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Title: Multiple SLC and ABC transporters contribute to the placental transfer of entecavir

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Running Title: The mechanism of placental transfer of entecavir

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Abbreviations: 5-HT: 5-hydroxytryptamine; **6-CF**: 6-carboxyl fluorescein; **ANOVA**: one-way analysis of variance; **BCRP:** breast cancer resistance protein ; **CNT**: concentrative nucleoside transporter ; **D22**: decynium-22; **DMEM**: Dulbecco's modified Eagle medium ; **ENT**: equilibrative nucleoside transporter; **ETV**: entecavir; **F12K**: Kaighn's Modification of Ham's F-12; **FBS**: Fetal bovine serum; **HEK293**: human embryonic kidney 293; **HBSS**: Hank's balanced salt solution; **LLC-PK1**: Lilly Laboratories Cell-Porcine Kidney 1; **MCT**: monocarboxylate transporter; **MDCK**: Madin–Darby canine kidney; **MRP**: multidrug resistance-associated protein; **NBTI**: S-(4-Nitrobenzyl)-6-thioinosine; **NMDG**: N-methyl-D-glucamine; **OAT**: organic anion transporter; **OCT**: organic cation transporter; **OCTN**: organic cation/carnitine transporter ; **PHTCs**: primary human trophoblast cells; **P-gp**: P-glycoprotein.

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Abstract

Entecavir (ETV), a nucleoside analogue with high efficacy against hepatitis B virus, is recommended as a first-line antiviral drug for the treatment of chronic hepatitis B. However, scant information is available on the use of ETV in pregnancy. To better understand the safety of ETV in pregnant women, we aimed to demonstrate whether ETV could permeate placental barrier and the underlying mechanism. Our study showed that small amount of ETV could permeate across placenta in mice. ETV accumulation in activated or non-activated BeWo cells (treated with or without forskolin) was sharply reduced in the presence of 100 μM of adenosine, cytidine and in Na^+ free medium, indicating that nucleoside transporters possibly mediate the uptake of ETV. Furthermore, ETV was proved to be a substrate of concentrative nucleoside transporter (CNT) 2 and CNT3, of organic cation transporter (OCT) 3 and of breast cancer resistance protein (BCRP) using transfected cells expressing respective transporters. The inhibition of ETV uptake in primary human trophoblast cells further confirmed that equilibrative nucleoside transporter (ENT) 1/2, CNT2/3, OCT3 and organic cation/carnitine transporter (OCTN) 2 might be involved in ETV transfer in human placenta. Therefore, ETV uptake from maternal circulation to trophoblast cells was possibly transported by CNT2/3, ENT1/2 and OCTN2, while ETV efflux from trophoblast cells to fetal circulation was mediated by OCT3, and from trophoblast cells to maternal circulation might be mediated by BCRP, multidrug resistance-associated protein 2 and P-glycoprotein. The information obtained in the present study may provide a basis for ETV's use in pregnancy.

Introduction

Worldwide, an estimated 240 million people are chronically infected with hepatitis B, which accounts for around 45% of cases of hepatocellular carcinoma and 30% of cirrhosis (Papatheodoridis et al., 2015). Perinatal transmission is the major route of hepatitis B virus (HBV) transmission. To reduce the risk of mother-to-infant transmission, American Association for the Study of Liver Diseases recommends the pregnant women with an HBV DNA level > 200,000 IU/ml to be treated with nucleoside analogues (NAs) (Terrault et al., 2016). Currently, tenofovir, entecavir, emtricitabine, telbivudine, lamivudine and adefovir have been applied to hepatitis B treatment; however, none of them are approved for use in pregnancy. (Lok et al., 2016). Women of reproductive age with hepatitis B infection or women already on therapy in pregnancy have to face the drug safety issue on the fetus (Giles et al., 2011). Lamivudine is the most used antivirals but with high risk of viral resistance, while tenofovir and telbivudine, classified as pregnancy category B drugs, were also reported with nephron-toxicity and rhabdomyolysis (Woodward et al., 2009; Zou et al., 2011; Hermans et al., 2016). It was reported that tenofovir would lower bone mineral content in infants if it was applied in late pregnancy (Siberry et al., 2015). Therefore, it is necessary to explore other potential antivirals to treat HBV infection in pregnancy.

Entecavir (ETV) is a synthetic deoxyguanosine analog, approved in 2005 by US Food and Drug Administration, and has been the first-line antiviral agent in general population based on the high potency against HBV and significant barrier to drug resistance (Hosaka et al., 2013). However, the safety of ETV in pregnancy has not reached consensus. To clarify whether ETV could be applied in pregnancy, it is essential to investigate whether ETV will cross the placental barrier which interferes with drug delivery to fetus (Prouillac and Lecoeur, 2010). ETV is a hydrophilic compound with a pKa value of

10.5, which implies that it is unlikely to cross the placenta by passive diffusion. However, various drug transporters, including solute carrier (SLC) transporters and ATP-binding cassette (ABC) transporters located in the single layer of polarized syncytiotrophoblasts in placenta, may mediate the ETV permeation across the placental barrier. Organic cation transporter (OCT) 3 and organic anion transporter (OAT) 4 are the highest OCTs and OATs in placenta, respectively. OCT3 and OAT4 are reported in basal side of the trophoblasts, which contribute to the transport of substrate drugs from fetus to placenta (Ugele et al., 2003; Lee et al., 2013a). Organic cation/carnitine transporter (OCTN) 1/2 are also expressed in human placenta, which may mediate the uptake of drugs from maternal blood into trophoblasts (Lahjouji et al., 2004; Grube et al., 2005). P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) 2 and breast cancer resistance protein (BCRP) are expressed in maternal side of the trophoblast layer and mediate drugs from fetal to maternal circulation (Pollex and Hutson, 2011; Berveiller et al., 2015). Previous researches on ETV renal clearance *in vivo* indicated that OAT1/3, OCT2 and peptide transporter (PEPT) 2 might be involved in the renal secretion and reabsorption of ETV (Chen et al., 2011; Xu et al., 2014). However, scant information is available with respect to the transporters involved in the placental transfer of ETV.

The aim of this study was to explore whether ETV could cross the human placenta and the underlying mechanism based on the role of drug transporters. Firstly, we studied placental permeation of ETV in pregnant mice to evaluate the fetal exposure of ETV. Secondly, we clarified which transporters were involved in placental transfer of ETV in forskolin treated or untreated BeWo cells (a human choriocarcinoma cell line), cell models high expressing the specific transporters, and primary human trophoblast cells. The results will provide the useful information to elucidate whether ETV could be applied in the pregnant women.

