Cytotoxicity of Antiviral Nucleotides Adefovir and Cidofovir Is Induced by the Expression of Human Renal Organic Anion Transporter 1

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Abstract. The transport of organic anions in proximal convoluted tubules plays an essential role in the active secretion of a variety of small molecules by the kidney. In addition to other anionic substrates, the human renal organic anion transporter 1 (hOAT1) is capable of transporting the nucleotide analogs adefovir and cidofovir. To investigate the involvement of hOAT1 in the mechanism of nephrotoxicity associated with these two clinically important antiviral agents, Chinese hamster ovary (CHO) cells were stably transfected with hOAT1 cDNA. The resulting CHOhOAT cells showed probenecid-sensitive and pH-dependent uptake of \( p \)-aminohippurate \( (K_m = 15.4 \mu M, V_{max} = 20.6 \text{ pmol}/10^6 \text{ cells} \cdot \text{min}) \), a prototypical organic anion substrate. In addition, the stably expressed hOAT1 mediated efficient transport of adefovir \( (K_m = 23.8 \mu M, V_{max} = 46.0 \text{ pmol}/10^6 \text{ cells} \cdot \text{min}) \) and cidofovir \( (K_m = 58.0 \mu M, V_{max} = 103 \text{ pmol}/10^6 \text{ cells} \cdot \text{min}) \) such that the levels of intracellular metabolites of both nucleotides were ~100-fold higher in CHO\textsuperscript{OAT}\textsuperscript{1} cells than in parental CHO. Consequently, adefovir and cidofovir were approximately 500-fold and 400-fold more cytotoxic, respectively, in CHO\textsuperscript{OAT}\textsuperscript{1} cells compared to CHO. The cytotoxicity of both drugs in CHO\textsuperscript{OAT}\textsuperscript{1} cells was markedly reduced in the presence of hOAT1 inhibitors. The cyclic prodrug of cidofovir, which exhibits reduced \textit{in vivo} nephrotoxicity, was a poor substrate for hOAT1 and showed only marginally increased cytotoxicity in CHO\textsuperscript{OAT}\textsuperscript{1} cells. In conclusion, these studies demonstrate that hOAT1 plays a critical role in the organ-specific toxicity of adefovir and cidofovir, and indicates that CHO\textsuperscript{OAT}\textsuperscript{1} cells may represent a useful \textit{in vitro} model to investigate the potential nephrotoxicity of clinically relevant organic anion agents.

Active renal secretion of a wide variety of small molecules occurs through the cooperative function of specific transport proteins located in the basolateral and apical membranes of the tubular epithelium (1). The renal organic anion transporter, which has been identified in many diverse animal species, is a component of a specific secretory pathway of organic anions. This basolateral transport system mediates the active uptake of a wide variety of endogenous metabolites, toxins, xenobiotics, therapeutics, and other negatively charged molecules from the systemic circulation into the proximal tubular epithelium (2). Once accumulated within the convoluted tubule, the anionic substances are subsequently secreted into the tubular lumen via a less well characterized membrane carrier(s) or channel(s) (2). Although the enormous physiologic importance of renal secretion of organic anions was recognized years ago, significant progress toward the molecular characterization of membrane proteins involved in this process has only recently occurred with the cloning, expression, and functional characterization of renal organic anion transporters from several animal species including rat (rROAT1, OAT1) (3,4), mouse (mOAT) (5), and winter flounder (fROAT) (6). In all cases, these transporters mediated uptake of \( p \)-aminomhippuric acid (PAH), a prototypical organic anion substrate, and they functioned as organic anion/dicarboxylate exchangers. Initial studies with OAT1 have demonstrated its broad substrate specificity with the capability of transporting urate, methotrexate, prostaglandins, and cyclic nucleotides (3). More recently, OAT1 has been reported to mediate uptake of nonsteroidal anti-inflammatory drugs such as salicylate, acetylsalicylate, and salicylurate (7). Consistent with the proposed function of OAT1, immunohistochemical analysis has demonstrated that it is specifically localized to the basolateral membrane of S2 segments of the proximal convoluted tubules (8).

