Multiple Drug Transporters Are Involved in Renal Secretion of Entecavir

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Entecavir (ETV) is a first-line antiviral agent for the treatment of chronic hepatitis B virus infection. Renal excretion is the major elimination path of ETV, in which tubular secretion plays the key role. However, the secretion mechanism has not been clarified. We speculated that renal transporters mediated the secretion of ETV. Therefore, the aim of our study was to elucidate which transporters contribute to the renal disposition of ETV. Our results revealed that ETV (50 μ M) remarkably reduced the accumulation of probe substrates in MDCK cells stably expressing human multidrug and toxin efflux extrusion proteins (hMATE1/2-K), organic cation transporter 2 (hOCT2), and carnitine/organic cation transporters (hOCTNs) and increased the substrate accumulation in cells transfected with multidrug resistance-associated protein 2 (hMRP2) or multidrug resistance protein 1 (hMDR1). Moreover, ETV was proved to be a substrate of the above-described transporters. In transwell studies, the transport of ETV in MDCK-hOCT2-hMATE1 showed a distinct directionality from BL (hOCT2) to AP (hMATE1), and the cellular accumulation of ETV in cells expressing hMATE1 was dramatically lower than that of the mock-treated cells. The accumulation of ETV in mouse primary renal tubular cells was obviously affected by inhibitors of organic anion transporter 1/3 (Oat1/3), Oct2, Octn1/2, and Mrp2. Therefore, the renal uptake of ETV is likely mediated by OAT1/3 and OCT2 while the efflux is mediated by MATEs, MDR1, and MRP2, and OCTN1/2 may participate in both renal secretion and reabsorption.

Chronic hepatitis B virus (HBV) infection, with a high rate of morbidity, is one of the most important health problems worldwide. It is a major risk factor for cirrhosis and liver cancer (1). Entecavir (ETV) is a novel and highly selective deoxyguanosine analog with a high antiviral efficacy and a high genetic barrier to viral resistance (2). Since ETV was approved in 2005 by the U.S. FDA, it has been unanimously recommended as the first-line antiviral agent for treatment of chronic HBV infection by global hepatology scientific societies, such as the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver (3, 4).

It was reported that about 73% of orally dosed ETV was eliminated in urine in an unchanged form. The renal clearance of ETV is independent of the dose and ranged from 360 to 471 ml/min in healthy volunteers, which is much greater than the glomerular filtration rate (GFR; 120 to 130 ml/min for normal humans) (5, 6), suggesting that tubular secretion accounts for the major part of the urinary excretion of ETV. Therefore, it is speculated that transporters expressed on the proximal tubular cells are involved in the process of tubular secretion of ETV. Furthermore, ETV is a hydrophilic weak base with a pK_a value of 10.5 (>99% of ETV is positively charged at pH 7.4) and a logD value of -1.1 (pH 4 to 10), which implies that ETV is unlikely to penetrate the cell membrane by passive diffusion. Thus, transporters might play a crucial role in ETV renal secretion.

Multiple transporters are expressed in the renal tubular cells, for instance, organic cation transporter 2 (OCT2) and organic anion transporters 1 and 3 (OAT1 and OAT3) are abundantly expressed on the basolateral (BL) membrane of renal proximal tubular fragments, while carnitine/organic cation transporters (OCTNs), multidrug and toxin efflux extrusion proteins (MATEs), multidrug resistance-associated protein 2 (MRP2), and multidrug resistance protein 1 (MDR1) are abundantly expressed

on the apical (AP) side. All of the renal transporters may play important roles in renal elimination of some drugs (7, 8).

Chen et al. found that cimetidine (a potent inhibitor of OCTs and MATEs) and probenecid (a potent inhibitor of OATs) reduced ETV renal clearance in rats by 50.5% and 67.8%, along with increasing the steady-state plasma concentration by 127.6% and 169.5%, respectively (9), which indicated OCT2, MATEs, OAT1, and OAT3 are involved in the renal secretion of ETV. ETV has been identified as a substrate of OAT1 and OAT3 (10); however, Mandikova et al. found that it was not a substrate of hOCT2 using a transiently transfected cell model (11). Since more than 99% of ETV is positively charged under physiological conditions, we conjectured that OCT2 is involved in the renal disposition of ETV. In addition to OAT1, OAT3, and OCT2, scant information was available on whether transporters located at the apical side of tubular cells, including solute carrier (SLC) transporters (MATE1, MATE2-K, OCTN1, and OCTN2) and ABC transporters (MDR1 and MRP2), contribute to the renal disposition of ETV.

With those in mind, the aim of the current study was to explore which transporters are involved in the renal disposition of ETV using stably transporter-transfected cell models, including single-transporter- and double-transporter-transfected cell models, and

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mouse primary renal tubular cells (*mPRTC*). The results will provide information to elucidate the molecular mechanism of tubular secretion of ETV and also help us predict the drug-drug interactions (DDIs) mediated by transporters.