Materials and methods

Materials

Fetal bovine serum (FBS), trypsin, Kaighn's Modification of Ham's F-12 (F12K) medium and Dulbecco's modified Eagle medium (DMEM) were from GIBCO (Invitrogen Life Technologies, USA). MK-571, Ko143, rhodamine123 and 6-carboxyl fluorescein (6-CF) were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-carnitine, L-ergothioneine, adenosine, phlorizin, 5-hydroxytryptamine (5-HT) and forskolin were from Aladdin Co., Ltd (Shanghai, China). Entecavir, cytidine, guanosine and uridine were provided by Meilun biological Co., Ltd. (Dalian, China). Bicinchoninic acid (BCA) protein assay kit was from Beyotime Institute of Biotechnology (Beyotime, China). Acetonitrile was obtained from Tedia (Fairfield, TX, USA). All other chemicals were of analytical grade.

Blank vector (pEnter), hCNT2 (SLC28A2) expression plasmid, hCNT3 (SLC28A3) expression plasmid and hOAT4 (SLC22A11) expression plasmid was obtained from ViGene Biosciences Inc. (Vigene, Shandong, China.)

Animals and the placental transfer of ETV in pregnant mice

Adult (8 – 10 week of age) specific pathogen free wild-type ICR mice were obtained from the Experimental Animal Center of the Zhejiang Academy of Medical Sciences, and housed in the specific-pathogen-free facility in the Zhejiang University. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University Medical Center. To obtain pregnant mice, we performed timed mating and checked the female early the following morning. The date that a vaginal plug observed was assigned as gestational day 0.5 (E=0.5). After overnight fast at E19.5, pregnant mice were administered with ETV by gavage at the dosage of 0.833 μg/g. The maternal blood samples (0.25 mL) were collected from the orbital venous sinus to

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heparinized tubes at 0.25, 1.0 and 4.0 h post-dosing, and the fetal blood samples were collected immediately after fetuses were sacrificed. The plasma was collected immediately after centrifugation at 8000 g for 10 min and then stored at -80°C until analysis.

Cell culture and transfection

BeWo cells were kindly provided by Prof. Ximei Wu, Zhejiang University School of Medicine, and cultured in F12K medium supplemented with 15% FBS, and 1% penicillin/streptomycin in a humidified air / CO_2 incubator (5% v/v) at 37°C. The Madin–Darby canine kidney II (MDCK) cells and human embryonic kidney 293 (HEK293) cells were acquired from Peking Union Medical College (Beijing, China) and kindly provided by Prof. Feng Han, College of Pharmaceutical Sciences, Zhejiang University, respectively. MDCK and HEK293 cells were cultured in DMEM medium with 10% FBS and 1% penicillin/streptomycin.

The MDCK and HEK293 cells were seeded at appropriate density in 24-well plates. At the following day, MDCK cells were transiently transfected with hCNT2/3 (MDCK-hCNT2/3) using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. HEK293 cells were transiently transfected with hOAT4 (HEK293-hOAT4) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA).

MDCK cells stably transfected with full length hOCT3 cDNA (MDCK-hOCT3) and Lilly Laboratories Cell-Porcine Kidney 1 (LLC-PK1) cells stably transfected with full length hBCRP cDNA (LLC-PK1-hBCRP) were established or kept in our laboratory (Tian et al., 2013; Sun et al., 2014). MDCK-hOCT3 cells were cultured in DMEM with 10% FBS, while LLC-PK1-hBCRP cells in M199 with 6% FBS.

RNA isolation, cDNA synthesis and quantitative Real-Time polymerase chain

reaction assays

Total RNAs were isolated using RNA simple Total RNA Kit (Tiangen, China), and then cDNAs were synthesized using PrimeScript™ RT reagent Kit (Takara Bio, Japan), followed by Real-Time PCR procedure using SYBR® Premix Ex Taq™ II (Takara Bio, Japan). Relative mRNA levels of target genes were calculated using the ΔCT method in which the ratio of target to GAPDH genes was equal to $2^{-\Delta CT}$, ΔCT= CT (target gene) – CT (GAPDH). All the primer pairs were showed in Table 1.

Cellular accumulation assays

BeWo cells were plated in 24-well plates at a destiny of 2×10^5 cells/well overnight and cultured with or without 20 μM of forskolin for 48 h to obtain activated or non-activated cells prior to the accumulation experiment. The cellular accumulation of ETV was performed as the method previously reported with minor modifications (Li et al., 2016) . Briefly, the cells were pre-incubated with Hank's balanced salt solution (HBSS) with or without the inhibitors of influx or efflux transporters for 10 or 30 min at 37°C or 4°C. The accumulation was initiated by adding HBSS containing ETV in the absence or presence of the transporter inhibitors, and terminated by removing the incubation buffer and adding ice-cold phosphate buffered saline at the designated times. Then cells were washed with ice-cold phosphate buffered saline for three times and lysed with 100 μL of 0.1% sodium dodecyl sulfate. The accumulation assay in MDCK-hOCT3 was similar to that in BeWo cells.

The accumulation of rhodamine 123 (5 μM, 1h) was also performed in non-activated BeWo cells similar to ETV uptake in BeWo cells. Then cells were lysed with 100 μL of 0.2 M sodium hydroxide and analyzed by microplate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 485 and 535 nm,

respectively. 6-CF (20 μM, 5 min) accumulation assay was performed in HEK-hOAT4 cells and then 6-CF in cell lysate was quantified by microplate reader with excitation and emission wavelengths of 490 and 525 nm, respectively.

To clarify whether ETV is a substrate of hCNT2/3 or hOAT4, the cellular uptake study of ETV was performed as the method for BeWo cells, except the buffer was replaced by upB (NaCl, 125 mM; D-glucose, 5.6 mM; KCl, 4.8 mM; $MgSO_4 \cdot 7H_2O$, 1.2 mM; KH_2PO_4, 1.0 mM; $CaCl_2$, 1.2 mM; HEPES, 25 mM; pH = 7.4) for hCNT2/3, or MES (NaCl, 140 mM; D-glucose, 5.6 mM; KCl, 5.4 mM; $MgSO_4 \cdot 7H_2O$, 0.8 mM; KH_2PO_4, 0.4 mM; $CaCl_2$, 1.3 mM; $NaHCO_3$, 4.2 mM; $Na_2HPO_4 \cdot 12H_2O$, 0.2 mM; MES, 10 mM; pH 6.0) for hOAT4, respectively. The cellular accumulation in the absence or presence of inhibitors was performed at 42 h after transfection. The cells transiently transfected with blank vector was used as the control.

ETV accumulation in primary human trophoblast cells (PHTCs)

Placental cytotrophoblasts cells were isolated from human uncomplicated placentae delivered at term (38 - 40 weeks) as the method reported previously with minor modifications (Wang et al., 2012). Briefly, villous from the maternal surface was cut away from vessels. The tissue was minced and transferred to 200 mL DMEM contained 25 mM glucose (DMEM-H-G) containing 0.07% trypsin (Gibico) and 0.2 mg/ml DNase I (Sigma; ≥ 400 Kunitz units/mg protein). The mixture was incubated in a shaking water bath for digestion for four times successively with 30, 40, 15 and 15 min each, and the third and fourth digestion were used to collect pellets after centrifuging at 1340 g for 10 min. The pellets were re-suspended in 10 ml DMEM-H-G, which was layered over a 5% to 65% Percoll gradient solution at step increments of 5%, and centrifuged at 1340 g for 20 min. After centrifugation, the middle layer containing cytotrophoblasts were removed to another tube and washed once with DMEM-H-G, and re-suspended subsequently in

medium contained 10% FBS and 1% penicillin/streptomycin. Finally, the cytotrophoblasts were seeded in 12 well plate with the density of 1.5×10^6.

