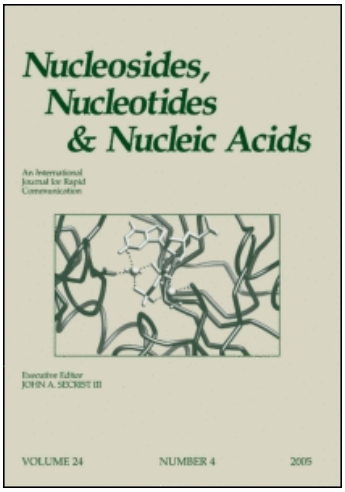

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HUMAN RENAL ORGANIC ANION TRANSPORTER 1 (hOAT1) AND ITS ROLE IN THE NEPHROTOXICITY OF ANTIVIRAL NUCLEOTIDE ANALOGS

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ABSTRACT

hOAT1 is a renal membrane protein able to efficiently transport acyclic nucleoside phosphonates (ANPs). When expressed in CHO cells, hOAT1 mediates the uptake and cytotoxicity of ANPs suggesting that it plays an active role in the nephrotoxicity associated with cidofovir CMV therapy and high-dose adefovir HIV therapy. Although efficiently transported by hOAT1, tenofovir did not show any significant cytotoxicity in isolated human proximal tubular cells, which correlates with the lack of nephrotoxicity observed in HIV-infected patients on prolonged tenofovir therapy.

INTRODUCTION

Cidofovir, adefovir, and tenofovir are acyclic nucleoside phosphonates (ANPs), a unique class of nucleotide analogs, which are currently being clinically utilized or investigated as antiviral therapeutics (Fig. 1). Cidofovir (HPMPC) has been approved for the treatment of CMV retinitis in AIDS patients [1] and has also shown activity in the treatment of progressive multifocal leukoencephalopathy [2] and papillomavirus-associated cutaneous diseases [3,4]. An intracellular cyclic

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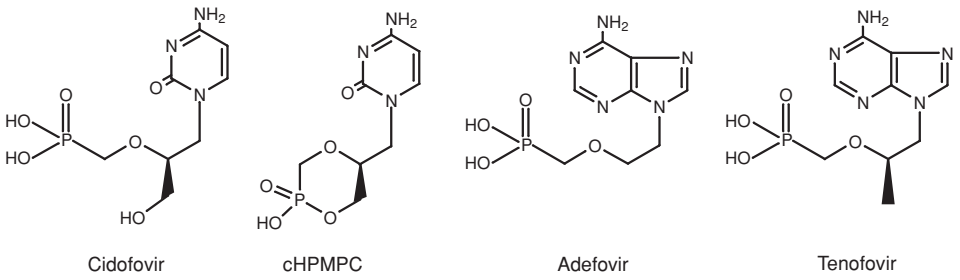

Figure 1. Acyclic nucleoside phoshonates.

prodrug of cidofovir (cHPMPC, Fig. 1) has been designed and shown to have similar *in vitro* antiviral efficacy as parental cidofovir [5]. A high dose of adefovir dipivoxil (60 to 120 mg), an oral prodrug of adefovir (PMEA), has been extensively studied as an anti-HIV therapy [6]. In addition, a reduced dose of adefovir prodrug (10 mg) is currently being evaluated for the treatment of hepatitis B virus infections [7]. Tenofovir disoproxil, a lipophilic prodrug of tenofovir (PMPA), is currently in Phase III clinical development for treatment of HIV-1 infection [8].

Nephrotoxicity manifested as changes in laboratory markers of renal tubular functions is the main clinical toxicity associated with cidofovir CMV therapy and the high-dose adefovir HIV therapy [1,6]. In contrast, HIV patients treated with tenofovir disoproxil therapy do not exhibit any significant signs of renal dysfunction [8]. ANPs undergo renal tubular secretion [9] indicating that the specific drug accumulation in renal proximal tubule cells may play a role in the etiology of the nephrotoxicity associated with cidofovir and high-dose adefovir therapy. It has been shown that both drugs are efficiently transported by the human renal organic anion transporter 1 (hOAT1) [10]. High-level expression of hOAT1 is specific to kidney [10] and the transporter has been localized to the basolateral membrane of human renal proximal tubules [11]. In addition, renal accumulation and nephrotoxicity of cidofovir is reduced by the co-administration of probenecid, an efficient inhibitor of hOAT1 [12,13]. In an attempt to understand the differences in the nephrotoxicity observed between ANPs and the involvement of hOAT1 in the drug-associated nephrotoxicity, we studied the hOAT1-mediated transport and cytotoxicity of ANPs. In addition, we evaluated the cytotoxic effects of ANPs in human renal proximal proximal tubule epithelial cells (RPTECs).