MATERIALS AND METHODS

Chemicals. 4',6-Diamidino-2-phenylindole (DAPI), MK-571, rhodamine (Rho123), 1-methyl-4-phenylpyridiniumiodide (MPP⁺), cimetidine, and rabbit polyclonal anti-SLC47A2 antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hygromycin B was purchased from Roche (Basel, Switzerland). Quinidine sulfate was provided by Nanjing De Bio-Chem Co., Ltd. (Nanjing, China). The bicinechoninic acid (BCA) protein assay kit was acquired from Beyotime Biotechnology Co., Ltd. (Shanghai, China). L-Carnitine, L-ergothioneine, cyclosporine, and metformin were purchased from Aladdin Co., Ltd. (Shanghai, China). Entecavir and mildronate were provided by Meilun Biological Co., Ltd. (Dalian, China). Lipofectamine 2000, the vector pcDNA3.1 (+), fetal bovine serum (FBS), trypsin, Dulbecco's modified Eagle medium (DMEM), DMEM with nutrient mixture F12 (DMEM-F12), insulin-transferrin-selenium, penicillin, streptomycin, and collagenase (type IV) were acquired from Gibco (Invitrogen Life Technologies, USA). All other chemicals or solvents were of the highest grade commercially available.

Animals. Male ICR mice, weighing 20 to 25 g, were supplied by the Experimental Animal Center of Zhejiang Academy of Medical Sciences. Animals were maintained at 20 ± 2°C with 50% ± 10% relative humidity and 12 h light–12 h dark cycles and were given free access to water and food, but they were fasted overnight with water available before the experiment. All of the animal experiments followed an approved animal protocol of Zhejiang University.

Cell culture and transfection. The parental Madin-Darby canine kidney II (MDCK) cells were acquired from Peking Union Medical College (Beijing, China). The full sequence of human MATE2-K cDNA was subcloned into expression vector pcDNA3.1(+)-Hygro in our laboratory and then transfected into MDCK cells by lipo2000. Cells were screened out by hygromycin B (400 µg/ml) for 14 days, and their transport function was preliminarily confirmed by DAPI accumulation study (12). The cell model was determined by comparing the positive single clones based on quantitative real-time PCR (qRT-PCR) and the probe substrate (MPP⁺ and metformin) accumulation experiment with or without typical inhibitor (cimetidine or quinidine at 100 µM) (13). The hMATE2-K protein was detected by Western blotting. MDCK cells stably transfected with pcDNA3.1(+)-hygro (mock), full-length hOCT2 cDNA (MDCK-hOCT2), hMATE1 cDNA (MDCK-hMATE1), hOCTN1 cDNA (MDCK-hOCTN1), hOCTN2 cDNA (MDCK-hOCTN2), hMDR1 cDNA (MDCK-hMDR1), and hMRP2 cDNA (MDCK-hMRP2), as well as MDCK-hOCT2-hMATE1 double-transfected cells, were established or kept in our laboratory (14–18), and the cells were cultured in DMEM with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin in a humid atmosphere of 5% CO₂ and 95% air at 37°C.

qRT-PCR of hMATE2-K. Total RNAs were extracted from mock-transfected cells and the selected MDCK-hMATE2-K-positive clones using the RNAsimple total RNA kit (Tian Gen, China). cDNAs were synthesized using PrimeScript RT reagent kit (TaKaRa Bio, Japan) followed by a qRT-PCR procedure using SYBR premix Ex Taq II (TaKaRa Bio, Japan). Expression of the target mRNAs was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primer pairs are listed in Table 1.

Western blot assay for hMATE2-K. Proteins were extracted from mock-transfected cells or MDCK-hMATE2-K cells with radioimmuno-precipitation assay (RIPA) lysis buffer, and the extracted samples were suspended in loading buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 100 mM dithiothreitol) (19). After boiling at 100°C for 5 min, aliquots of denatured protein were separated by electrophoresis on SDS-PAGE (Bio-Rad, Hercules, CA) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The mem-

TABLE 1 Primers used in qRT-PCR for hMATE2-K

Gene	Direction	Sequence (5'→3')	Product length (bp)
GAPDH	Forward	AGGAGCGAGATCCCGCCAACA	112
	Reverse	CCGCCTTCAAGTGAGCCCCA	
hMATE2-K	Forward	TGGGGCATATTTTACCAATG	114
	Reverse	GAATCCGCCATAGACACAAC	

branes were blocked overnight at 4°C with TBST buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk and then probed with the primary antibody (hMATE2-K at 1:1,500, GAPDH at 1:2,000) in blocking buffer, followed by horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000) incubation at room temperature for another 1 h. The signals of proteins were visualized by chemiluminescence using the enhanced chemiluminescence (ECL) Western blotting detection system (LI-COR Biosciences, Lincoln, NE).

Isolation of mouse renal tubular cells. Mouse primary renal proximal tubular fragments were isolated from ICR mice with DMEM-F12 containing 0.1% collagenase (IV)–0.1% trypsin as the method previously reported (20). The fragments were plated in gelatin-coated 12-well plates (Costar Corning Inc., Corning, NY, USA), cultured in DMEM-F12 medium supplemented with 10% FBS, 1% insulin-transferrin-selenium, 100 µg/ml streptomycin, and 100 U/ml penicillin, and incubated in a humidified CO₂-air incubator (5:95, vol/vol) at 37°C. After 4 days, the cells formed confluent monolayers.

