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Cidofovir peptide conjugates as prodrugs

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Abstract

Cidofovir (HPMPC, Vistide®) is a broad-spectrum anti-viral agent that is used to treat AIDS-related CMV retinitis. Currently, cidofovir is of particular interest as a potential therapy for orthopox virus infections, including smallpox. An important limitation of cidofovir and analogous nucleotide drugs in a therapeutic role is their low oral bioavailability and poor transport into cells. In principle, bioavailability of a drug can be improved by structural modification targeting transporters expressed in human intestine. To be effective, the transported prodrug must be cleaved by endogenous enzymes to its parent compound. In this work, three examples of novel cyclic cidofovir (cHPMPC) prodrugs incorporating dipeptides were synthesized and evaluated in a rat oral bioavailability model, in which the prodrugs showed significantly enhanced transport vs. HPMPC and cHPMPC. The prodrugs inhibited Gly–Sar uptake in a competitive binding assay using DC5 cells over-expressing hPepT1.

Keywords: Nucleotide analogues; Antiviral prodrugs; Cidofovir; Transporter

1. Introduction

The prodrug approach has been utilized widely since the late 1950s for increasing drug bioavailability as well as drug targeting after oral administration [1]. A prodrug is a compound that has to undergo transformation within the body before eliciting its therapeutic action [2]. This strategy is based on chemically modifying an active substance by attaching pro-moieties to pharmacophores, which ideally should overcome the biochemical and physical barriers impeding drug transport of the parent substance. Limited oral bioavailability is usually attributed to poor membrane permeability, low aqueous solubility (in the gastrointestinal fluids), or extensive first-pass metabolism. It was long thought that intestinal absorption of most drugs proceeded by passive diffusion, in which the lipid solubility of the drug molecule was the determining factor. However, many water-soluble compounds have been shown to move well across cell membranes utilizing specialized carrier-mediated transport mechanisms. These membrane transporters play a key role in determining exposure of cells or organisms to a variety of solutes including nutrients and cellular byproducts, as well as drug molecules. Many efforts have been made to improve drug bioavailability by using different pro-moieties targeting various active transportation systems present in the small intestine. Important intestinal transporters are shown schematically in Fig. 1 [3].

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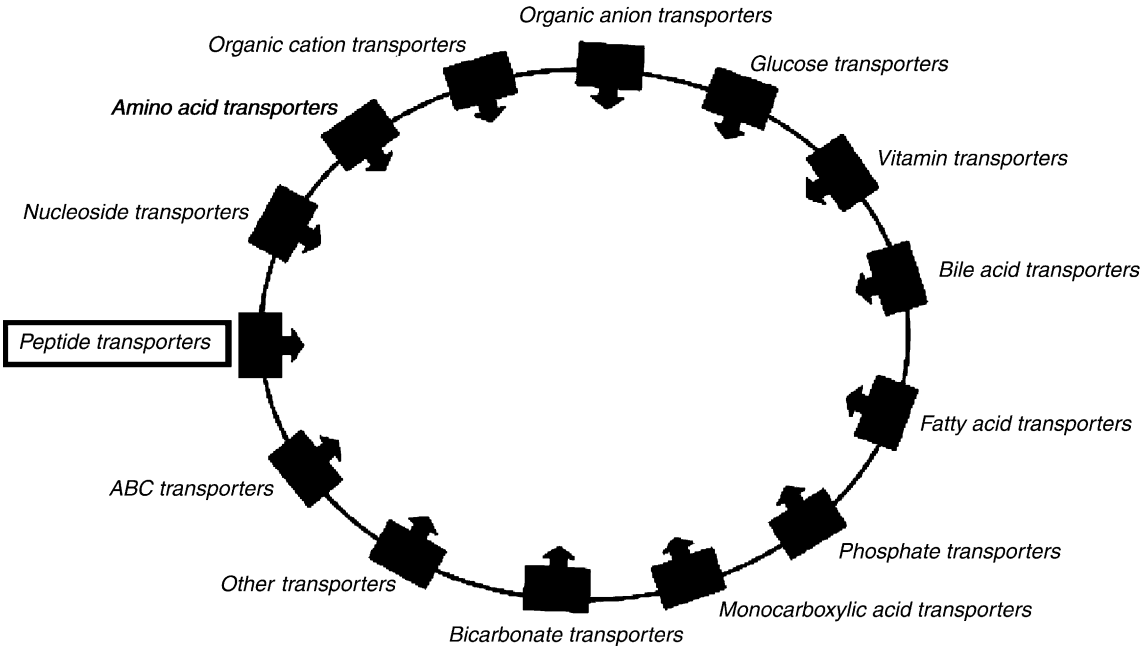

Fig. 1. Overview of known membrane transporters located in the gastrointestinal tract. Based on [3].