The syncytiotrophoblasts were spontaneously syncytialized from cytotrophoblasts after 72 h cultivation, and then the accumulation assay was performed as the method for BeWo cells.

Quantification of ETV by LC-MS/MS

The quantification of ETV in cell lysates was determined by an Agilent 1290-6460 LC-MS with a triple quadrupole mass spectrometer (Agilent, CA, USA) as the method established in our lab (Yang et al., 2016).

The concentration of ETV in plasma was determined as the method above with a minor modification. Briefly, 600 μl acetonitrile containing 10 nM of phenacetin (internal standard) were added to 100 μl of the plasma for protein precipitation. After vortexing for 5 min, the mixture was centrifuged at 16000 g for 15 min, and 600 μL of the supernatant were transferred to another tube and then evaporated to dryness at 38◦C in a vacuum concentrator system (Labconco, Kansas, MO, USA). The residue was reconstituted in 100 μL of acetonitrile / water (9:1, v/v) and the aliquot of 10 μL was injected into LC-MS/MS system. The chromatographic separation was performed on a X-Bridge™ BEH HILIC column (2.5 μm, 2.1×50 mm) at 30°C with a gradient elution (0-1.6 min, 95% of B; 1.6-2.0 min, 95-60%; 2.0-4.0 min, 60% of B) at 0.25 ml/min, where mobile phase A and B were 0.1% formic acid in 10 mM ammonium formate-water and 0.1% formic acid in acetonitrile, respectively. Mass spectrometric analysis was performed using an ESI source in positive ion mode. Quantification was obtained using multiple reaction monitoring mode at m/z transitions of 278 > 152 for ETV and 180 > 110 for phenacetin. Fragmentor voltage was set at 110 V for both ETV and phenacetin, and collision energy was 16 V and 20 V for ETV and phenacetin, respectively. The lower limit of

quantitation was 1 nM. The method was validated according to Food and Drug Administration guidelines and satisfied specificity, precision, recovery, matrix effect and accuracy was demonstrated.

Statistical analysis

The data were presented as mean ± standard deviation (SD) in triplicate of at least two independent experiments. One-way analysis of variance (ANOVA) followed by Dunnett significant difference test and unpaired Student's t-test were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). A value of $P < 0.05$ was considered to be significant.

Results

The placental transfer of ETV in pregnant mice

To study whether ETV could cross the mouse placenta, the concentrations of ETV in the maternal and fetal plasma were determined. According to the pharmacokinetic characteristics in human, the plasma concentration of ETV reached maximum at 0.5-1.5 h after oral administration of 1 mg ETV /individual (Scott and Keating, 2009). The plasma concentration was determined at 0.25, 1.0 and 4.0 h post-dosing with ETV at 0.833 μg/g mouse body weight. As shown in Fig. 1, the mean concentrations of ETV were 87.7, 96.0 and 10.7 ng/ml in maternal plasma, and 2.1, 17.4 and 6.8 ng/ml in fetal plasma, respectively. The mean fetal to maternal concentration ratio of ETV was 2.9 %, 25.6% and 72.9%, respectively.

ETV accumulation in BeWo cells

BeWo cells were treated with or without 20 μM of forskolin for 48 h prior to being applied to mimic the human placenta transfer of ETV. The mRNA expressions of relevant transporters in BeWo cells treated with or without 20 μM of forskolin were depicted in Fig. 2A. ETV accumulations at 37°C in BeWo cells treated with or without forskolin were significantly higher than that at 4°C, indicating transporters mediated the transfer of ETV into BeWo cells. ETV accumulation in non-activated BeWo cells displayed a time-dependent increase within 60 min, but decreased after 60 min at 37°C. ETV accumulation in activated BeWo cells was higher than that in non-activated cells (Fig. 2B). In addition, the accumulation (2 min) was increased within the concentration from 5 to 2000 μM. ETV uptake in activated BeWo cells was higher than that in non-activated cells within 20 μM but not at higher concentrations (Fig. 2C).

Roles of uptake and efflux transporters in ETV accumulation in BeWo cells

Since ETV was reported to be a substrate of hPEPT2 (Xu et al., 2014) and mRNA

expression of PEPT2 is detected in human placenta (Berveiller et al., 2015), we compared ETV accumulation in activated or non-activated BeWo cells in the presence or absence of carnosine (a substrate and inhibitor of PEPT2). Our data showed that carnosine (5 mM) significantly reduced the ETV accumulation in non-activated ($P < 0.05$) but not in activated BeWo cells. Additionally, activated or non-activated BeWo cells showed a high expression of OCTN2, and 100 μM of carnitine (a substrate and inhibitor of OCTN2) markedly reduced ETV accumulation in non-activated ($P < 0.05$) but not in activated BeWo cells. Additionally, salicylic acid (an inhibitor of monocarboxylate transporter, MCT) did not depress ETV accumulation (Fig. 3A).

Placenta also expresses the efflux transporters, such as P-gp, MRP2 and BCRP. Our results showed verapamil (100 μM) or GF120918 (10 μM), MK571 (50 μM) and Ko143 (5 μM), the inhibitors of P-gp, MRP2 and BCRP, respectively, increased ETV accumulation in BeWo cells (Fig. 3B), which suggests that P-gp, MRP2 and BCRP might contribute to the ETV efflux from the cells. Although mRNA expression of P-gp in BeWo cells was fairly low, the accumulation of rhodamin 123 (a substrate of P-gp) was significantly inhibited by verapamil and GF120918, indicating that P-gp is functionally expressed in BeWo cells (Fig. 3C). In addition, ETV accumulation (5 μM, 1h) in LLC-PK1-hBCRP cells was 63% of that in mock cells, and GF120918 (10 μM) markedly increased ETV accumulation in LLC-PK1-hBCRP cells further confirmed that ETV was a substrate of BCRP ($P < 0.01$) (Fig. 3D).