On day 5, the mRNA expression levels of target genes encoding Oct2, Octn1, Octn2, Oat1, Oat3, Mate1, Mdr1a, and Mrp2 in *mPRTC* were quantified by qRT-PCR. All of the primers are listed in Table 2. Relative mRNA levels of target genes were normalized by GAPDH using the ΔC_T method (where C_T is threshold cycle) and described as $2^{-\Delta C_T}$ ($\Delta C_T = C_{T \text{ target gene}} - C_{T \text{ GAPDH}}$). The mRNA levels ranked in the order of Mrp2 > Oct2 > Octn2 > Oat1 > Mate1 > Oat3 > Octn1 > Mdr1a, with values of 0.0041, 0.0025, 0.0021, 0.0017, 0.0016, 0.0013, 0.0011, and 0.0010, respectively, indicating that the *mPRTC* could be employed to study the role of transporters in renal disposition of ETV.

Cellular accumulation and inhibition assays in transporter-transfected cells. MDCK cells stably expressing hOCT2, hOCTN1, hOCTN2, hMATE1, hMATE2-K, hMDR1, hMRP2, or mock-transfected cells were seeded in 24-well plates (Costar Corning Inc., Corning, NY, USA) at a density of 2×10^5 /well. On day 3 after seeding, the accumulation studies were performed according to methods developed in our laboratory (14–16, 21). The effectiveness of the transgenic cell models was verified using probe substrates including MPP⁺ (1 µM) for hOCT2 and hMATE1/2-K, L-ergothioneine (3 µM) for hOCTN1, mildronate (2 µM) for hOCTN2, and Rho123 (4 µM) for hMDR1 and calcein AM (1 µM) for hMRP2.

For accumulation in cells stably transfected with hOCT2, hOCTN1, hOCTN2, hMDR1, or hMRP2, the medium was removed, and the cells were washed with prewarmed phosphate-buffered saline (PBS) and preincubated with Hanks' balanced salt solution (HBSS; pH 7.4) for 10 min or 30 min (for hMDR1 and hMRP2) prior to the accumulation assays. The accumulation was initiated by adding HBSS (pH 7.4) containing ETV or probe substrates of studied transporters with or without inhibitors at 37°C for 3 min, 60 min (for hMDR1), and 90 min (for hMRP2). At the end of the incubation, the medium was drawn off, and the accumulation was actually terminated when the cells were rinsed with ice-cold PBS immediately three times, followed by adding 100 µl of 0.1% SDS to lysis the cells. Lysate aliquots of 80 µl were exposed to acetonitrile at a ratio of 1:3 for protein precipitation.

For accumulation mediated by hMATEs, MDCK-hMATE1 and MDCK-hMATE2-K cells and mock-transfected cells were preincubated with buffer containing 30 mM ammonium chloride for 20 min to make the intracellular acidification for evaluation of transport by uptake mea-

TABLE 2 Primers used in qRT-PCR for multiple transporters expressed in mouse (Mus) kidney

Primer	Direction	Sequence (5'→3')	Product length (bp)
Mus gapdh	Forward	GCCTGCTTCTTACTCGGCTA	106
	Reverse	AAAGTCCGTGGAGACCACCT	
Mus mate1	Forward	ATCGCGCTGATGTTTGCT	60
	Reverse	GATGATGATGCCTGACCACA	
Mus oat1	Forward	CCTATGCTGTGCCCCACT	95
	Reverse	GGCTGACTCAATGAAGAACCA	
Mus oat3	Forward	GTGGCGCTGGCTACAGTT	90
	Reverse	ACCAGCCAGCGTATGGACT	
Mus oct2	Forward	AGGCTCTGCCTGAGACCAT	96
	Reverse	GTTCTGCTTTCTTAACCTTGGAGGT	
Mus octn1	Forward	CAGCAGGGATATTCGATCCT	82
	Reverse	CTGAACAGGTCCAGGATTATGA	
Mus octn2	Forward	AGGGTCATCACCATCATGTCT	95
	Reverse	ATGTCCCATGCAAGTTAGG	
Mus mdr1a	Forward	GGGCATTTACTTCAAACCTTGTC	73
	Reverse	TTTACAAGCTTCATTTCTTAATTCAA	
Mus mrp2	Forward	GTCACACGGATTGACCAAGA	73
	Reverse	TTTCTTCTTTTTCGAGTCTTCCA	

surement (12), followed by incubation with uptake buffer for 5 min prior to the accumulation experiment. The accumulation was initiated by adding the medium containing typical substrate or ETV in the absence or presence of inhibitors as described above.

Kinetic studies were performed with a series concentration of ETV for a 3-min incubation, and the background counts of mock-transfected cells were subtracted from the data. The concentration of ETV or probe substrates (MPP⁺, L-ergothioneine, and mildronate) in the cell lysate was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The level of intracellular DAPI (excitation wavelength [λ_{ex}], 358 nm; emission wavelength [λ_{em}], 461 nm), Rho123 (λ_{ex}, 485 nm; λ_{em}, 535 nm), or Calcein (λ_{ex}, 490 nm; λ_{em}, 515 nm) was determined by a microplate reader (Spectra Max M2; Molecular Devices, USA). The accumulation was normalized to the total protein content in the lysates with a BCA protein assay kit.