All these transporters are mainly located in the brush border membrane with variable distribution along the gastrointestinal tract and show diverse substrate specificities.

The peptide transporter-1 (PepT1) is known to play a critical role in the absorption of diverse drugs and prodrugs from the intestinal tract. PepT1 is located in the apical enterocytic membrane of the upper small intestine where it serves as a symporter, using an electrochemical proton gradient as its driving force [4]. Human PepT1 (hPepT1) contains 708 amino acids oriented in 12 membrane-spanning domains [5]. The hPepT1 carrier protein is stereoselective, with peptides that contain L-amino acid residues having higher affinity for binding and transport than peptides containing one or more D-amino acids. The transporter absorbs many peptide-like drugs including β-lactam antibiotics such as penicillins and cephalosporins [6], ACE-inhibitors [4,7,8], renin inhibitors [9], thrombin inhibitors [10] and the dipeptide-like antineoplastic drug bestatin [11] as well as prodrugs of ganciclovir [12], L-dopa [13] and pamidronate [14]. These studies indicate that the presence of a peptide bond is not essential for transport. The L-valyl ester prodrug of the antiviral nucleoside acyclovir exhibits three- to fivefold enhanced oral bioavailability [15–18]. hPepT1 displays extremely broad substrate specificity, making it an attractive target for design of diverse prodrugs.

Cidofovir (HPMPC, Vistide®) [19] has been identified as one of the most promising drugs active against variola and other orthopoxviruses but its very limited oral bioavailability (<5%) has prompted widespread interest in design of cidofovir analogues exhibiting improved transport properties [20–22]. In this paper, we describe the design of novel cyclic cidofovir (cHPMPC) prodrugs incorporating X–L-Ser dipeptides (X = CO_2 Me ester of Val, Ala or Leu) and report their preliminary evaluation in two biological transport models.

2. Results and discussion

2.1. cHPMPC prodrugs

In principle, amino acids could be conjugated at any of several points of attachment to the HPMPC or cHPMPC scaffolds, for example as shown in Fig. 2. Modification of HPMPC via a (potentially labile) amino acid carboxyl ester linkage to the HPMPC propyl-3′-hydroxy group as in **4**, **5** or **6** (Fig. 3) would leave an unmasked phosphonic diacid group that would ionize under physiological conditions as in the parent drug, disfavoring transport. The amido-linked derivatives **1**,

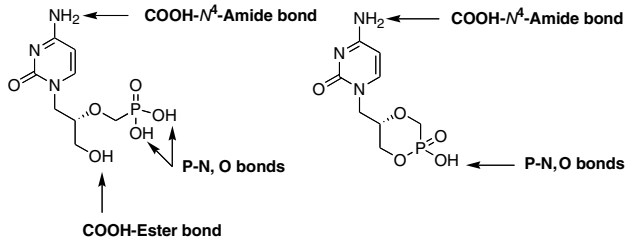

Fig. 2. Some HPMPC (left) and cHPMPC (right) amino acid conjugate linking strategies.