Nucleoside transporters played roles in the uptake of ETV in BeWo cells

The inhibitors of PEPT2 and OCTN2 even at high concentration did not reduce ETV accumulation in activated BeWo cells, and slightly depressed the ETV accumulation in

non-activated BeWo cells, indicating other transporters must be involved in ETV uptake in BeWo cells. Based on the structure of ETV and expression of nucleoside transporters (NTs) in BeWo cells (Fig. 2A), we speculated that the NTs might play significant roles in the uptake of ETV in BeWo cells. To test our speculation, the effects of Na^+ and S-(4-Nitrobenzyl)-6-thioinosine (NBTI) (an ENT-specific inhibitor) on ETV accumulation in BeWo cells were studied. Our data demonstrated that NBTI markedly reduced ETV accumulation in non-activated BeWo cells at 100 μM (a concentration at which ENT1 and ENT2 were inhibited completely), but not at 1 μM (a concentration at which ENT1 but not ENT2 could be inhibited). The inhibition of NBTI (100 μM) was aborted in activated BeWo cells (Fig. 4A), which was consistent with decreased mRNA expression of ENT2 (Fig. 2A). Moreover, 100 μM of adenosine and cytidine (inhibitors of NTs) and 200 μM of phlorizin (a CNT2/3 inhibitor) strongly inhibited the ETV accumulation both in activated and non-activated BeWo cells. In addition, Na^+ free buffer reduced ETV accumulation to 66% and 25% of the control, respectively, in activated and non-activated BeWo cells (Fig. 4A). The results above strongly indicate that CNTs, sodium-dependent transporters, play key roles in ETV uptake in BeWo cells. The accumulation of other antivirals like adefovir, emtricitabine and tenofovir was much lower than that of ETV (data not shown). The NBTI inhibited the uptake of emtricitabine, and the accumulation of adefovir was slightly reduced in Na^+ free buffer, whereas neither NBTI nor Na^+ free buffer affected the accumulation of tenofovir in non-activated BeWo cells (Fig. 4B).

ETV was confirmed to be a substrate of hCNT2/3

To further confirm the contribution of hCNT2/3 to ETV transport, ETV uptake was performed in MDCK cells transiently transfected hCNT2/3. MDCK-hCNT2/3 cells were evaluated by functional activity with the accumulation of guanosine (a probe substrate of

hCNT2/3) and mRNA expression level (Fig. 5A and Fig. 5B). The accumulation of ETV (10 μM, 5 min) in MDCK-hCNT2 and MDCK-hCNT3 was 4 and 34 folds of that in mock cells, which could be significantly inhibited by 200 μM of phlorizin (Fig. 5C and Fig. 5D). ETV accumulation in MDCK-hCNT3 followed typical dynamics, and the *Km* and *Vmax* values were 23.1 μM and 0.3 nmol/mg protein/min, respectively (Fig. 5F). In contrast, the kinetics of ETV uptake in MDCK-hCNT2 cells displayed atypical dynamics under Eadie-Hofstee analysis. At low concentration (5 - 80 μM), the *Km* and *Vmax* values were 53.2 μM and 0.034 nmol/mg protein/min, respectively; while at high concentration (200 - 2000 μM), the *Km* and *Vmax* values were 1083 μM and 0.215 nmol/mg protein/min, respectively (Fig. 5E).

hOCT3 contributed to the transport of ETV while hOAT4 not

ETV was reported to be a substrate of OAT1 and OAT3 (Xu et al., 2013). Considering the overlap of substrates for OATs and the high expression of OAT4 in placenta, we speculated that OAT4 might be involved in the transport of ETV in human placenta. However, our results revealed the accumulation of ETV in HEK293-hOAT4 cells was not different with that in mock cells, although ETV (100 μM) inhibited the accumulation of 6-CF (20 μM, 5 min), a typical substrate of OAT, in HEK293-hOAT4 cells ($P < 0.01$) (Fig. 6B).

Because hOCT3 is high expressed in human placenta, but it is rarely expressed in BeWo cells, we applied MDCK-hOCT3 cells to study whether hOCT3 would mediate ETV transport. ETV (100 μM) and D22 (4 μM, an inhibitor of OCT3) significantly ($P < 0.001$) inhibited the accumulation of 5-HT (a substrate of OCT3). ETV accumulation in MDCK-hOCT3 cells was 1.7 folds of that in mock cells (Fig. 6D), which could also be reduced by D22. The above results indicate that ETV is a substrate of OCT3, which imply that OCT3 is likely to mediate ETV transport in placenta.

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Inhibition of ETV uptake into PHTCs

To further confirm and predict whether ETV could transport across human placenta, ETV accumulation in PHTCs was studied with or without respective inhibitors of transporters. The cellular accumulation of ETV (20 μM) was significantly reduced in the presence of inhibitors of ENT1 (1 μM of NBTI) ($P < 0.05$), of ENT2 (100 μM of NBTI) ($P < 0.001$), of CNT2/3 (200 μM of phlorizin) ($P < 0.001$), of OCTN2 (100 μM of L-carnitine) ($P < 0.01$), of OCT3 (10 μM D22) ($P < 0.001$) and in the Na^+ free buffer ($P < 0.01$), but not in the presence of 10 μM of L-ergothioneine (an inhibitor of OCTN1) (Fig. 7A). Unexpectedly, the inhibitors of P-gp (verapamil, 100 μM of or GF120918, 10 μM), MRP2 (MK571, 50 μM), and BCRP (Ko143, 5 μM) did not obviously increase ETV accumulation in PHTCs (Fig. 7B).

Discussion

This study investigated the trans-placental passage of ETV and the underlying mechanism, and found that only small amount of ETV crossed mouse placenta, indicating fetal exposure of ETV in human might be very low. The study further reveals that the SLC transporters, including CNT2/3, ENT1/2, OCTN2 and OCT3, and the ABC transporters, including P-gp, BCRP and MRP2 might contribute to ETV transport across human placenta.

Tenofovir and lamivudine, for treatment of HBV as well as immunodeficiency virus infection, could permeate human placenta in immunodeficiency virus-infected women with the median ratio of cord to maternal blood 60-70% and almost 100%, respectively (Mandelbrot et al., 2001; Hirt et al., 2009a; Mirochnick et al., 2014). Our study showed the ratio of fetal to maternal plasma concentration of ETV was 2.9%, 25.6% and 72.9% at 0.25,1.0 and 4.0 h after dosing, respectively (Fig. 1). However, the absolute concentration in fetal plasma was lower than 22.7 ng/ml. In consideration of the low therapeutic dose of ETV (0.5 mg/day) and similar overall structures between mice and human (Watson and Cross, 2005), we deduced that the fetal exposure of ETV in human is very low.

The cellular accumulation of ETV in BeWo cells at 37°C was extremely higher than that at 4°C within 60 min (Fig. 2B and 2C), which strongly suggests that transporters are involved in the ETV uptake. ETV accumulation was increased at concentrations up to 2000 μM but without saturation. We speculated some transporters like ENT1/2 mediated ETV uptake characterized by atypical dynamics similar to ETV accumulation in MDCK-hCNT2 (Fig. 5E). Interestingly, ETV accumulation in non-activated BeWo cells was decreased after 60 min at 37°C (Fig. 2B), thus we deduce that some efflux

transporters probably play roles in ETV transport. Subsequently, we confirmed P-gp, MRP2 and BCRP mediated ETV efflux in BeWo cells by comparing its accumulation in the presence and absence of the respective inhibitors (Fig. 3B). Moreover, ETV was proved to be a substrate of BCRP in present study and a substrate of P-gp and MRP2 in our lab (Yang et al., 2016). Together with the high expressions of P-gp, BCRP and MRP2 in human placenta and their location in apical membrane, we deduce that they may contribute to ETV transport from trophoblast cells to maternal blood. The efflux transporters, P-gp, BCRP and MRP2, were expressed in PHTCs (Fig. 7D). Unexpectedly, the inhibitors of P-gp, BCRP and MRP2 did not increase the ETV accumulation in PHTCs (Fig. 7B), which were not consistent with the results in BeWo cells (Fig. 3B). The exact reasons for the inconsistent results in BeWo cells and PHTCs were not clearly clarified. We speculated that the roles of efflux transporters might be eclipsed because of the co-existed high-affinity influx transporters which mediated ETV into PHTCs.