Transcellular transport experiments. The transcellular transport studies were performed as reported previously (22). Cells were seeded on transwell inserts (0.4 μm, diameter of 12 mm) at a density of 2 × 10⁵ cells per insert for MDCK-hOCT2-hMATE1, MDCK-hOCT2, and MDCK-hMATE1 and at 1 × 10⁵ cells per insert for mock-transfected cells to unify protein concentrations for different amounts of cell growth. After growing for 72 h to form cell monolayers, cells were washed with prewarmed PBS and equilibrated with Hanks' balanced salt solution (pH 7.4) for 30 min. The integrity of the cell monolayers was measured by transepithelial electrical resistance (TEER) using a commercial apparatus (Millicell-ERS equipment; Millipore, MA, USA), and cell monolayers with values over 250 Ω were used for the following experiments. For apical (AP; corresponding to tubular lumen)-to-basal (BL; corresponding to renal blood plasma) transport, 1.5 ml HBSS (pH 7.4) was added to the basal compartment, and the transport was initiated by adding 0.5 ml HBSS (pH 6.0; to increase MATE1-mediated export) containing 10 μM ETV to the apical compartment. For BL-to-AP transport, 0.5 ml HBSS (pH 6.0) was added to the apical side and the transport was initiated by adding 1.5 ml HBSS (pH 7.4) containing 10 μM ETV to the basal side. A 100-μl aliquot was taken from the receiver compartment at the designated time for determi-

nation of ETV transport, and another equivalent volume of fresh buffer was added. At the end of the incubation, the medium was removed and the inserts were rinsed with ice-cold PBS three times. The cells then were lysed with 0.1% SDS to determine the intracellular accumulation of ETV.

ETV accumulation in isolated mouse renal tubular cells. The mPRTC seeded in 12-well plates were used to further study the transporter-mediated renal disposition of ETV. On day 5, the accumulation studies were performed as described above, except that the preincubation buffer was replaced with 500 μl of HBSS (pH 7.4). The accumulation of ETV (10 μM) was measured at 5, 10, 15, and 20 min at 4°C or 37°C. The time courses demonstrated that the accumulation of ETV increased in a linear manner within 10 min. Thus, the inhibitory effect of cimetidine (100 μM), quinidine (100 μM), L-ergothioneine (10 μM), and L-carnitine (100 μM) on the accumulation (10 min) were conducted to explore whether Oct2, Octn1, Octn2, Oat1, and Oat3 contributed to the renal secretion of ETV. The inhibitory effects of cyclosporine (20 μM) and MK-571 (50 μM) on the accumulation of ETV were investigated within 60 min to evaluate the role of ABC transporters. The accumulation was terminated by removing the incubation medium and washing the cells with ice-cold PBS, and then the cells were lysed with 0.1% SDS and the intracellular concentration of ETV was measured by LC-MS/MS.

LC-MS/MS assay. The concentrations of MPP⁺, L-ergothioneine, and mildronate in the samples were determined by an Agilent 1290/6460 LC-MS with a triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) according to the method described previously (21, 23). For ETV determination, 80 μl of the cell lysate was mixed with 240 μl acetonitrile containing the internal standard (5 ng/ml MPP⁺) for 3 min, and then the mixture was centrifuged for 15 min at 16,000 × g and the supernatant was analyzed by LC-MS/MS. Isocratic chromatographic separation was performed on an XBridge HILIC-C₁₈ column (3.0 μm, 2.1 by 50 mm) at 30°C. The mobile phase consisted of 10% solvent A, 0.1% formic acid in 20 mM ammonium formate-water, and 90% solvent B, 0.1% formic acid in acetonitrile at a flow rate of 0.2 ml/min. The compounds were detected by mass spectrometry with an electrospray ionization source under the following conditions: positive ion mode; ion source

FIG 1 Characterization of MDCK cells stably expressing hMATE2-K. (A) The mRNA levels of hMATE2-K expression in MDCK-hMATE2-K and mock-transfected cells. (B) Western blot analysis of hMATE2-K. GAPDH was monitored to ensure equal protein loading. (C and D) The accumulation of MPP⁺ (C) and MET (D) in MDCK-hMATE2-K and mock-treated cells. CIME, cimetidine; QUI, quinidine; MET, metformin. Data represent means $\pm$ SD from three independent experiments conducted in triplicate. ***, $P < 0.001$ compared with accumulation in mock-transfected cells; ###, $P < 0.001$ compared with accumulation in the absence of inhibitors.

temperature, 140°C; desolation temperature, 350°C; capillary voltage, 4.5 kV; collision energy, 11 eV; multiple-reaction-monitoring (MRM) scan mode monitored the ion pair of ETV at m/z 278 > 152 and MPP⁺ at m/z 230 > 112 (A > B indicates A is the precursor ion and B is the product ion). The assay was validated over concentrations of 10 to 1,000 nM, the inter- and intraday precisions (percent relative standard deviation [RSD]) were $\leq 10.5\%$, the recovery ranged from 108.5% to 116.3%, and the relative matrix effect (%RSD) was $\leq 4.2\%$. The lower limit of quantification was 10 nM with an RSD of $\leq 10.8\%$.