Recently, we (9) and others (10,11) have independently reported the cloning and expression of the human renal organic anion transporter 1 (hOAT1 or hPAHT). Similar to the organic anion transporters from other species, hOAT1 functions as a PAH/dicarboxylate exchanger when transiently expressed in \textit{Xenopus laevis} oocytes or HeLa cells. Despite one study suggesting a much narrower substrate specificity of hOAT1 compared to rROAT1 (11), we have shown that hOAT1 efficiently transports various cyclic nucleotide analogs including adefovir [9-(2-phosphonylmethoxyethyl)adenine] and cidofovir [(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine], two clinically important antiviral therapeutics (9). Interestingly,
hOAT1 showed markedly higher affinity toward this type of molecule than did rROAT1 (9).

Both adefovir and cidofovir are nucleoside phosphonate analogs, a class of novel antivirals structurally related to natural nucleotides (Figure 1). Cidofovir has been approved for the treatment of cytomegalovirus retinitis in AIDS patients (12), and adefovir dipivoxil, an orally available prodrug of adefovir, is currently undergoing clinical evaluation as an anti-HIV and anti-hepatitis B virus agent (13). Both drugs are actively secreted by the kidney (14,15), presumably via hOAT1, and for both of them, the main dose-limiting toxicity is nephrotoxicity (16,17). To reduce this adverse event, cidofovir is used in conjunction with probenecid, an inhibitor of organic anion transport (12). The organic anion transport system has also been implicated in the nephrotoxicity of other agents, for example, cephaloridine and cephaloglycin, two cephalosporin antibiotics (18), suggesting that hOAT1 may also be directly involved in the induction of nephrotoxicity associated with adefovir and cidofovir.

In an attempt to understand the involvement of hOAT1 in drug-induced nephrotoxicity, we studied the effect of hOAT1 expression on the transport, intracellular metabolism, and cytotoxicity of the two antiviral nucleotide analogs. Using a cell line stably expressing hOAT1, we have been able to demonstrate a direct role of hOAT1 in the etiology of organ-specific toxicity associated with adefovir and cidofovir.

Materials and Methods

Stable Expression of hOAT1 in Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cells (American Type Culture Collection [ATCC] CCL61, Manassas, VA) were grown in F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Plasmid pRES-hOAT used for stable transfection of hOAT1 cDNA into CHO cells was constructed as follows. The hOAT1 coding sequence was amplified by PCR from plasmid pOAT-8 (9) under standard conditions using Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN). Oligonucleotides 5'-ACGGTCTAGAATTC- TTTTATTTTTAATTTTTCTTTCAAATACGTGGCCATTTAATGACCCTCTGACAGAAGG-3' and 5'-TACTCAGCTGATCCCTTTAAATCTCAGTGTTCTAGGCCCTTTAAGG-3' were used as a sense and antisense primer to introduce EcoRI and BamHI restriction sites (in bold), respectively. Using the sense primer, a truncated 5'-untranslated sequence (5'-UTR) of alfalfa mosaic virus (19) (underlined) and a favorable Kozak consensus (20) (in italic) were introduced upstream from the 5' end of the hOAT1 open reading frame to optimize initiation of translation. Plasmid pRES-hOAT was generated by the cloning of the EcoRI/BamHI-digested PCR product into a pIREShneo expression vector (Clontech, Palo Alto, CA). Upon completion of the cloning, the correct nucleotide sequence of the entire fragment generated by PCR was verified. For the transfection, CHO cells were seeded into a 100-mm Petri dish (6 × 10⁶ cells). After 24 h, medium was aspirated and 6 ml of fresh growth medium containing 12 μg of pRES-hOAT and 60 μg of Cytofectin GSV (Glen Research, Sterling, VA) was added to the cells followed by an additional 6 ml of medium 4 h later. After an overnight incubation, stably transfected cells were selected in phenol red-free growth medium supplemented with 1 mg/ml G418 (Clontech). Growing colonies were isolated and tested for PAH uptake in the presence and absence of 1 mM probenecid as described above. A clone showing the highest probenecid-sensitive accumulation of PAH was designated CHOhOAT. Under the same conditions, CHO cells were also transfected with the empty pIREShneo vector. The pool of cells harvested after G418 selection (CHO-IREShneo) was used as the control for initial experiments. In the cytotoxicity experiments, V-79 cells (ATCC CCL-93) stably transfected with hOAT1 cDNA were used in addition to CHOhOAT cells. The transfection and cultivation of V-79 cells were carried out under the same conditions as defined for the CHO cells.

