMV) retinitis in AIDS patients. Cidofovir also has therapeutic potential in the treatment of other herpes and DNA viruses including polyoma-, papilloma-, adeno-, and poxvirus infections.[1,2] Recently, cidofovir has become of particular interest as a potential emergency therapy for orthopox virus infections.[3] An important limitation of cidofovir and analogous nucleotide drugs in a therapeutic role is their low oral bioavailability and poor transport into cells, necessitating intravenous administration. In an emergency situation such as a smallpox outbreak, an orally available form of cidofovir would thus be highly advantageous.

Our current research is focused on improving cidofovir's oral bioavailability. The phosphonic diacid group of cidofovir is ionized under physiological conditions,

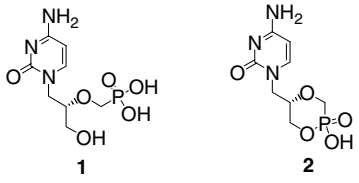

Figure 1. Chemical structures of cidofovir (HPMPC) **1** and cyclic cidofovir (cHPMPC) **2**.

which substantially accounts for its low bioavailability (<5%).[4,5] Cyclic cidofovir (cHPMPC, **2**, Fig. 1) undergoes biotransformation when exposed to cCMP phosphodiesterase to generate the parent drug, HPMPC.[6,7] Severe nephrotoxic effects have been associated with cidofovir treatment[8], while cHPMPC has been reported to be significantly less nephrotoxic.[6] cHPMPC also exhibits low oral bioavailability, indicating a need to mask its remaining anionic charge at physiological pH.

Prodrugs[9] have proven valuable for increasing drug bioavailability as well as for drug targeting after internal absorption.[10] The prodrug strategy is based on chemical

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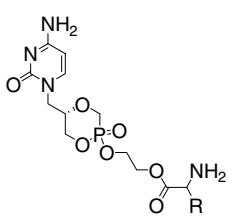
modification of the drug, often achieved by conjugating a pro-moiety to the pharmacophore. The modification should not only confer enhanced transport, but must also be reversible via biotransformation in vivo to release the parent drug.

We have previously reported the synthesis of dipeptide conjugates of cidofovir as prodrugs targeting a peptide transporter (PepT1) present in the small intestine.[11–13] Several of these conjugates showed significantly enhanced intestinal uptake in a rat model.[11–13] Glysar, a known PepT1 substrate, inhibited uptake in the same model. Attaching the pro-moiety of the prodrug through the phosphonic acid group on cyclic cidofovir achieves complete masking of cidofovir's original phosphonic diacid group. On exposure of these cyclic cidofovir dipeptide conjugates to biological fluids, the parent drug was rapidly released from the biologically benign dipeptide pro-moiety.[11–13] Other research groups have recently published different types of cidofovir prodrugs designed for enhanced absorption in the intestine,[14,15] such as ether-lipid-conjugates of cidofovir and cyclic cidofovir, in which the pro-moiety resembles a natural lipid.[16] Alternative prodrug approaches have been documented in the literature.[17–21]

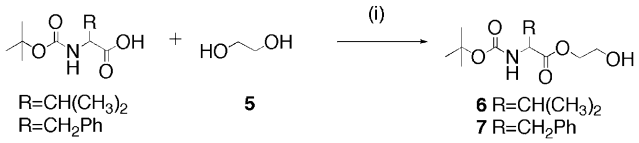
The successful valyl ester prodrug of acyclovir (valacyclovir)[22] stimulated our interest in exploring the chemical and biological properties of a single amino acid conjugate of cyclic cidofovir. Several structural requirements must be satisfied in masking the phosphonic diacid group of cidofovir and targeting the active transporter PepT1 present in the small intestine. One crucial structural requirement for optimal recognition by PepT1 is a free $-NH_2$ group.[23,24] Therefore, the synthetic strategy presented herein involves P–O conjugation between cyclic cidofovir and an ethylene glycol ester-linked amino acid, which masks the negative charge on the cyclic cidofovir (and the amino acid carboxyl group), leaving the N-terminal of the amino acid free for possible involvement as a substrate recognition site for PepT1 (Fig. 2).

(*S*)-HPMPC was synthesized by modification[11] of a previously described[25] method. The ethylene glycol-linked amino acids were synthesized by conjugating *t*-Boc-protected amino acids (L-Val, **3**, or L-Phe, **4**, 1 equiv) to ethylene glycol (4 equiv) using DCC in the presence of a catalytic amount of DMAP (Scheme 1).



3 R=CH(CH$_3$)$_2$
4 R=CH$_2$Ph

Figure 2. Structure of the synthesized ethylene glycol-linked amino acid conjugates of cyclic cidofovir **3** and **4**.