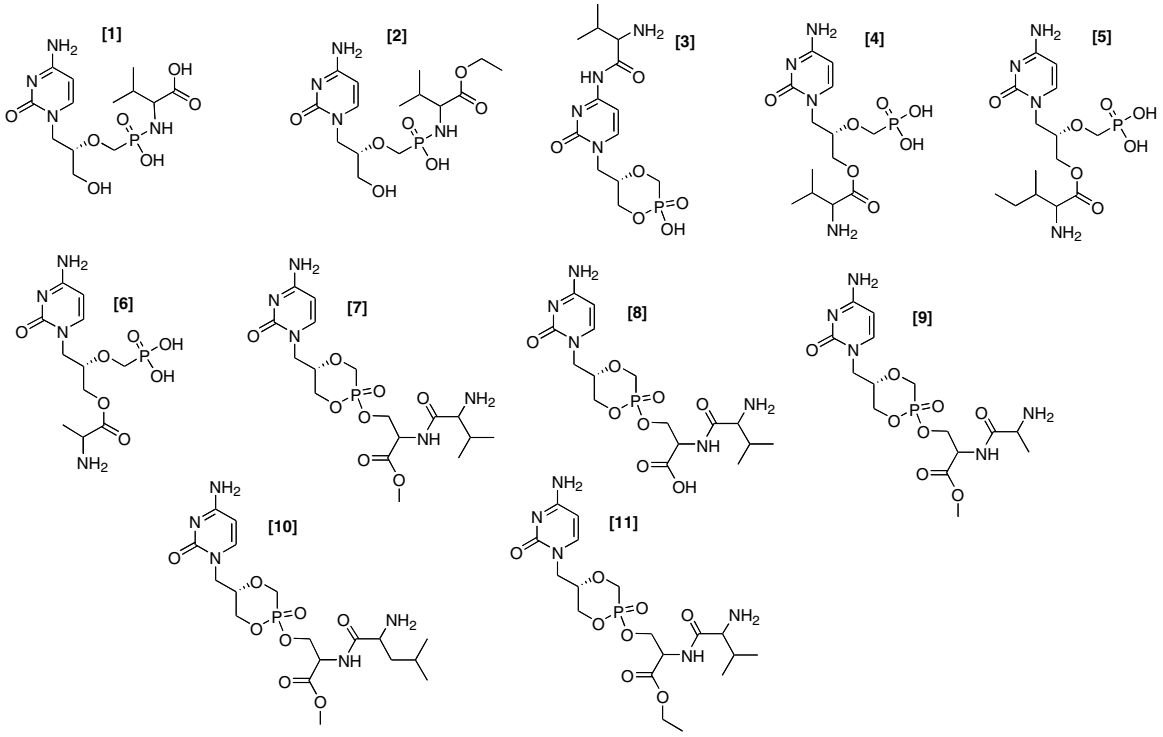

Fig. 3. Structures of HPMPC and cHPMPC peptide analogues discussed in the text.

2 and **3**, which have all proven synthetically accessible (unpublished work from McKenna laboratory), retain a phosphonic monoacid group.

In this report, we focus on the novel cHPMPC X–L-Ser dipeptide conjugates, where X = L-Val, L-Ala or L-Leu, exemplified in **7**–**11**. The cyclic form of HPMPC is only a monoanion at physiological pH, eliminating one site of negative charge relative to HPMPC itself. Potently anti-viral in vitro, cHPMPC is also converted to HPMPC in cells by cyclic CMP phosphodiesterase and thus is a prodrug of HPMPC [23]. By conjugation of cHPMPC to serine via the side chain hydroxy group of the amino acid, a potential 'pro-prodrug' of HPMPC is formed in which all charge has been masked in the parent drug, while leaving both the amino and carboxylic acid functions of the amino acid free for formation of di- or higher peptide moieties. These might be left in their native, zwitterionic state as in the Val–Ser conjugate **8**, or preferably further modified as in the model terminal peptide carboxyl esters **7** and **9**–**11** to achieve a 'pro-pro-prodrug' of HPMPC. These compounds might be subject to significant transport by either passive or active mechanisms, with PepT1 mediation a reasonable possibility in the latter case, owing to the incorporation of the dipeptide component with a free terminal amino group, protonated at physiological pH. The Ser CO_2-Me esters **7**, **9** and **10** where amino acid component X has a hydrophobic side chain (Val, Ala, or Leu) were therefore synthesized and compared to HPMPC and cHPMPC in a rat oral bioavailability model, and also in a competitive binding assay using Gly–Sar in DC5 cells over-expressing hPepT1. The 'pro-prodrug' version of **7**, **8** and the homologous ethyl ester of **7**, **11** have also been synthesized but were not evaluated in the present study.

In vitro transport evaluations of **7**, **9** and **10** vs. HPMPC and cHPMPC in a single pass intestinal perfusion with portal vein blood collection the in rat showed ~10–20× increase in permeability for the prodrugs relative to HPMPC and cHPMPC (Table 1). The greatest enhancement was seen with **7**, which contains valine as the variable amino acid.