Northern Blot Analysis

Total RNA was extracted from CHO and CHOhOAT cells using Trizol Reagent (Life Technologies, Rockville, MD). After separation on 1.2% agarose gel, RNA was transferred onto a Hybrid-Membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and hybridized with hOAT1-specific 32P-labeled probe (9) for 1 h at 68°C in ExpressHyb hybridization buffer (Clontech). Subsequently, the membrane was washed twice in 2× SSC with 0.05% sodium dodecyl sulfate for 30 min at room temperature followed by a single wash in 0.1× SSC with 0.1% sodium dodecyl sulfate at 50°C. After autoradiography, the membrane was stripped and reprobed with a β-actin control probe (Clontech) under the same conditions.

Transport Assays

The assays were carried out in 12-well plates with nearly confluent cells seeded 48 h before each experiment. On the day of the experiment, growth medium was aspirated and the cells were washed twice with phosphate-buffered saline (PBS). The uptake of radiolabeled substrates was determined at 37°C in Waymouth buffer (135 mM

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**Figure 1.** Structure of adefovir, cidofovir, and cyclic prodrug of cidofovir.
NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 0.8 mM MgSO₄, 28 mM glucose, and 13 mM Hepes, pH 7.2). At the end of incubation, the cells were washed 3 times with ice-cold PBS (2 ml/well) and lysed directly on the plate by adding 0.3% Triton X-100 (0.5 ml/well) for 15 min. Subsequently, the wells were washed with an additional 0.5 ml of the detergent, the lysate and wash were combined, and the radioactivity in each sample was determined after addition of scintillation fluid (Beckman Instruments, Fullerton, CA). In parallel plates, the number of cells was determined and the substrate uptake was expressed as pmol/10⁶ cells. For determination of transport kinetics, the assays were performed at various substrate concentrations, and the kinetic constants were estimated by the linear regression from double reciprocal plots using Enzyme Kinetics software (ChemSW, Fairfield, CA). Inhibition experiments were carried out in the presence of various inhibitor concentrations at the substrate concentration equal to its Kᵣ. Fifty percent inhibitory concentration (IC₅₀) values were estimated from semilogarithmic plots of inhibitor concentration versus percentage of uptake relative to uninhibited control.

### Intracellular Metabolism

Confluent CHO and CHO<sup>hOAT1</sup> cells in 6-well plates were incubated in phenol red-free growth medium containing 5 μM [³H]adefovir or 10 μM [¹⁴C]cidofovir. After 12 h at 37°C, the cells were washed 3 times with 3 ml of ice-cold PBS and extracted with 0.5 ml 5% TCA as described previously (21). The number of cells was determined in parallel samples incubated with unlabeled drugs under identical conditions. The extracts were analyzed using a Separan SGX C18 HPLC column (Merck Technologies, Sunnyvale, CA) with a linear gradient of acetonitrile (0 to 20% over 40 min at 1 ml/min) in the presence of 50 mM potassium phosphate, pH 7.0, and 3 mM L-lysine. One-milliliter fractions were collected, and the amount of [³H]- or [¹⁴C]-radioactivity was determined after addition of 6 ml of scintillation fluid (Beckman Instruments). The identity of each radioactive metabolite was confirmed by comparison of its retention time with that of a corresponding unlabeled standard, and by HPLC analysis of extracts treated with alkaline phosphatase or phosphodiesterase I as described previously (22).

### Drug Cytotoxicity Assays

CHO and CHO<sup>hOAT1</sup> cells were seeded in parallel into 96-well plates at a density of 3 × 10⁴ cells/well. After 24 h, various concentrations of the tested drugs were added in triplicate, and the cells were incubated for an additional 12 h. At the end of the incubation, cell viability was determined by a modified colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) (23). Briefly, medium with the drug was removed and replaced by fresh medium (200 μl/well) containing 0.1 mg/ml MTT. After a 90-min incubation at 37°C, the medium was aspirated and the cells were extracted with 150 μl/well of DMSO. The concentration of the extracted formazan metabolite was determined by measurement of the absorbance at 560 nm (A<sub>560</sub>) in a 96-well plate reader. Average A<sub>560</sub> values were calculated from triplicates after subtraction of the blank values, and the 50% cytotoxic concentration of each drug (CC₅₀) was determined from a semilogarithmic plot of drug concentration versus percentage of A<sub>560</sub> relative to untreated control.