Statistical analysis. The data were expressed as means $\pm$ standard deviations (SD). The experiments were conducted at least twice with three replicates for each experiment. The Michaelis-Menten constants $V_{\max}$ and K_m were calculated using GraphPad Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA) by fitting the data to the Michaelis-Menten equation $V = V_{\max} [S]/K_m + [S]$ (24), where V is the uptake velocity and $[S]$ is the substrate concentration. One-way analysis of variance (ANOVA) with the Dunnett's test or two-way ANOVA followed by the Bonferroni posttest was used to evaluate the statistical differences among different groups; Student's t test was used to compare two groups. Differences between groups were considered significant if P values were < 0.05 .

RESULTS

Stable expression of hMATE2-K in MDCK cells. The SLC47A2 mRNA (encoding hMATE2-K) expression in stably transfected MDCK cells was verified using qRT-PCR. The results (Fig. 1A) demonstrated that the mRNA expression of SLC47A2 in MDCK-hMATE2-K cells was 5,000-fold higher than that in mock-transfected cells. The hMATE2-K protein level in mock-transfected cells was negligible to the naked eye, whereas it was much higher in MDCK-hMATE2-K cells (Fig. 1B). The intracellular accumulation of probe substrate MPP⁺ or metformin in MDCK-hMATE2-K cells was approximately 45-fold and 20-fold higher than those in mock-transfected cells. The typical inhibitors (cime-

tidine or quinidine) significantly reduced ($P < 0.001$) MPP⁺ or metformin accumulation in MDCK-hMATE2-K cells but not in mock-transfected cells (Fig. 1C and D). These results indicated that the MDCK cell model stably expressing hMATE2-K was successfully established and could be applied to evaluate hMATE2-K-mediated drug transport and drug-drug interactions.

Interaction of ETV with hOCT2, hMATE1/2-K, and hOCTN1/2. To estimate the role of SLC transporters in renal secretion of ETV, MDCK cells stably expressing human transporters were incubated with probe substrates in the absence or presence of ETV. As shown in Fig. 2, 50 μ M ETV markedly reduced ($P < 0.001$) the MPP⁺ accumulation in hOCT2-, hMATE1-, or hMATE2-K-transfected cells, L-ergothioneine accumulation in hOCTN1-transfected cells, and mildronate accumulation in hOCTN2-transfected cells.

The accumulation of ETV in the above-described cell models and respective mock-transfected cells was also determined. As shown in Fig. 3, the accumulation of ETV in transfected cells was 2-fold (hOCT2), 5-fold (hMATE1), 10-fold (hMATE2-K), 4-fold (hOCTN1), and 3-fold (hOCTN2) higher than that in mock-transfected cells, which could be substantially reduced by the typical inhibitors (for hOCT2, 5 μ M decynium-22; for hMATEs, 100 μ M cimetidine or quinidine; for hOCTN1, 100 μ M quinidine; for hOCTN2, 100 μ M L-carnitine).

The kinetics of ETV uptake in hOCT2- and hOCTN1/2-transfected cells followed typical dynamics. The K_m and $V_{\max}$ values were 0.30 ± 0.02 , 2.4 ± 0.3 , 1.9 ± 0.2 mM and 0.9 ± 0.02 , 4.3 ± 0.3 , 3.0 ± 0.2 nmol/mg protein/min, respectively (Fig. 4 and Table 3). In contrast, the kinetics of ETV uptake in MDCK-hMATE1 and MDCK-hMATE2-K cells displayed atypical dynamics under Eadie-Hofstee analysis. Under low concentrations (50 to 400

FIG 2 Accumulation of probe substrates in the absence or presence of ETV or specific transporter inhibitors in mock-treated cells and MDCK cells stably expressing hOCT2, hMATE1/hMATE2-K (A), hOCTN1 (B), and hOCTN2 (C). Cimetidine was applied as a typical inhibitor for hMATEs and hOCT2, quinidine for hOCTN1, and L-carnitine for hOCTN2. EGT, L-ergothioneine; THP, mildronate; L-Car, L-carnitine. Data represent means $\pm$ SD from three independent experiments conducted in triplicate. ***, $P < 0.001$ compared with accumulation in mock-transfected cells; ###, $P < 0.001$ compared with accumulation in the absence of inhibitors.

μM), the K_m value for both hMATE1 and hMATE2-K was 0.20 mM and the $V_{\max}$ values were 0.70 and 3.0 nmol/mg protein/min, respectively, while under high concentration (400 to 3,000 μM), the K_m values were 1.6 and 0.9 mM and the $V_{\max}$ values were 3.0 and 6.5 nmol/mg protein/min, respectively (Fig. 4 and Table 3). These results indicated that ETV was a substrate of hOCT2, hMATE1, hMATE2-K, hOCTN1, and hOCTN2. The rank of uptake clearance expressed as $V_{\max}/K_m$ was hMATE2-K > hMATE1 > hOCT2 > hOCTN1 > hOCTN2.