EXPERIMENTAL

Materials. [^{14}C]cidofovir, [^{3}H]adefovir, [^{3}H]tenofovir, and [^{14}C]cHPMPC were purchased from Moravek Biochemicals. Non-radioactive ANPs were synthesized at Gilead Sciences [14].

Cells. Chinese hamster ovary (CHO) cells stably expressing hOAT1 (CHO^{hOAT} cells) and the control cells (CHO^{pIRES}) lacking hOAT1 are described

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elsewhere [15]. RPTECs were provided by Dr. Kenneth McMartin (Louisiana State University, Shreveport) and maintained on collagen-coated plastic as described previously [16].

Transport Assays. After the incubation of CHO^{hOAT} and CHO^{pIRES} cells with radiolabeled substrates in 12-well plates at 37°C, the cells were washed with ice-cold PBS, lysed with 0.3% Triton X-100, and the radioactivity in the lysates was counted [15]. The transport kinetic constants were estimated by linear regression from double reciprocal plots.

Drug Cytotoxicity Assays. CHO^{pIRES} and CHO^{hOAT} cells were seeded into 96-well plates and after 24 hours, various concentrations of ANPs were added. Cell viability was determined after 5-day incubation using MTT [15] and the 50% cytotoxic concentration of each drug (CC_{50}) was estimated. Experiments with RPTECs were carried out in 96-well plates coated with bovine collagen (Collagen Biomaterials). CC_{50} values were determined using the MTT-based assay after 4-day incubation with ANPs. The effect of ANPs on the maintenance of RPTECs tight junctions was tested on collagen-coated membrane inserts (Millicel-PCF, 12 mm; Millipore) by measurement of the transepithelial electrical resistance (TER) using the EVOM instrument (World Precision Instruments) [16]. After the cells formed tight junctions, ANPs at various concentrations were added to both basolateral and apical compartments and TER was measured following a 10-day incubation. The concentration reducing TER by 50% ($CTER_{50}$) was determined for each drug.

RESULTS AND DISCUSSION

Expression of hOAT1 Induces Cytotoxicity of Cidofovir and Adefovir. CHO^{hOAT} cells expressing hOAT1 can accumulate cidofovir and adefovir to a level 30 to 50-fold higher than that detected in the control CHO^{pIRES} cells which lack the transporter (data not shown). We investigated whether the enhanced drug uptake due to hOAT1 expression would translate into increased susceptibility of CHO^{hOAT} cells towards the two drugs. Both CHO^{pIRES} and CHO^{hOAT} cells were incubated with adefovir or cidofovir, and the cytotoxic effect was determined. As shown in Figure 2, expression of hOAT1 enhanced the cytotoxicity of both ANPs by approximately 400-fold. In contrast, cHPMPC was only 4-fold more cytotoxic to CHO^{hOAT} cells than to CHO^{pIRES} control cells. This finding correlates with the observed low transport efficiency of cHPMPC by hOAT1 (see below).

Probenecid, which inhibits the uptake of cidofovir and adefovir by hOAT1 with IC_{50} value of 5 and 7.5 μM, respectively, markedly reduced the cytotoxicity of both ANPs in CHO^{hOAT} cells. While the CC_{50} of cidofovir and adefovir in CHO^{hOAT} cells was 0.5 and 0.2 μM in the absence of probenecid, the presence of 1 mM probenecid reduced the cytotoxicity of the two ANPs in CHO^{hOAT} cells by 50- to 80-fold (Fig. 2). In contrast, cytotoxicity of the two drugs in CHO^{pIRES} cells did not decrease in the presence of probenecid (data not shown). This specific

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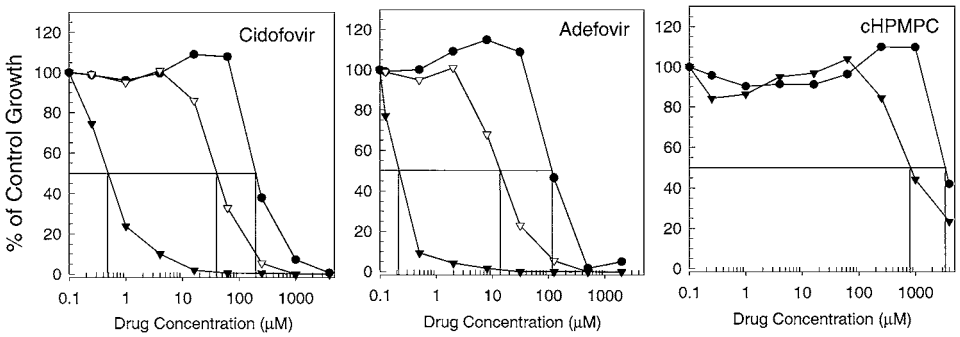

Figure 2. Cytotoxicity of cidofovir, adefovir, and cHPMPC in CHO^{pIRES} cells (solid circles) and in CHO^{hOAT} cells in the absence of probenecid (solid triangles) and in the presence of 1 mM probenecid (open triangles).

cytoprotective effect of probenecid in cells expressing hOAT1 provides a molecular explanation for its *in vivo* nephroprotective activity when co-administered with cidofovir [13].