### Materials

[³H]PAH (3.7 Ci/mmol) was obtained from Dupont New England Nuclear (Boston, MA). [³H]-2-(phosphonomethoxy)adenine (adefovir; 30 Ci/mmol), [¹⁴C]S-(3-hydroxy-2-phosphonylme-

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### Results

#### Stable Expression of hOAT1 in CHO Cells

Initially, appropriate cells for the expression and study of hOAT1 were selected. From a number of established cell lines suitable for transfection, CHO cells showed the lowest uptake of both PAH and adefovir. The uptake of both compounds was not saturable and was insensitive to probenecid, indicating the absence of a carrier-mediated transport system that could potentially interfere with the determination of hOAT1-specific transport.

After transfection with the pRES-hOAT plasmid, a number of G418-resistant CHO clones were isolated and assayed for PAH uptake in the presence and absence of probenecid. A transfecant of CHO cells capable of accumulating PAH to a level more than 30-fold higher than that determined in CHO cells was identified and designated CHO<sup>hOAT1</sup>. Northern analysis with a hOAT1-specific DNA probe detected a 5-kb bicistronic transcript (containing both the hOAT1 and G418-resistance marker sequences) in the RNA extract from CHO<sup>hOAT1</sup>, but not CHO cells, indicating the specific expression of hOAT1 in the stably transfected cells (Figure 2A). The uptake of PAH into CHO cells stably transfected with the empty pRESneo vector (CHO<sup>pRES</sup>) was similar to that determined for parental CHO cells (Figure 2B). Preloading CHO<sup>hOAT1</sup> cells with 5 mM glutarate, a counterion accepted by the renal organic anion transporter (1), trans-stimulated PAH uptake; thus, the stably expressed hOAT1 functions as a PAH/dicarboxylate exchanger (Figure 2B).

Inhibition experiments (Figure 2C) showed that probenecid efficiently blocks the uptake of PAH by CHO<sup>hOAT1</sup> cells with an IC₅₀ value of 6.5 ± 1.0 μM (n = 3). Betamipron (N-benzoyl-β-alanine) also inhibited hOAT1-mediated PAH uptake, but was less potent than probenecid with an IC₅₀ of 16.2 ± 2.9 μM (n = 3).

### High Efficiency of Adefovir and Cidofovir Transport Mediated by hOAT1

In addition to PAH, CHO<sup>hOAT1</sup> cells exhibited efficient intracellular accumulation of adefovir and cidofovir. As shown in Figure 3A, hOAT1-mediated uptake of adefovir was linear during the initial 20 min. After 60 min, adefovir accumulated in CHO<sup>hOAT1</sup> cells to a level almost 80-fold higher than that detected in parental CHO cells under the same conditions. Uptake of cidofovir into CHO<sup>hOAT1</sup> cells showed similar characteristics (data not shown). Efficiency of hOAT1-mediated uptake of nucleotide analogs and PAH was compared in transport kinetic experiments. To approximate the initial rate of uptake, the experiments were carried out within 3 min. Under
these conditions, $K_m$ values of 15.4, 23.8, and 58.0 $\mu$M were determined for PAH, adefovir, and cidofovir, respectively, indicating a slightly lower affinity of hOAT1 for nucleotide analogs compared to PAH (Table 1). In contrast, the $V_{\text{max}}$ values for the uptake of the nucleotide analogs were higher than that for PAH, resulting in comparable efficiencies (i.e., $V_{\text{max}}/K_m$ ratio) of hOAT1-mediated uptake of all substrates. Interestingly, the cyclic prodrug of cidofovir (Figure 1) was also a substrate for hOAT1, even though one of the negative charges in the molecule is blocked by an intramolecular ester bond. However, compared with cidofovir, hOAT1-mediated uptake of the cyclic prodrug showed a significantly higher $K_m$ and lower $V_{\text{max}}$, resulting in an almost 15-fold decrease in the transport efficiency for this molecule (Table 1).