Transport of ETV by MDCK cells transfected with hOCT2 and/or hMATE1. Since the transcellular transport by hOCT2 (BL) in combination with efflux via hMATE1 (AP) has been rec-

ognized as an essential system for renal elimination of cationic drugs (25), MDCK-hOCT2-hMATE1 cells were applied to assess functional interplay between uptake mediated by hOCT2 and efflux by hMATE1. As shown in Fig. 5A, the transport of ETV from BL to AP was much greater than that from AP to BL across MDCK-hOCT2-hMATE1 cell monolayers, and cimetidine markedly reduced ETV transport from BL to AP. Moreover, intracellular accumulation was much lower in AP-to-BL transport as a result of hMATE1 expressed on the AP side to export ETV (Fig. 5B). In addition, the transcellular transport of ETV (10 μM) from BL to AP was significantly higher ($P < 0.001$) in MDCK-hOCT2-hMATE1 cells than in MDCK-hMATE1 cells, followed by

FIG 3 Accumulation of ETV in mock-treated cells and MDCK cells transfected with hOCT2 (A), hOCTN1 or hOCTN2 (B), and hMATE1 or hMATE2-K (C) in the absence or presence of typical inhibitors. Cells were incubated at 37°C for 3 min with incubation buffer containing 10 μM ETV with or without inhibitors (for hOCT2, decynium-22/D22; for hOCTN1, QUI; for hOCTN2, L-Car; for hMATEs, CIME and QUI). Data represent means $\pm$ SD from three independent experiments conducted in triplicate. ***, $P < 0.001$ compared with the accumulation in mock-transfected cells; ###, $P < 0.001$ compared with accumulation in the absence of inhibitors.

FIG 4 Kinetics of ETV uptake mediated by hOCT2 (A and D), hOCTN1 or hOCTN2 (B and E), or hMATE1 or hMATE2-K (C and F). Cells were incubated with a series concentrations of ETV for 3 min. Panels A, B, and C are Michaelis-Menten plots, and panels D, E, and F are Eadie-Hofstee plots. In v/s , v is the uptake velocity and s is the substrate concentration.

MDCK-hOCT2 and mock-transfected cells, which implied the importance of hMATE1 in ETV transport (Fig. 5C). The ranking of intracellular accumulation of ETV was in the order of MDCK-hOCT2 > mock-transfected > MDCK-hOCT2-hMATE1 > MDCK-hMATE1 cells (Fig. 5D).

Interaction of ETV with ABC transporters. To test the possibility of hMDR1 and hMRP2 participating in the renal secretion of ETV, we evaluated the interaction of ETV with hMDR1 and hMRP2. As shown in Fig. 6A and B, ETV (50 μ M) rose the accumulation of the typical substrates of hMDR1 (Rho123) and hMRP2 (Calcein AM) in MDCK-hMDR1 and MDCK-hMRP2 cells, respectively. In addition, the cellular accumulation of ETV (10 μ M for 1 h) in MDCK-hMDR1 cells was only about 85% of that in mock-transfected cells, and cyclosporine (20 μ M; an in-

hibitor of hMDR1) markedly raised ($P < 0.05$) the accumulation (Fig. 6C). The accumulation of ETV (10 μ M for 1.5 h) in MDCK-hMRP2 cells was about 50% of that in mock-transfected cells, and MK-571 (20 μ M; an inhibitor of hMRP2) obviously increased ($P < 0.001$) the accumulation (Fig. 6D). Thus, ETV was a substrate of hMDR1 and hMRP2.

ETV accumulation in mPRTC. We applied mouse primary renal proximal tubular cells (mPRTC) to further study the transporter-mediated renal secretion of ETV. As shown in Fig. 7A, the accumulation of ETV in mPRTC at 37°C was obviously higher than that at 4°C, which indicated that transporters were involved in the process of ETV disposition. Furthermore, the accumulation of ETV (10 μ M) in mPRTC was significantly reduced by inhibitors of Oct2 (100 μ M cimetidine or quinidine), of Oat1 and Oat3 (100

TABLE 3 Kinetic parameters of ETV uptake in MDCK cells stably transfected with hOCT2, hMATEs, and hOCTNs^a

Cell type and calculation range ^b	K_m (mM)	V_{max} (nmol/mg protein/min)	CL_{int} (ml/g/min)
hOCT2	0.30 ± 0.02	0.9 ± 0.02	3.0
hMATE1			
1	0.20 ± 0.03	0.70 ± 0.05	3.5
2	1.6 ± 0.2	3.0 ± 0.2	1.9
hMATE2-K			
1	0.20 ± 0.03	3.0 ± 0.2	15
2	0.9 ± 0.1	6.5 ± 0.3	7.2
hOCTN1	2.4 ± 0.3	4.3 ± 0.3	1.8
hOCTN2	1.9 ± 0.2	3.0 ± 0.2	1.6

^a Data are expressed as means $\pm$ SD ($n = 6$).

^b The kinetics of ETV uptake in MDCK-hMATE1 and MDCK-hMATE2-K cells displayed atypical dynamics, and the parameters were calculated in the range of 50 to 400 μ M (1) and 400 to 3,000 μ M (2).