Recently, a number of nonsteroidal anti-inflammatory drugs have been identified as potent inhibitors of hOAT1-specific transport of ANPs. Some of them, e.g. ketoprofen and naproxen, reduced the hOAT1-mediated cytotoxicity of adefovir 2 to 3-fold more efficiently than probenecid [17].

ANPs are Transported by hOAT1 with a Similar Efficiency. The above results emphasize the active role of hOAT1 in the mechanism of nephrotoxicity associated with cidofovir and high-dose adefovir therapy. However, it remains to be explained why tenofovir does not induce any significant renal dysfunction. Reduced renal tubular uptake of tenofovir could be one potential explanation. Therefore, the efficiency (i.e. V_{max}/K_m ratio) of hOAT1-mediated transport of ANPs was compared. The steady-state transport kinetic experiments revealed that the transport of tenofovir is at least as efficient as that of cidofovir and adefovir suggesting that the absence of tenofovir nephrotoxicity is not due to its reduced renal tubular uptake via hOAT1 (Table 1). In contrast, cHPMPC was transported by hOAT1 with an efficiency at least 10-fold lower than that of cidofovir, presumably because of its reduced negative charge. Hence, the minor change in cHPMPC cytotoxicity upon hOAT1 expression shown above as well as the reduced nephrotoxicity of cHPMPC observed *in vivo* [18] can be explained by its limited cellular transport. Consistently, cHPMPC exhibited approximately 20-fold reduced renal accumulation compared to parental cidofovir [19].

Cytotoxicity of ANPs in Human Renal Proximal Tubule Cells (RPTECs). In order to understand the differences in the nephrotoxicity of ANPs, we compared the *in vitro* effects of cidofovir, adefovir, and tenofovir on RPTECs. Cidofovir exhibited the most pronounced inhibition of the growth of RPTECs with a CC_{50} of 260 μM. Adefovir was less inhibitory with CC_{50} of approximately 500 μM and

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Table 1. Kinetics of hOAT1-Mediated Transport of Antiviral Nucleotide Analogs[a]

Substrate	K_m [μM]	V_{max} [pmol/10^6 cells · min]	Transport Efficiency (V_{max}/K_m)
Cidofovir	58.0 ± 5.7	103 ± 10	1.77
Adefovir	23.8 ± 4.2	46.0 ± 4.4	1.93
Tenofovir	33.8 ± 3.4	110 ± 12	3.26
cHPMPC	309 ± 106	42.2 ± 10.3	0.137

[a]CHO^{hOAT} cells were incubated with labeled substrates at various concentrations and kinetic constants for each substrate were estimated from a double-reciprocal plot.

tenofovir did not show any significant inhibition of the cell growth at concentrations as high as 2 mM (Fig. 3). When the antiviral potency of the three drugs was taken into account, the *in vitro* therapeutic index of adefovir (considered as anti-HBV drug) was 7 to 8-fold higher than that of cidofovir and in a similar range as tenofovir, which appears to be in accordance with the improved toxicity profile of low dose adefovir HBV therapy.

In vivo, RPTECs form differentiated polarized epithelium, which selectively separates peritubular plasma from tubular lumen, the compartment where urine is formed. When cultured on collagen-coated microporous membranes, RPTECs undergo *in vitro* differentiation and form tight junctions, which are an important characteristic of the integrity of tubular epithelium [20]. This process can be followed by the measurement of transepithelial electrical resistance (TER) [16]. We examined the effects of ANPs on the ability of RPTECs from two independent donors to maintain the tight junctions for the period of 10 days. As shown in Figure 4, cidofovir affected the tight junctions most profoundly with $CTER_{50}$ of 100 to 120 μM. In contrast, adefovir exhibited only mild effect with $CTER_{50}$ of 1,000 to