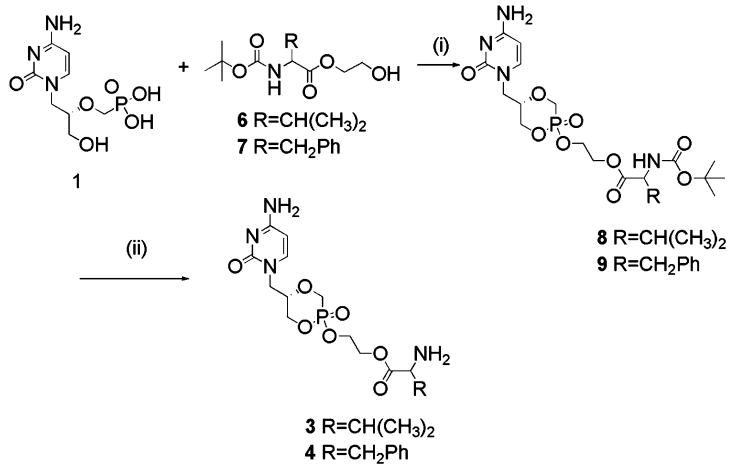


Scheme 1. Synthesis of ethylene glycol-linked amino acids **6** and **7**. Reagents and conditions: (i) DMAP (0.15 equiv), DCC (1.3 equiv), CH_2Cl_2, rt, 18 h, 48–84%.

After purification of **6** (48%) and **7** (84%), by silica gel column chromatography, the ethylene glycol-linked amino acids were conjugated to cidofovir using 2.5 equiv PyBOP as the condensing agent (Scheme 2). Concurrent conversion of HPMPC to cHPMPC occurs in a 1-pot reaction.[11] The reaction is easily monitored by ^{31}P NMR: cyclic cidofovir has a chemical shift of ~5 ppm, while the ethylene glycol-linked amino acid conjugates show resonance between 13 and 15 ppm. The *t*-Boc-protected conjugates (**8** and **9**) were purified by silica gel column chromatography (CH_2Cl_2/acetone/methanol, 6:3:1) and deprotected by exposure to trifluoroacetic acid (TFA). The final compounds, **3** and **4**, were obtained as a diastereoisomeric mixture (diastereomeric ratios of 1:0.8), after purification by preparative TLC on silica in overall yields of 8% and 25%, respectively.[26,27]

The stability of cHPMPC, **3**, and **4** in buffered aqueous solution at 37 °C was evaluated by incubating each compound (500 μM) in 50 mM phosphate buffer in which the pH was adjusted by HCl to 3.0, 4.0, 5.0, 6.0, or 7.0. Aliquots (0.25 mL) were removed at 0, 1, 2, and 3 h and immediately combined with an equal volume of ice-cold 10% TFA to minimize degradation prior to HPLC analysis. The rate of disappearance was estimated by HPLC and the half-life ($t_{1/2}$) was determined from apparent first order rate constant derived from linear regression analysis ($r^2 \leqslant 0.95$) of pseudo-first order plots of prodrug concentration versus time (Table 1).

The ethylene glycol-linked valine and phenylalanine conjugates of cyclic cidofovir (**3** and **4**) showed no evidence of hydrolysis over 3 h at pH values ⩽5.0. Although **3** and **4** were stable in moderately acidic solutions (3.0–5.0), noticeable but slow hydrolysis was observed at pH 6.0–7.0. These observations were in agreement with the chemical stability observed for the cyclic cidofovir dipeptide derivatives previously reported.[11] Further investigations focused on the L-Val conjugate **3**. The activation of **3** in the presence of endogenous hydrolases was investigated by exposing the compound (control cHPMPC) to cellular (Caco-2 and HFF) and tissue homogenates (rat liver and intestine) as well as to an intestinal perfusate obtained from Sprague–Dawley rats. cHPMPC and **3** were exposed to the various homogenates at 37 °C and samples taken at several time intervals between 0 and 3 h were analyzed by HPLC. The half-lives of cHPMPC and **3** when exposed to homogenates were determined as described for the buffer pH stability data. The results are summarized in Table 2.



Scheme 2. Synthesis of **3** and **4**: ethylene glycol-linked amino acid conjugates of cyclic cidofovir. Reagents and conditions: (i) PyBOP (2.5 equiv), DIEA, DMF, 40 °C, 2.5–4 h, 30–56%; (ii) TFA, CH_2Cl_2, rt 4 h.

Table 1. Half-lives ($t_{1/2}$ in hours) for cHPMPC, **3** and **4** presented in hours at various pHs[a]

	$t_{1/2}$(h)				
	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0
cHPMPC	st	st	st	st	st
3	st	st	st	16.0	3.5
4	st	st	st	nd	3.7[b]

[a] $t_{1/2}$ of 500 μM cHPMPC, **3** and **4** in an aqueous solution at 37 °C as a function of pH. (st, stable; nd, not done.)
[b] pH 6.5.

Table 2. Half-lives ($t_{1/2}$ in minutes) for HPMPC and **3** in cellular and tissue homogenates[a]

	$t_{1/2}$[a] (min)				
	Caco-2	HFF	Liver	Intestine	Perfusate[b]
cHPMPC	st	st	st	st	st
3	20.2	69.9	3.7	12.5	141.4

[a] Estimated $t_{1/2}$ of cidofovir and **3** in homogenate solutions at 37 °C. (st, stable.)
[b] Perfusate represents intestinal luminal contents and is prepared by passing buffered solution (pH 6.5) through an isolated intestinal

When the $t_{1/2}$ values for **3** obtained from the buffer pH and homogenate stability studies are compared, it is apparent that although **3** is stable in moderately acidic conditions and only slowly hydrolyzed in neutral buffer, it is rapidly metabolized by endogenous liver, intestinal, and cellular enzymes. The activation pathway for **3** and the relevant enzymes involved are not yet known.