FIG 5 Transcellular transport of ETV in MDCK cells stably expressing hOCT2 and/or hMATE1 or in mock-transfected cells. Time courses of ETV (10 μ M) transcellular transport (A) and intracellular accumulation (B) in direction of BL to AP and AP to BL in the presence or absence of cimetine in MDCK-hOCT2-hMATE1 cells within 120 min. ***, $P < 0.001$ compared with AP-to-BL transport; $P < 0.01$ (##) and $P < 0.001$ (###) compared with transport in the absence of inhibitors. (C and D) ETV (10 μ M) transcellular transport (C) and intracellular accumulation (D) from BL to AP in MDCK-hOCT2, MDCK-hMATE1, and MDCK-hOCT2-hMATE1 and in mock-transfected cell monolayers within 60 min. ***, $P < 0.001$ compared with mock-transfected cells; ###, $P < 0.001$ compared with MDCK-hOCT2 cells; $P < 0.05$ (Δ) and $P < 0.01$ ($\Delta\Delta$) compared with MDCK-hMATE1 cells. All data represent means $\pm$ SD from two independent experiments conducted in triplicate.

μ M probenecid), of Octn1 (10 μ M L-ergothioneine) ($P < 0.001$), and of Octn2 (100 μ M L-carnitine) ($P < 0.01$), which was in accordance with our findings that ETV was a substrate of the above-described transporters (Fig. 7C). Mrp2 inhibitor (MK-571; 20 μ M) significantly increased ($P < 0.05$) the ETV accumulation in mPRTC (Fig. 7B); however, cyclosporine (20 μ M), an inhibitor of Mdr1a, did not show the inhibitory effect.

DISCUSSION

The present study investigated the role of transporters in renal disposition of ETV and found that hOCT2, hOATs, hOCTNs, hMATEs, hMDR1, and hMRP2 were involved in the renal tubular secretion of ETV. hOCT2 and hOAT1/3, abundantly expressed on the basolateral membrane of renal epithelial cells, contributed to the uptake of substrate drugs from systemic circulation. Our study proved that ETV is a substrate of hOCT2. In contrast, the study of Mandikova et al. revealed that ETV (100 μ M) increased the accumulation of MPP⁺ in MDCK cells transiently transfected with hOCT2 (11), which might be attributed to the alteration or loss of the function of hOCT2 of the applied cell model during the experiment. Our study found that the K_m of ETV for hOCT2 was 300 μ M, which approximates the value for hOAT1 in the published studies (316.5 and 250 μ M, respectively) and is much higher than that of hOAT3 (23 μ M) (10, 11). Moreover, the intrinsic clearance (CL_{int}) value of (V_{max}/K_m) for hOCT2 was 2.7 ml/g/min, much lower than that of hOAT3 (95 ml/g/min) (10), indicating that hOAT3 contributes more than hOAT1 or hOCT2 to the uptake of ETV from circulation to renal epithelial cells.

The renal epithelial cells highly express efflux transporters on the apical side, including hMATE1, hMATE2-K, hMDR1, and hMRP2, which could also be involved in renal tubular secretion of drugs. The hMATEs, H⁺/organic cation antiporters discovered in 2005 (26), coupled with ABC efflux protein in the brush border membrane of proximal epithelial cells (27), assist some uptake transporters (hOAT1, hOAT3, and hOCT2) located in the basolateral membrane to transport the cationic compounds, even neutral and anionic substances, into urine (28–30). Our study found the accumulation of ETV (10 μ M) in MDCK-hMATE1 and MDCK-hMATE2-K cells was 5-fold and 10-fold higher, respectively, than that in mock-transfected cells after cells were preincubated with 30 mM ammonium chloride, indicating that hMATE1 and hMATE2-K participate in renal secretion of ETV. Considering the inevitable differences under nonphysiological and physiological conditions, transcellular transport of ETV at physiological pH was performed, and a distinct directionality transport from hOCT2 (BL) to hMATE1 (AP) was found in MDCK-hOCT2-hMATE1 cells that was inhibited by cimetine. The cellular accumulation of ETV was the lowest in cells singly expressing the efflux transporter hMATE1 and the highest in cells merely expressing the influx transporter hOCT2, suggesting that hOCT2 and hMATE1 transport ETV cooperatively. In addition to hMATEs, hMDR1 and hMRP2 have been demonstrated to be the pivotal transporters contributing to some nucleoside reverse transcriptase inhibitor (NRTI; such as adefovir and tenofovir) efflux into urine (31–33). Our results demonstrated that ETV was a substrate

FIG 6 Interaction of ETV with hMDR1 and hMRP2. Inhibitory effect of ETV on cellular accumulation of Rho123 in MDCK-hMDR1 cells (A) and calcein in MDCK-hMRP2 cells (B) and cellular accumulation of ETV in MDCK-hMDR1 (C) and MDCK-hMRP2 cells (D) with or without typical inhibitors. CsA, cyclosporine. All data represent means $\pm$ SD from three independent experiments conducted in triplicate. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) compared with accumulation in mock-transfected cells; $P < 0.05$ (#) and $P < 0.001$ (###) compared with accumulation in the absence of inhibitors.

of hMDR1 and hMRP2, indicating the involvement of hMDR1 and hMRP2 in ETV efflux.