A competitive binding assay performed in DC5 cells over-expressing hPepT1 showed that **7**, **9** and **10** have significant affinity for PepT1 (Table 2). Gly–Sar uptake inhibition by **7**, **9** and **10** gave K_i values of 2.7, 3.4 and

Table 1
Permeability for cHPMPC methyl-ester dipeptide conjugates determined from the mesenteric plasma during the in situ single pass perfusion

Compound	$P_{e\text{-}mes}$ (cm/s × 10^5)
HPMPC	0.052
cHPMPC	0.003
7	1.18
7 + Gly–Sar	0.006
9	0.98
10	0.48

Table 2
Competitive binding assay of cHPMPC methyl-ester dipeptide conjugates in DC5 cells over-expressing hPepT1

Compound	Gly–Sar inhibition (mM), K_i
HPMPC	10.8 ± 0.3
cHPMPC	10.7 ± 0.2
7	2.7 ± 0.1
9	3.4 ± 0.2
10	5.4 ± 0.2

5.4 mM, respectively, while HPMPC and cHPMPC had K_i values of 10.8 and 10.7 mM, respectively. These results indicate that hPepT1 might be involved, at least partly, in transportation of the prodrugs into the blood circulation.

To further examine this idea, **7** was coperfused with Gly–Sar, a known competitive substrate of hPepT1 in the in situ perfusion model. As seen in Table 1, Gly–Sar almost completely suppressed the transport of **7**.

3. Conclusion

Consideration of different approaches to masking charge in cidofovir while incorporating dipeptide moieties to enhance drug transport led us to create novel X-Ser dipeptide prodrugs of cidofovir (HPMPC) formed via a cHPMPC phosphonate ester link to the serine side chain hydroxy group. The serine CO_2-Me ester cHPMPC conjugates **7**, **9** and **10** displayed enhanced oral bioavailability relative to cHPMPC and HPMPC in a rat model and exhibited significant affinity for the hPepT1 transporter in a competitive binding assay in DC5 cells over-expressing hPepT1. Further studies of these and related cidofovir prodrugs are in progress.

4. Experimental

4.1. General considerations

Compound structures and purity were verified by ^{1}H, ^{13}C, and ^{31}P NMR, HPLC, and HRMS. Full details of the syntheses summarized here will be presented elsewhere.

4.2. Cidofovir synthesis

The literature procedure [24] for preparation of (*S*)-HPMPC was utilized, however in the reaction of two intermediates, tritylated (*R*)-glycidol and *N*-benzoyl cytosine, we encountered very low yields. Modifying the published reaction sequence, we found it is advantageous to react tritylated (*R*)-glycidol directly with unprotected cytosine to achieve regiospecific opening of the epoxide ring, followed by reaction with benzoic anhydride to obtain the desired *N*-benzoyl intermediate.

4.3. Cidofovir prodrug syntheses

Compounds **1** and **2** were obtained by EDC coupling of L-valine methyl or ethyl ester, respectively, with *S*-HPMPC in H_2O. Purification of **1** and **2** was accomplished by preparative reverse phase HPLC (C-18 column, mobile phase: 0.1 M triethylammonium carbonate with 7% or 13% acetonitrile, respectively, and a pH of 7.4); 35–40% yields were obtained for **1** and **2**, estimated by ^{31}P NMR. When utilizing PyBOP to form conjugates, HPMPC was converted into cHPMPC via an intra-molecular reaction before the desired condensation with the appropriate *t*-BOC-protected amino acid or dipeptide. The dipeptides were synthesized via standard DCC or EDC coupling (CH_2Cl_2) of the methyl or ethyl ester of L-serine with the appropriate *t*-BOC-protected amino acid in the presence of HOBt (44–98% yields). Compounds **3** and **8** were purified by preparative reverse phase HPLC (C-18 column, mobile phase: 0.1 M triethylammonium carbonate with 7% acetonitrile, pH 7.4 and 0.1 M triethylammonium acetate with 3.5% acetonitrile, pH 6.7, respectively) while **7** and **9–11**, as *t*-BOC protected compounds, were purified by silica gel column chromatography [CH_2Cl_2, CH_2Cl_2:acetone (2:1), CH_2Cl_2:acetone:MeOH (6:3:1)] as well as preparative TLC on silica gel (20 × 20 cm, 1000 microns, [CH_2Cl_2:MeOH (6:1)]) after removal of the *t*-BOC group (TFA in CH_2Cl_2). The prodrugs were the major products of these reactions based on ^{1}H NMR analysis. Isolated yields ranged from 10% to 30% and were not optimized.