Similar to what was observed with PAH, the hOAT1-mediated uptake of adefovir was trans-stimulated by preloading CHO hOAT cells with glutarate. Optimization of the preloading conditions showed maximal stimulation of adefovir uptake after a 2-h preincubation of CHO hOAT cells in Waymouth buffer containing 5 mM glutarate. Under these conditions, preloading resulted in an approximately threefold increase in the efficiency of hOAT1-mediated uptake of adefovir (Figure 3B).

Based on the observation that hOAT1 is able to transport molecules that differ in terms of their net charge, we studied the pH dependence of hOAT1-mediated uptake of adefovir and PAH by performing the transport assay at pH values ranging from 5 to 9. As shown in Figure 4, PAH uptake was highest at pH 5 to 6. In contrast, adefovir showed highest accumulation in CHO hOAT cells at pH 6.5 to 7.5. At pH $> 8$, there was only very limited uptake (<15% of that at pH optimum) of both PAH and adefovir by CHO hOAT cells.

Metabolism of Adefovir and Cidofovir in CHO hOAT Cells

As analogs of nucleoside monophosphates, both adefovir and cidofovir are intracellularly metabolized to the corresponding mono- and diphosphoryl derivatives (22,25,26). In addition, cidofovir forms an adduct with choline, cidofovir-phos-
phocholine (22). Although the metabolism of both nucleotide analogs has been extensively studied in a variety of cell types, the mechanism of cellular uptake in the vast majority of these models was fluid-phase endocytosis (27–29), and none of the investigated cells apparently expressed hOAT1. In addition, highly efficient uptake of adefovir and cidofovir via hOAT1 may result in the saturation of intracellular metabolism with subsequent changes in the proportions of metabolites.

To explore the effect of hOAT1 expression on the intracellular metabolism of the two nucleotide analogs, CHO and CHOhOAT cells were incubated for 12 h with 5 μM [3H]adefovir or 10 μM [14C]cidofovir followed by analysis of the metabolites. The total intracellular accumulation of adefovir and cidofovir was almost 140-fold and 190-fold higher, respectively, in CHOhOAT cells compared with CHO cells (Table 2). The levels of all adefovir metabolites in CHOhOAT increased proportionally to the total drug accumulation, with adefovir-diphosphate being the most abundant metabolite both in CHOhOAT and CHO cells. In contrast, the most abundant metabolite of cidofovir was cidofovir-phosphocholine. A more than 100-fold increase in the level of each cidofovir and adefovir metabolite upon hOAT1 expression indicated that nucleotide analogs, and presumably also the other substrates taken up via hOAT1, are subjected to the intracellular metabolism similar to that observed in hOAT1-negative cells.

Selectivity Cytotoxicity of Adefovir and Cidofovir in Cells Expressing hOAT1

Consistent with the limited intracellular accumulation of adefovir and cidofovir, CHO cells exhibited a relatively low susceptibility to the cytotoxic effects of the two drugs. Thus, we investigated whether the enhanced intracellular uptake and metabolism of both nucleotide analogs due to hOAT1 expression would translate into increased susceptibility of CHOhOAT cells toward the two drugs. Both CHO and CHOhOAT cells were incubated for 5 d with various concentrations of adefovir or cidofovir, and the cytotoxic effect was determined by MTT colorimetric assay. As shown in Figure 5A, both nucleotide analogs were markedly more cytotoxic in CHOhOAT cells compared with CHO cells. In the case of adefovir, expression of hOAT1 enhanced its cytotoxicity approximately 500-fold. Similarly, cidofovir showed 400-fold higher cytotoxicity in CHOhOAT cells compared with CHO cells. In contrast, CHO hOAT cells were only fourfold more susceptible to the cyclic prodrug of cidofovir, which corresponds with the low efficiency of hOAT1-mediated uptake of this compound.