BeWo cells consists of most cytotrophoblasts and few syncytialized cells under non-activated condition, and could be induced by forskolin and differentiated to syncytiotrophoblasts. In terms of fetal-maternal barrier, it is mainly the syncytialized trophoblasts. Therefore, the activated BeWo cells are more appropriate to be used as an *in vitro* model to mimic drug placental transfer. ETV (5 μM) accumulation in non-activated BeWo was higher than that in activated BeWo (Fig. 2B), however this difference was not obvious at higher concentrations (Fig. 2C), which might be attributed to the saturation of some transporters with high affinity like CNT3. The result of ETV accumulation in the non-activated BeWo cells indicates ENT2 and CNT3 contribute to ETV uptake based on the mRNA expressions of NTs. The mRNA expressions of ENT2 and CNT3 were decreased in activated BeWo cells (Fig. 1A), corresponding to the lower inhibition of NBTI (100 μM) and phlorizin in the ETV accumulation than that in non-activated

condition (Fig. 4A). The inhibitions of carnosine and carnitine in ETV accumulation were aborted in activated BeWo cells (Fig. 3A), which might be attributed to the down-regulation of PEPT2 and reduced affinity of OCNT2 due to protein phosphorylation (Huang et al., 2009). Our study confirmed that mRNA expression of PEPT2 in cytotrophoblasts is higher than that in syncytiotrophoblasts (Berveiller et al., 2015). The results in PHTCs showed that ENT1 also be involved in the placental transfer (Fig. 7A), which might be attributed to the higher expression of ENT1 in PHTCs (Fig. 7C). ENT1 is definitely expressed in placenta and located in brush-border membrane (Barros et al., 1995), which suggests ENT1 facilitates the transport of ETV across the membrane. ENT2 is also identified in chorionic villi region but its location is not clearly clarified (Govindarajan et al., 2007).

ETV was proved to be a substrate of hCNT2 and hCNT3 (Fig. 5C and Fig. 5D), which was accorded with the recent research showing that CNT2/3 might be involved in the renal reabsorption of ETV (Trejtnar et al., 2016). Together with the results in PHTCs, we demonstrated that CNT2 and / or CNT3 could contribute to trans-placental passage of ETV. Previous researches (Lu et al., 2004; Yamamoto et al., 2007) and our results (data not shown) demonstrated the mRNA expressions of CNT1/2/3 in human and mouse placenta. However, protein expression of CNTs in human placenta is far less well documented and a consensus has not been reached. Govindarajan et al. reported that no appreciable hCNT1 protein staining is detected in trophoblast layer by in situ bybridization (Govindarajan et al., 2007), whereas Errasti-Murugarren et al. identified that CNT1 is the only CNT-type protein functionally expressed in human syncytiotrophoblast (Errasti-Murugarren et al., 2011). However, our results showed the uptake of ETV, a proved substrate of CNT2/3, could be markedly inhibited by phlorizin in PHTCs (Fig. 7A), which provide the evidence that CNT2 and/ or CNT3 are expressed

in human placenta. The above discrepancy might be attributed to individual variation and small sample size used in studies.

Both human and mouse placenta extremely high express OCT3 but with low expressions of OCT1/2 (Kekuda et al., 1998; Lee et al., 2013a), and OCT3 was reported to be located at the basal membrane of human trophoblasts (Sata et al., 2005). Our study revealed that ETV is a substrate of hOCT3 (Fig. 6D). Furthermore, D22 obviously reduced ETV accumulation in PHTCs (Fig. 7A). Therefore, we concluded that OCT3 contributes to the ETV transfer from fetal to maternal blood. OCTN1/2 are also expressed in human placenta and ETV is a substrate of OCTN1/2 (Yang et al., 2016). The results in PHTCs indicate that OCTN2 but not OCTN1 contributes to the placental transfer of ETV, which might be ascribed to the higher protein expression of OCTN2 in placenta and higher affinity of ETV to OCTN2 (Yang et al., 2016). hOAT4 is the highest OATs expressed in placenta whereas hOAT1 and hOAT3 are minimally expressed or even absent (Ugele et al., 2003). Our study in transfected hOAT4 cells demonstrated that ETV was not a substrate of OAT4, thus OAT4 was unlikely to mediate ETV transport.

The accumulations of adefovir, emtricitbine and tenofovir in BeWo cells, was less than that of ETV (data not shown), however emtricitabine and tenofovir were reported with high permeation ratio in human placenta (Hirt et al., 2009a; Hirt et al., 2009b). It might be attributed to the scarce expressions of OATs and OCTs in BeWo cells, because all three drugs were reported to strongly interact with OATs or OCTs (Nakatani-Freshwater and Taft, 2008; Kohler et al., 2011; Maeda et al., 2014). Therefore, various cell models including BeWo cells, transporter transfected cells, PHTCs and *in vivo* studies should be comprehensively employed to draw the reasonable conclusion.

Even though ETV is classified as pregnancy category C drug, it is still used in pregnancy and no evidence shows that birth defect is associated with maternal use of

ETV during pregnancy. Our results indicate that ETV could cross human placenta but the fetal exposure of ETV in human is low. Therefore, ETV probably could be a candidate for HBV treatment to reduce perinatal transmission at delivery. Our study revealed that multiple transporters are involved in the ETV transport across placenta. The expressions of P-gp and OCT3 at term were reported to be lower than that at preterm (Sun et al., 2006; Lee et al., 2013b). Therefore, it is necessary to pay attention to the effect of gestation on placental transfer of ETV although antivirals are recommended to reduce perinatal transmission in the third term.

In summary, we showed a comprehensive study of the placental transfer of ETV and revealed that CNT2/3, ENT1/2 and OCTN2 contributed to the ETV uptake from maternal circulation to trophoblast cells, while OCT3 contributed the ETV efflux from trophoblast cells to fetal circulation, and BCRP, MRP2 and P-gp might be involved in the efflux of ETV from trophoblast cells to maternal circulation (Fig. 8). To further verify the extent of human placental transfer of ETV, *ex vivo* dually perfused human placenta model should be considered. The safety use of ETV in pregnancy to reduce intrauterine transmission ratio should be concluded from more data from clinical trials in pregnant women.