4.4. Binding studies

K_i values for the cidofovir compounds were determined in the hPepTl over-expressing cell line (DC5) using ^{3}H glycine–sarcosine (GS). DC5 cells were plated (12,000 cells/well) in 96-well tissue culture plates (Falcon) and allowed to grow for 4 days. The cells were washed once with 200 μl of uptake buffer and aspirated. The plates were cooled to 4 °C and 25 μl of uptake buffer containing 50 μM Gly–Sar (0.5 μCi/ml) was added. The uptake buffer also contained the test cidofovir compounds over a range of concentrations. Uptake was initiated by placing the plate in a shaker water bath (37 °C) and terminated at 10 min by rapid washing with multiple changes of 4 °C PBS (Sigma). The radioactive peptide was extracted from the cell layer with 200 μl of methanol:water (1:1) and counted in 4 ml of CytoScint ESTM scintillation cocktail (ICN). Non-linear regression analysis of the data was used to determine the IC_{50} using the solver function in Microsoft Excel.

4.5. Single pass perfusion and portal vein blood collection analysis

Male albino Sprague–Dawley rats, 9–10 weeks old and weighing 250–400 g were used in the study. Prior to each experiment, the rats were fasted for 18 h with free access to water. The rats were anesthetized with an i.m. injection of ketamine/xylazine/butorphanol (87, 6, and 0.2 mg/kg body weight, respectively). The abdomen was opened by a midline incision of 4–5 cm. A suitable intestinal segment of approximately 10–20 cm was cannulated on two ends and connected to a syringe pump that guided the solution through a water bath of 37 °C. This isolated segment was then rinsed with buffer at the respective pH at a flow-rate of 0.5 ml/min in order to clean out any residual debris. A 10 mM HEPES (pH 7.5) buffer containing 135 mM NaCl, 5 mM KCl and 0.01% PEG 4000 was used for the perfusion buffer. The intestinal segments were perfused at a flow rate of approximately 0.2 ml/min with these buffers containing test drug and ^{14}C-PEG 4000 as a non-absorbable marker for measuring water flux. After steady-state was reached in the segment, typically 30 min after the start of perfusion, perfusate samples were taken in 10 min intervals for an hour. Sample analysis for ^{14}C (PEG-4000) was performed by scintillation counting. For these studies, we also took blood from the portal vein (prehepatic) as described below. After a segment of intestine was cannulated, the portal vein was then immediately cannulated using a 24-gauge Angeocath catheter (Becton Dickinson, Sandy, UT). The cannula was secured in place using cyanoacrylate glue or surgical tape and connected to an approximately 35 cm long Silastic tubing, which allowed blood to flow into vials placed about 25 cm below the mesenteric vein. The single-pass intestinal perfusion was initiated at this time. Blood flowing out of the mesenteric vein from the isolated intestinal segment was collected directly into vials, centrifuged immediately and plasma was separated and stored at −80 °C.

Rat plasma samples were acidified and processed with solid phase extraction. Briefly, a cation exchange solid-phase cartridge (MCX, 30 mg/i cc, Waters) was activated with 1.0 ml of methanol and equilibrated with 1.0 ml of water. A 500 μl aliquot of rat plasma was acidified and loaded onto the cartridge. After washing with 1.0 ml of 0.1 N HC1 and 1.0 ml methanol, the compounds were eluted with 1 ml of 5% NH_4OH in methanol. The solvent was evaporated under vacuum and the residue was reconstituted in 200 μl of water. Samples were analyzed using a LC–MS/MS system (Micromass Quattro II, HP 1100). Ten μL of sample was separated with a C8 column (2.1 × 150 mm) using a mobile phase of 5–10% acetonitrile:water containing 0.5% formic acid.

From the plasma concentration of compound, the mesenteric permeability was determined by substitution into Eq. (1).

$$\text{Flux} = (A)(P_{\text{e-mes}})(C_{\text{i}}), \quad (1)$$

where Flux is equal to the portal blood flow (estimated at 1 ml/min in the rat) × the steady state mesenteric blood concentration of cidofovir prodrugs, A is the absorptive area of the intestine (estimated at 12.56 cm^2 in the rat intestine), $P_{\text{e-mes}}$ is the mesenteric permeability (cm/s), and C_{i} is the starting concentration of the drug in the perfusate (μg/ml).

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