To address whether hOAT1 expression can also induce the cytotoxicity of adefovir and cidofovir in other cell types, Chinese hamster lung fibroblasts (V-79) were also stably transfected with hOAT1 cDNA. A transfectant of V-79 cells exhibiting 50-fold enhancement in the uptake of adefovir was isolated and its susceptibility to nucleotide analogs was compared with parental V-79 cells. Figure 5B shows that hOAT1 expression in V-79 cells also caused a marked shift in the susceptibility to adefovir and cidofovir, but not to the cyclic prodrug of cidofovir.

hOAT1-Mediated Cytotoxicity Is Reduced by Probenecid and Betamipron

Similar to PAH, the uptake of nucleoside phosphonate analogs by CHOhOAT cells was inhibited in the presence of the hOAT1 inhibitors probenecid and betamipron (Figure 6). Both compounds showed similar inhibitory potency with IC50 values...
from CHO cells, since probenecid has been shown previously to interfere with cellular efflux of various organic anions (30,31). Consequently, in the presence of probenecid, adefovir and cidofovir were only 2.5-fold and 4-fold more cytotoxic, respectively, in CHO hOAT cells than in CHO cells. Betamipron, in accordance with its inhibitory effect on hOAT1-specific transport, also markedly reduced hOAT1-mediated cytotoxicity of both nucleotide analogs (Table 3).

**Discussion**

A recent study utilizing protein expression in *Xenopus laevis* oocytes has demonstrated that both adefovir and cidofovir are high-affinity substrates for hOAT1 (9). Based on this finding, an active role of hOAT1-mediated uptake in the mechanism of nephrotoxicity associated with these two clinically important antivirals has been suggested (9). However, although the *Xenopus laevis* system is widely used for the study of membrane transport proteins because it offers fast and efficient expression as well as low background in the transport assays, one of its limitations is the inability to study the effects of the expressed transport protein on other cellular functions. Therefore, we stably expressed hOAT1 in CHO cells, which have been chosen because of their low background accumulation of hOAT1 substrates. To maximize the efficiency of expression, hOAT1 cDNA was cloned downstream from the truncated version of AMV 5’-UTR, which has been shown previously to stimulate *in vitro* protein expression (19). This approach resulted in 30- to 100-fold enhancement in the uptake of hOAT1 substrates in stably transfected CHO cells, i.e., similar to that achieved by the expression of hOAT1 in *Xenopus* oocytes (9).

Kinetic experiments carried out using CHO<sup>hOAT</sup> cells demonstrated that the efficiency (V<sub>max</sub>/K<sub>m</sub> ratio) of hOAT1-specific uptake of adefovir and cidofovir is comparable to that of PAH. Consistent with the proposed function of hOAT1 as an organic anion/dicarboxylate exchanger, hOAT1-specific uptake of PAH and adefovir in CHO<sup>hOAT</sup> cells was trans-stimulated by glutarate. Further functional characterization of hOAT1 demonstrated a marked dependence of its transport activity on extracellular pH. The uptake of PAH and adefovir showed different pH optima, most likely reflecting the differences in the ionization of the two substrates. Comparison of pK<sub>a</sub> estimates for the carboxyl moiety of PAH (pK<sub>a</sub> = 3.2) and the phosphonate moiety of adefovir (pK<sub>a</sub> = 7.8) (32) is consistent with the lower pH optimum of PAH uptake and supports
previous findings demonstrating a direct relationship between the pK_a values of various organic anions and their interference with PAH uptake in isolated kidney proximal tubules (33). Notably, both the uptake of adefovir and PAH were almost completely suppressed at pH >8. This effect is opposite to that observed with some of the organic cation transporters. For example, transport activity of placental organic cation transporter type 3 is highest at pH 8. This effect is opposite to that observed with some of the organic cation transporters. For example, transport activity of placental organic cation transporter type 3 is highest at pH 8, but is almost completely abolished at pH <6 due to the electrogenic character of the transporter, which makes it strongly sensitive to changes in the membrane potential induced by a shift to acidic pH (34). Because the sharp decline in hOAT1 transport activity at higher pH could not be explained only by the suppression of charge-charge interactions between the substrate and hOAT1, a further electrophysiologic characterization of hOAT1 is necessary to explore its sensitivity to membrane potential and fully explain the observed pH dependence.

The finding that the efficiency of hOAT1-mediated uptake of adefovir and cidofovir is as high as that of PAH supports the proposed role of hOAT1 in the mechanism of nephrotoxicity associated with the two antiviral nucleotides. However, in this study, we have also obtained direct evidence of the involvement of hOAT1 in the organ-specific toxicity induced by adefovir and cidofovir. After comparing the cytotoxic effect of both drugs in parental cells and cells stably expressing hOAT1, we concluded that hOAT1 expression markedly increases the sensitivity of cells to the cytotoxic effect of the two antivirals. This sensitivity increase appears to be proportional to the elevation of intracellular metabolites of adefovir and cidofovir in cells expressing hOAT1.