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Authorship Contributions

Participated in research design: Ma, Yang, T. Jiang, Sun, Zheng, Zeng and H. Jiang

Conducted experiments: Ma, Yang, T. Jiang and Bai

Performed data analysis: Ma, Yang, Sun, and Zheng

Wrote or contributed to the writing of the manuscript: Ma, Sun, H. Jiang

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Footnotes

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Legends for Figures

Figure 1 The concentration of ETV in fetal and maternal plasma at 0.25, 1.0 and 4.0 h post-dosing with 0.833 μg/g of ETV. Data are expressed as mean ± SD., n = 8.

Figure 2 The mRNA expressions of relevant transporters in BeWo cells treated with or without 20 μM of forskolin for 48 h (A). Compared with mRNA expression in BeWo cells treated without forskolin, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; The time-dependent accumulation of ETV (5 μM) in activated or non-activated BeWo cells at 4°C or 37°C (B); ETV accumulation in activated or non-activated BeWo cells at concentrations up to 2000 μM for 2 min at 4°C or 37°C and the comparison of ETV (5 or 20 μM) uptake in BeWo cell treated with or without forskolin (C). All data represent mean ± SD of two independent experiments in triplicate.

Figure 3 The effects of SLC (A) and ABC transporter inhibitors (B) on the ETV accumulation in BeWo cells. Carnosine, carnitine and salicylic acid were used as the inhibitors of PEPT2, OCTN2 and MCT, while verapamil or GF120918, MK571 and Ko143 as the inhibitors of P-gp, MRP2 and BCRP, respectively. Compared with non-activated BeWo cells treated with ETV group, *$P < 0.05$ and ***$P < 0.001$. Compared with activated BeWo cells treated with ETV group, ### $P < 0.001$. Compared with the control, △ $P < 0.05$, △△△ $P < 0.001$. The accumulation of rhodamine 123 (5 μM, 1 h) in non-activated BeWo cells (C). Compared with the control, * $P < 0.05$, ** $P < 0.01$. The accumulation of ETV (5 μM, 1 h) in LLC-PK1-hBCRP and LLC-PK1 (mock) cells (D). Compared with the mock cells, ***$P < 0.001$; compared with LLC-PK1-hBCRP treated with ETV group, ## $P < 0.01$. All data represent mean ± SD from three

independent experiments conducted in triplicate.

Figure 4 The effect of general inhibitors (100 μM of adenosine and cytidine) of NTs , specific inhibitors of ENT or CNT (NBTI and 200 μM of phlorizin) and Na^+ free medium (Na^+ was replaced by NMDG) on the accumulation of ETV (5 μM, 2 min) in BeWo cells treated with or without 20 μM of forskolin for 48 h (A). Compared with non-activated BeWo cells treated with ETV group, ***P < 0.001. Compared with activated BeWo cells treated with ETV group, #P < 0.05, ###P < 0.001. Compared with the control, △△ P < 0.01, △△△ P < 0.001. The effect of NBTI and Na^+ on the accumulation of adefovir, emtricitabine and tenofovir (20 μM, 2 min) in non-activated BeWo cells (B). Compared with control, *P < 0.05, ***P < 0.001. All data represent mean ± SD from three independent experiments conducted in triplicate.

Figure 5 The interaction of ETV with hCNT2/3. The MDCK cells transiently expressing hCNT2 or hCNT3 were verified by functional activity with the accumulation of guanosine (A) and mRNA expression level (B). Compared with mock cells, *** P < 0.001; compared with the accumulation without inhibitor, ### P < 0.001. The accumulation of ETV (10 μM, 5 min) in MDCK-hCNT2 (C) or MDCK-hCNT3 cells (D) was compared to that in mock cells. 200 μM of phlorizin was used as an inhibitor of hCNT2/3. Concentration-dependent profiles of ETV uptake in MDCK-hCNT2 (E) or MDCK-hCNT3 cells (F) and Eadie-Hofstee plot. All data represent mean ± SD from two independent experiments conducted in triplicate.

Figure 6 The accumulations of probe substrates in mock cells and HEK293-hOAT4 (A) or MDCK-hOCT3 cells (C) in the absence or presence of ETV or probenecid (OAT4 inhibitor) or D22 (OCT3 inhibitor). The accumulation of ETV in mock cells and

HEK293-hOAT4 (B) or MDCK-hOCT3 cells (D) with or without probenecid or D22. Data are represented by means ± SD. (n=3). Compared with the accumulation in mock cells, ***P<0.001; compared with the accumulation without inhibitor, ##P<0.01 and ###P<0.001. All data represent mean ± SD from three independent experiments conducted in triplicate.

Figure 7 The effects of SLC (A) and ABC (B) transporter inhibitors on the accumulation of ETV in PHTCs. Compared with the accumulation without inhibitors, *P<0.05 , **P<0.01 and ***P< 0.001. The mRNA expressions level of SLC (C) and ABC (D) transporters in PHTCs. Data represent mean ± SD from three independent experiments conducted in triplicate.

Figure 8 Schematic diagram of the placental transfer of ETV. CNT2/3, ENT1/2 and OCTN2 contributed to the ETV uptake from maternal circulation to trophoblast cells, while OCT3 contributed the ETV efflux from trophoblast cells to fetal circulation, and BCRP, MRP2 and P-gp might be involved in the efflux of ETV from trophoblast cells to maternal circulation.

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Table 1 Primers used in Real-time quantitative PCR

Gene	Direction	Sequence(5'→3')	Product length (bp)
hENT1	Forward	CAGAATGTGTCCTTGGTCACT	512
	Reverse	ATGATAACAGCACAGGCTGTG	
hENT2	Forward	CCTCCGTCTGCTTCATCAACT	470
	Reverse	CTGGAAGACAGTGAAGACTGA	
hCNT1	Forward	TGGAAGGTCTGGGACATGGAGAA	612
	Reverse	ATGATGCTTTGAGCAGGCAA	
hCNT2	Forward	AAGAAGTAGAGCCTGAGGGAA	386
	Reverse	AACCAAGGAGACTCCTGCAAA	
hCNT3	Forward	GAGAACGAGAACACATCAGGA	438
	Reverse	CCAGAACCAATGGCTGTTTAG	
hOCT3	Forward	TCGCTCTGTTCAGGTCTGTG	115
	Reverse	TGGATGCCAGGATACCAAAG	
hOCTN1	Forward	CGGAATATTGCCATAATGACC	72
	Reverse	CAGAGCAAAGTAACCCACTGAG	
hOCTN2	Forward	GCAGCATCCTGTCTCCCTAC	91
	Reverse	GCTGTCAGGATGGTCAGACTT	
hOAT4	Forward	CTGTGGAAAGTACCTCGCTCT	120
	Reverse	CTTGAAGTCGCCCAACTCG	
hBCRP	Forward	CCACTCCCACTGAGATTGAGA	73
	Reverse	TGCGTTCCTAAATCCTACCC	
hP-gp	Forward	GAAATTTAGAAGATCTGATGTCAAACA	110
	Reverse	ACTGTAATAATAGGCATACCTGGTCA	
hMRP2	Forward	AGTGAATGACATCTTCACGTTTG	63
	Reverse	CTTGCAAAGGAGATCAGCAA	
hGADPH	Forward	GCACCGTCAAGGCTGAGAAC	138
	Reverse	TGGTGAAGACGCCAGTGGA	