To obtain a marker for its anti-viral activity, **3** was evaluated in a HCMV plaque reduction assay using the Towne strain, plaque purified isolate P_o of HCMV (kindly provided by Dr. Mark Stinski, University of Iowa). HFF cells seeded in 24-well cluster dishes were infected with approximately 100 pfu of HCMV per cm^2 cell sheet. Following virus adsorption, the compounds, prepared as 10 mg/mL stock solutions in DMSO, were diluted with growth medium and added to duplicate wells at 4–8 concentrations. After incubation at 37 °C for 7–10 days, cell sheets were fixed, stained with crystal violet, and the microscopic plaques enumerated.[29] Drug effects were calculated as a percentage of reduction in the number of plaques present at each drug concentration compared to the number observed in the absence of the drug. Fifty percent inhibitory concentrations (IC_{50}) were calculated from the linear portions of the regression lines and are presented in Table 3.[30] Compared to the control drug (ganciclovir), **3** was found to be fourfold more potent.

The cytotoxicity of **3** was evaluated in KB cells. Cells were grown logarithmically for two days in the presence of each compound (in triplicate) and evaluated by spectrophotometric quantitation of crystal violet dye eluted from stained KB cells.[29] It was found that cHPMPC as well as **3** showed little to no cytotoxicity in KB cells up to a concentration of 100 μM.

The HPMPC compounds were next tested directly for oral availability by direct injection of the drugs into the gastrointestinal tract of rat (Table 4). Compound **3** is efficiently converted to cHPMPC in this in vivo system: >90% of the sample detected after dosing of **3** was cHPMPC. After direct injection of **3** into the GI tract of rat, the oral availability of **3** was estimated to be 1.8%, which was not significantly different from cHPMPC (2.2%). Since improved oral bioavailability was not achieved, separation and evaluation of the **3** individual diastereoisomers were not pursued.

In conclusion, we have synthesized two novel ethylene glycol-linked amino acid conjugates of cyclic cidofovir.

Table 3. IC_{50} data for HPMPC and **3** obtained in a HCMV plaque reduction assay conducted in HFF cells

Drug	IC_{50} (μM)
3	0.68
Control[a]	3.0

[a] Ganciclovir served as the control.

Table 4. Bioavailability of HPMPC and **3** conducted in rat

	Dose normalized mean $AUC_{0-\infty}$ (ng/mL h)	% Bioavailability[a]
cHPMPC-iv	472.2	100
3	258.5	1.8
cHPMPC	304.6	2.2

[a] The AUC for **3** was determined by combining the contributions from both the generated cHPMPC and the prodrug. The bioavailability was calculated from the ratio of the dose normalized oral AUC divided by the intravenously (iv) administered cHPMPC AUC data, and a factor to account for the difference in the iv dose versus oral dose (0.1 mg iv vs 3 mg oral).

One, the valine conjugate (**3**) was investigated in detail. Compound **3** was actively metabolized releasing the parent drug in vivo. Compound **3** was also highly active against HCMV in a HFF cell assay showing an IC_{50} value in the submicromolar range, fourfold lower than ganciclovir, the positive control. Compound **3** was not cytotoxic at a concentration of 100 μM when evaluated in KB cells. However, the estimated oral bioavailability in rat of **3** was similar to that of the parent drug. This indicated that this *ethylene glycol*-linked *mono*-L-Val derivative of cHPMPC, while susceptible to biotransformation in vivo, lacks a structural requirement for enhanced oral bioavailability that is present in the Val-Ser-side chain-linked dipeptide conjugates of cHPMPC.[11]

Acknowledgments

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Supplementary data

(I) Detailed synthetic procedures for compounds **3** and **4**, (II) NMR spectra for **3** and **4**, and (III) methodology for evaluation of the oral bioavailability of **3**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.11.012.

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26. Compound **3**: ^{1}H NMR (CD_3OD, ppm): 0.98 (6H, m), 2.21 (1H, m), 3.67–4.43 (12H, m), 5.85 (1H, m), 7.54–7.62 (1H, 2d, $J = 7.1$). ^{31}P NMR (CD_3OD, ppm): 13.91 (1P, s) and 15.16 (1P′, s). Compound **4**: ^{1}H NMR (CD_3OD, ppm): 3.04–4.40 (14H, m), 5.82 (1H, d, $J = 7.2$), 5.86 (1H, d, $J = 8.28$), 7.15–7.30 (5H, m), 7.53 (1H, d, $J = 7.2$), 7.63 (1H, d, $J = 8.28$). ^{31}P NMR (CD_3OD, ppm): 13.87 (1P, s) and 15.23 (1P′, s).
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