Based on the observed differences in the metabolism of adefovir and cidofovir, the intracellular target associated with their toxicity may be unique for each compound despite their close structural similarity. In the case of cidofovir, the major metabolite in CHO^hOAT cells is cidofovir-phosphocholine, an analog of the phospholipid synthesis intermediate cytidine 5'-diphosphocholine. At high intracellular concentrations generated upon hOAT1 expression, this metabolite may possibly interfere with synthesis or degradation of membrane phospholipids, similar to what has been shown with arabinofuranosyl-cytosine 5'-diphosphocholine (35). In contrast, adefovir, which is an adenine nucleotide analog, undergoes only mono- and diphosphorylation in all cells tested thus far (13). Hence, following the accumulation via hOAT1, the drug itself or some of its metabolites may interfere with ATP synthesis, specific ATP-dependent processes, and/or transport of adenine nucleotides in various cellular compartments, e.g., the Golgi (36) or mitochondria (37).

It is important to recognize that in vivo, the hOAT1-mediated cytotoxic effect of adefovir and cidofovir in proximal tubules may not be as dramatic as that seen in our cell culture model due to the secretion of these two drugs from the tubular epithelium into the lumen as demonstrated by pharmacokinetic studies (14,15). Recently, the presence of multidrug resistance protein 2 (MRP2) in the apical membrane of human proximal tubules has been demonstrated (38). MRP2 is an ATP-dependent efflux pump for organic anion substances, and thus may mediate the apical efflux of adefovir and cidofovir into the lumen. For most substrates of the classical organic anion transport system, basolateral uptake appears to be the rate-limiting step in the entire tubular secretion pathway. However, an accumulation of cidofovir in the superficial kidney cortex, where proximal tubules are located, has been detected previously (39), indicating that the luminal efflux is most likely the

**Table 2. Comparison of intracellular metabolism of adefovir and cidofovir in CHO and CHO^hOAT cells**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Intracellular Level (pmol/10^6 cells)</th>
<th>CHO</th>
<th>CHO^hOAT</th>
<th>Fold Change^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adefovir treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>4.5</td>
<td>620</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>ADV</td>
<td>1.4 (31%)^c</td>
<td>155 (25%)</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>ADV-phosphate</td>
<td>0.7 (16%)</td>
<td>130 (21%)</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td>ADV-diphosphate</td>
<td>2.4 (53%)</td>
<td>335 (54%)</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Cidofovir treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>total</td>
<td>10.0</td>
<td>1860</td>
<td>186</td>
<td></td>
</tr>
<tr>
<td>CDV</td>
<td>3.7 (37%)</td>
<td>428 (23%)</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>CDV-phosphate</td>
<td>0.5 (5%)</td>
<td>108 (6%)</td>
<td>216</td>
<td></td>
</tr>
<tr>
<td>CDV-diphosphate</td>
<td>0.9 (9%)</td>
<td>353 (19%)</td>
<td>392</td>
<td></td>
</tr>
<tr>
<td>CDV-phosphocholine</td>
<td>4.9 (49%)</td>
<td>969 (52%)</td>
<td>198</td>
<td></td>
</tr>
</tbody>
</table>

^a CHO and CHO^hOAT cells were treated for 12 h with 5 μM [3H]adefovir (ADV) or 10 μM [14C]cidofovir (CDV). Cell extracts were separated by ion-pair HPLC, radioactivity in collected fractions was counted, and the amount of each metabolite was quantified. Cell numbers were determined in separate samples treated with unlabeled drugs under the same conditions. The data represent the average of two independent experiments.

^b Fold increase in the intracellular level of each metabolite in CHO^hOAT cells relative to that in CHO cells incubated under the identical conditions.

^c Numbers in parentheses represent percent contribution of each metabolite to the total amount of the intracellular drug.
limiting step in the tubular secretion of nucleoside phosphonates. Thus, in addition to highly efficient basolateral uptake, the slower rate of apical secretion may be another factor involved in the mechanism of nephrotoxicity associated with cidofovir and adefovir.