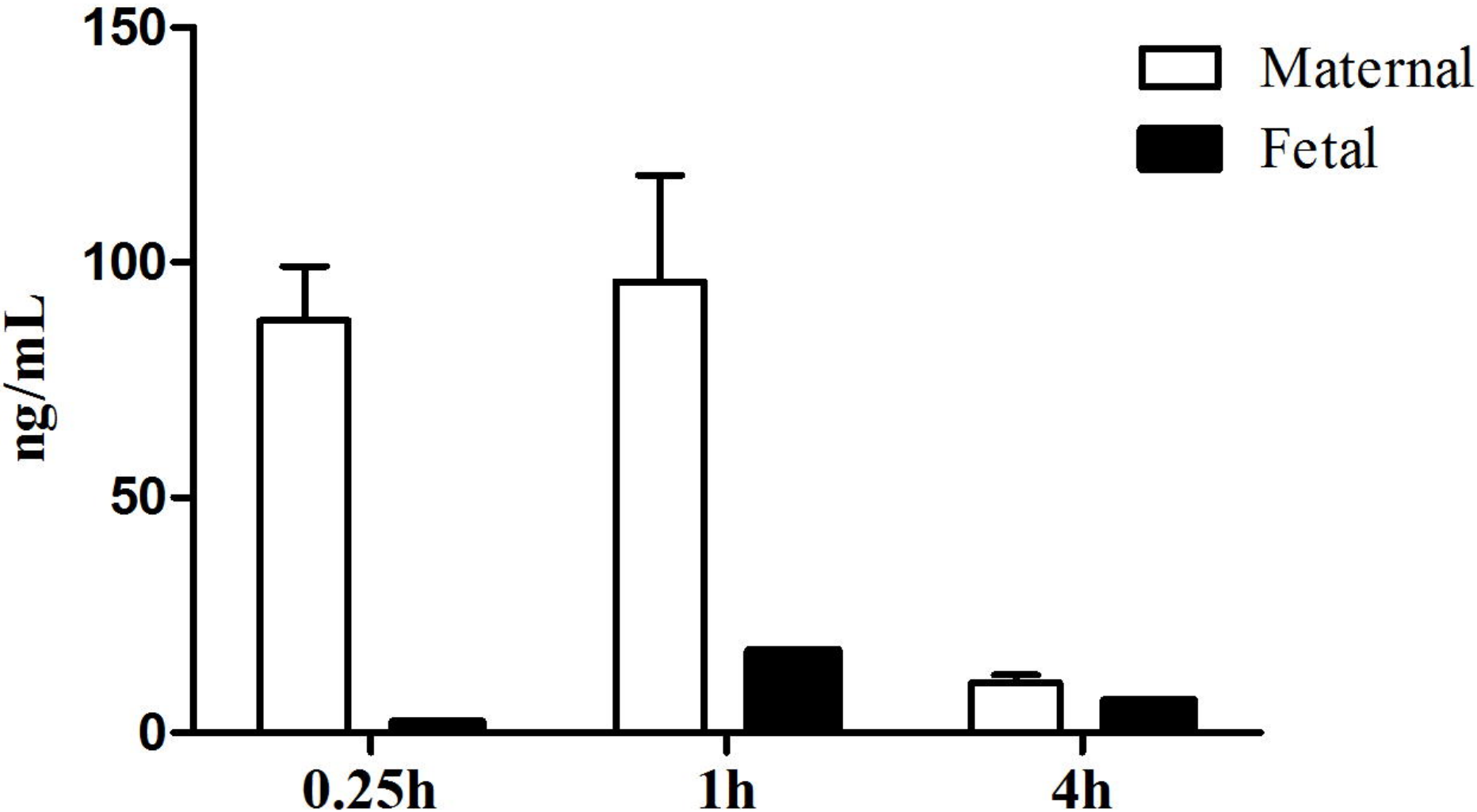
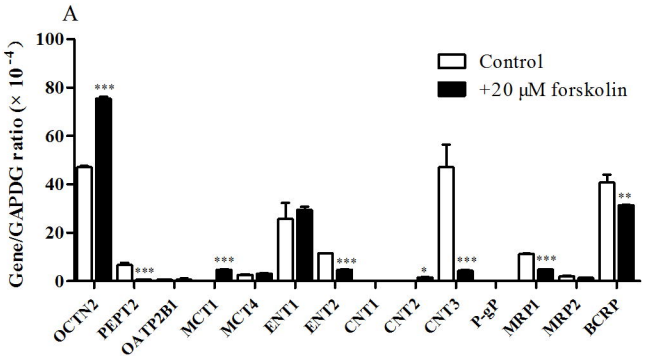
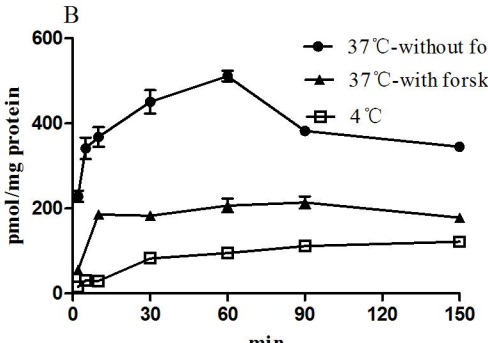
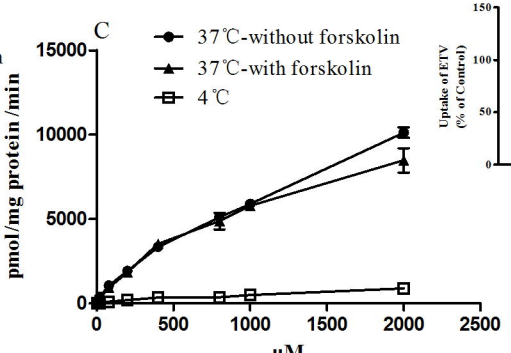


Figure 1


A
Gene/GAPDG ratio (× 10⁻⁴)
Control
+20 μM forskolin
OCTN2
PEPT2
OATP2B1
MCT1
MCT4
ENT1
ENT2
CNT1
CNT2
CNT3
P-gP
MRP1
MRP2
BCRP