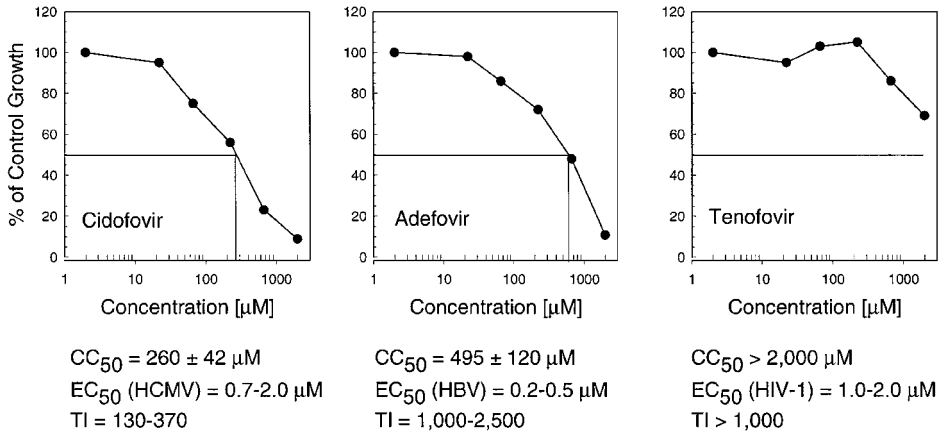

Figure 3. Inhibition of the *in vitro* growth of human renal proximal tubule epithelial cells in the presence of cidofovir, adefovir, and tenofovir.

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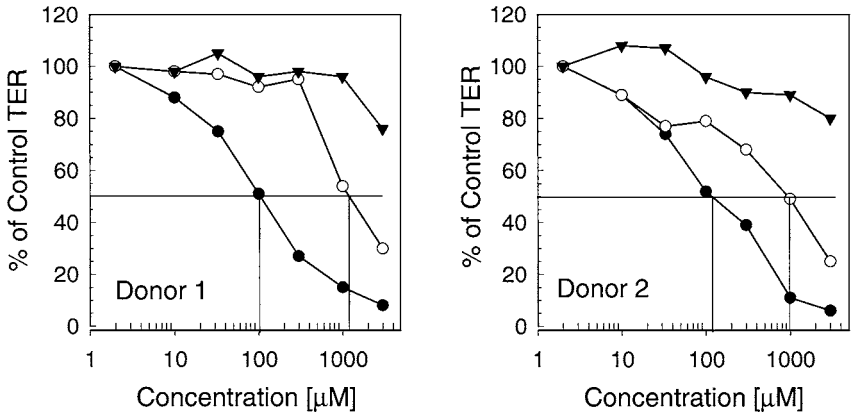

Figure 4. Effect of cidofovir (solid circles), adefovir (open circles), and tenofovir (solid triangles) on the *in vitro* integrity of renal proximal tubule epithelium after 10-day incubation in the presence of drugs. The two panels show results from the same experiment with RPTECs isolated from two independent donors. TER, transepithelial electrical resistance.

1,200 μM and tenofovir did not significantly change the integrity of the epithelium. The effects of ANPs were similar in RPTECs from the two donors, which formed tight junctions characterized by markedly different TER. This suggests that the degree of the epithelium disruption in the presence of ANPs does not directly depend on the ability of the cells to form tight junctions.

The more pronounced cytotoxicity of cidofovir to RPTECs may be associated with the formation of cidofovir-phosphocholine, a unique intracellular metabolite, which is not synthesized from other ANPs. Cidofovir-phosphocholine is an analog of the phospholipid synthesis intermediate cytidine 5′-diphosphocholine and appears to be the most abundant cidofovir metabolite formed in different cell types after prolonged incubation with the drug [21,22]. At high intracellular concentrations generated in hOAT1-expressing cells [15], this metabolite may have an effect on membrane phospholipids, similar to what has been shown with arabinofuranosylcytosine 5′-diphosphocholine [23].

In summary, we have shown that hOAT1, a membrane protein specifically localized in renal proximal tubules, is able to efficiently transport ANPs. When expressed in non-renal mammalian cells, hOAT1 induced cellular uptake and cytotoxicity of cidofovir and adefovir. The hOAT1-mediated cytotoxicity was markedly reduced in the presence of probenecid, which is clinically used in conjunction with cidofovir to reduce its nephrotoxicity. These observations support an active role of hOAT1 in the etiology of nephrotoxicity associated with these two antivirals. While cidofovir showed significant effects on the growth of RPTECs and the maintenance of tight junctions formed by these cells, the effects of adefovir were relatively mild and dose-dependent, which suggests that the safety profile for the low dose adefovir HBV therapy may be favorable. Notably, high concentrations of tenofovir showed minimal effects on the growth of RPTECs as well as the *in vitro* integrity of renal

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proximal tubule epithelium. Since all three ANPs were transported by hOAT1 with similar efficiency and the degree of their *in vivo* tubular secretion appears to be also similar [24,25,26], our data suggest that a lack of interference with essential intracellular function(s) rather than a difference in renal transport is presumably responsible for the improved nephrotoxicity profile of tenofovir.

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