We demonstrated that the cytotoxicity enhancement upon hOAT1 expression is cell type-independent, because a similar effect was observed both in the cells of epithelial origin (CHO) and in fibroblasts (V-79). In vivo, high-level expression of hOAT1 has been detected specifically in kidney (9,10) corresponding with the kidney-specific toxicity of adefovir and cidofovir. However, based on sensitive detection using reverse transcription-PCR, the presence of a hOAT1-specific transcript has also been detected in skeletal muscles and brain (9). In skeletal muscles, only a truncated form of hOAT1-specific mRNA has been found, suggesting the expression of a modified form of hOAT1 that may lack or may have altered transport activity. This hypothesis correlates with the observed lack of accumulation of cidofovir in skeletal muscles as well as with the lack of the muscle-specific toxicity of the two antiviral nucleotides (39). In contrast, detection of the full-length hOAT1-specific mRNA in brain suggests expression of the active transporter. Indeed, probenecid-sensitive active transport of PAH and a variety of other organic anions has been detected previously in the choroid plexus of several mammalian species (40,41). However, both cell culture (42) and in vivo experiments (43) suggest that the organic anion transport activity in the choroid plexus is localized apically rather than basolaterally and participates in the active secretion of anionic drugs from cerebrospinal fluid into the systemic circulation. The apical membrane localization of hOAT1 would preclude its active role in organ-specific drug toxicity correlating with the lack of neurotoxicity of adefovir and cidofovir. Nevertheless, the precise localization and function of hOAT1 in brain remains to be confirmed by molecular and immunohistochemical analysis.

Characterization of the effect of hOAT1 expression on uptake and cytotoxicity of the cyclic prodrug of cidofovir provided additional support for the involvement of hOAT1 in the mechanism of drug-associated nephrotoxicity. The efficiency of hOAT1-mediated uptake of the prodrug was 15-fold lower than that of cidofovir, presumably due to differences in the polarity of the two molecules. Accordingly, the cytotoxicity of the prodrug was enhanced only three- to fourfold by hOAT1

Figure 5. Effect of hOAT1 expression on cytotoxicity of adefovir and cidofovir. Parental and hOAT-1-transfected cells in 96-well plates were incubated in parallel for 5 d with various concentrations of tested drugs. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay was performed, and drug concentration inhibiting cell growth by 50% (CC50) was determined. (A) Cytotoxicity in parental CHO cells (○) and in CHOHOAT cells (●). (B) Cytotoxicity in parental V-79 cells (△) and in V-79HOAT cells (▲). Data are from representative experiments carried out simultaneously for the parental and hOAT1-transfected cells.
expression compared to 400-fold enhancement determined for cidofovir under identical conditions. This observation strongly correlates with significantly lower renal accumulation of the cyclic prodrug as well as with its much lower nephrotoxic potential in various species compared to cidofovir (44,45).

Finally, the uptake of adefovir and cidofovir in CHO hOAT cells was sensitive to probenecid and betamipron, two potent hOAT1 inhibitors, which have exhibited in vivo nephroprotective effects against various nephrotoxic organic anions (46–48). Both compounds protected CHO hOAT cells from hOAT1-induced cytotoxicity of both nucleotide analogs. In fact, probenecid showed a corresponding nephroprotective effect in cynomolgus monkeys treated with cidofovir (49) and is currently used in conjunction with cidofovir therapy in AIDS patients to reduce the potential for nephrotoxicity.

In conclusion, using a cell line stably expressing hOAT1, we have extended the functional characterization of this physiologically important renal transport protein and have provided compelling evidence that hOAT1 plays a key role in the mechanism of organ-specific toxicity associated with adefovir and cidofovir antiviral therapy. In addition, the presented data support the utility of this in vitro model to explore the involvement of hOAT1 in the nephrotoxicity of other agents, to study potential drug-drug pharmacokinetic interactions and mechanisms of renal drug secretion, and to screen for inhibitors that could serve as efficient nephroprotectants.

Acknowledgments

We thank Norbert Bischofberger, Mick Hitchcock, and Jay Toole of Gilead Sciences for helpful discussions and critical reading of the manuscript.

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