hOCTN1 and hOCTN2 are also abundantly expressed in apical membrane of renal proximal tubules. With the characteristics of bidirectional transport, hOCTN1/2 would play roles in both tubular efflux and reabsorption of the substrates from urine (34). Currently, no information about their roles in secretion of ETV (even other NRTIs) is available. Here, we identified that ETV was a substrate of hOCTN1 and hOCTN2, suggesting hOCTNs also are involved in renal disposition of ETV. Recently, it has been proved that ETV was a substrate of hCNT2 and hCNT3, with K_m of 1,522 and 99.02 μ M and V_{max} of 68.88 and 45.84 nmol/mg protein/5 min, respectively (11). Although hCNT2 and hCNT3 are expressed on the apical side of renal epithelial cells, considering their low expression in human kidney, we deduced that they were unlikely to play a critical role in renal disposition of ETV.

The PRTC of mice were utilized to further confirm the roles of the above-described transporters in ETV secretion because of expressing transporters similar to those of human (35, 36). Probenecid, quinidine, l-ergothioneine, or L-carnitine reduced the ETV accumulation in mPRTC, whereas MK-571 increased the accumulation, which was in agreement with the findings in our transfected cell models. Meanwhile, cimetidine, a MATE1 predominant inhibitor, rather than OCT2 causes an increase of ETV accumulation in MDCK-hOCT2/hMATE1 from BL to AP. However, cimetidine reduced the ETV accumulation in mPRTC, and it

might be attributed to the lower expression of MATE1 than OCT2 in cells under the present culture conditions. The above-described data indicated that Oct2, Oats, Octns, and Mrp2 were involved in renal disposition of ETV in mPRTC. Although ETV might be a weak substrate of MDR1, CsA (a typical inhibitor of MDR1) did not increase the ETV accumulation, which could be ascribed to reduction of Mdr1a expression in mPRTC during isolation and cultivation. Because there were no rodent isoforms corresponding to hMATE2-K and MATEs sharing the common inhibitors with OCT2, the function of MATEs in ETV secretion is hard to mimic by mPRTC. Due to the low concentrations of ETV in plasma at the regular clinical dosage and the CL_{int} values mediated by hMATE2-K (15 ml/g/min) or by hMATE1 (3.5 ml/g/min) in the transfected cells, it seems that hMATE2-K plays a more important role than hMATE1 in human renal secretion of ETV.

Hepatitis B disease is an inflammation of the liver which can cause multiple-organ injury. The comprehensive treatment, including antiviral, immune regulation (37, 38), and anti-inflammatory and liver function improvement, is recommended. Thus, ETV may be combined with various antivirals or other drugs, which may result in DDIs mediated by transporters. Considering the low dose of ETV (1 mg/day) (39), the probability of DDIs caused by ETV is low. In contrast, the coadministered drugs might affect ETV disposition *in vivo* via inhibiting transporters. For instance, the pharmacokinetic properties of ETV were changed when cimetidine, JBP485, or diammonium

FIG 7 Effect of multiple drug transporters on the accumulation of ETV in *mPRTC*. (A) The accumulation of ETV at 4°C and 37°C. (B) The effects of CsA and MK-571 on the accumulation of ETV. (C) The effects of SLC transport inhibitors on the accumulation of ETV. For panels B and C, cells were incubated with 10 μM ETV at 37°C for 60 min and 10 min, respectively, with or without inhibitors. Prob, probenecid. All data represent means ± SD from two independent experiments conducted in triplicate. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ compared with the absence of the accumulation of inhibitors.

glycyrrhizinate was coadministered with ETV (9, 10, 40). Additionally, based on the proton gradient mechanism of hMATEs and hOCTN1, it is noteworthy that some drugs and foods may acidify or alkalize urine and subsequently affect the renal secretion of ETV.

Even though ETV showed an excellent suppression of HBV

FIG 8 Schematic representation of ETV renal excretion. ETV elimination in the kidney, including glomerular filtration, renal secretion, and reabsorption. Transporters noted are involved in secretion and reabsorption at the proximal tubules.

replication without significant side effects (41), monitoring for ETV toxicity in long-term treatment was also recommended (42). We speculated that the accumulation of ETV in renal cells mediated by transporters in a long-period administration cause potential adverse effects. It has been noted that chronic exposure to some antiretroviral agents could regulate the expression and activities of several drug transporters (43) which dispose of not only drugs but also physiological endogenous substances. Therefore, further study is required to clarify whether ETV will alter the expression of transporters during chronic treatment.

In summary, our study demonstrated that ETV is a substrate of OCT2, MATE1/2-K, OCTN1/2 MDR1, and MRP2 for the first time and revealed that OAT1/3, OCT2, MATE1/2-K, OCTN1/2, MDR1, and MRP2 are involved in the renal disposition of ETV, in which OCT2, OAT1, and OAT3 contributed to the uptake of ETV from bloodstream to proximal tubular cells, while MATEs, MDR1, and MRP2 mediated the efflux from proximal tubular cells to renal lumen, and perhaps OCTN1/2 participated in both renal secretion and reabsorption of ETV (Fig. 8).

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