B
pmol/mg protein
37℃-without forskolin
37℃-with forskolin
4℃
min


C
pmol/mg protein /min
37℃-without forskolin
37℃-with forskolin
4℃
μM

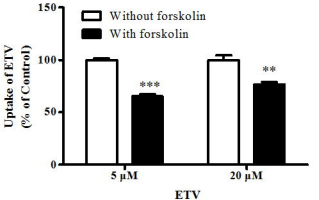

Without forskolin
With forskolin
Uptake of ETV (% of Control)
5 μM
20 μM
ETV

Figure 2

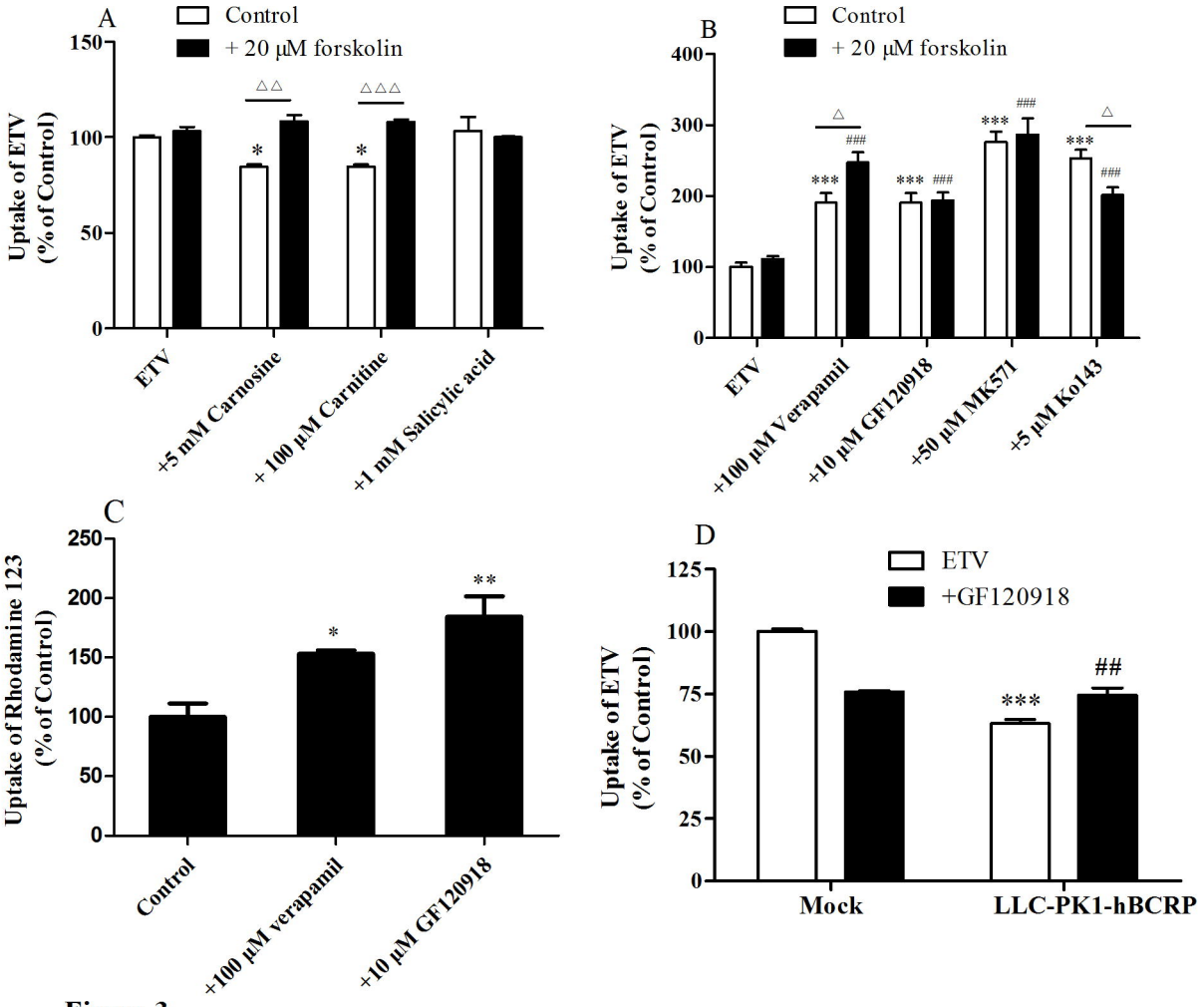
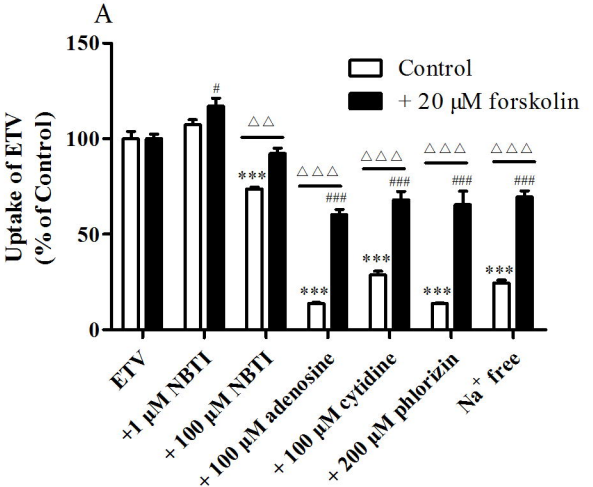

A
Control
+ 20 μM forskolin
Uptake of ETV
(% of Control)
150
100
50
0
△△
△△△
*
*
ETV
+5 mM Carnosine
+ 100 μM Carnitine
+1 mM Salicylic acid
B
Control
+ 20 μM forskolin
Uptake of ETV
(% of Control)
400
300
200
100
0
△
△
***
***
***
***
###
###
###
###
ETV
+100 μM Verapamil
+10 μM GF120918
+50 μM MK571
+5 μM Ko143
C
Uptake of Rhodamine 123
(% of Control)
250
200
150
100
50
0
*
**
Control
+100 μM verapamil
+10 μM GF120918
D
ETV
+GF120918
Uptake of ETV
(% of Control)
125
100
75
50
25
0
***
##
Mock
LLC-PK1-hBCRP

Figure 3


A
Uptake of ETV
(% of Control)
150
100
50
0
Control
+ 20 μM forskolin
ETV
+1 μM NBTI
+ 100 μM NBTI
+ 100 μM adenosine
+ 100 μM cytidine
+ 200 μM phlorizin
Na+ free

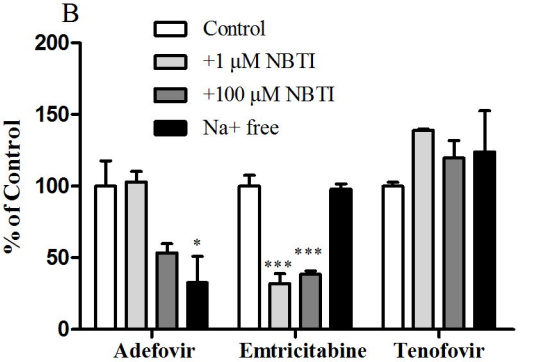

B
% of Control
200
150
100
50
0
Control
+1 μM NBTI
+100 μM NBTI
Na+ free
Adefovir
Emtricitabine
Tenofovir

Figure 4

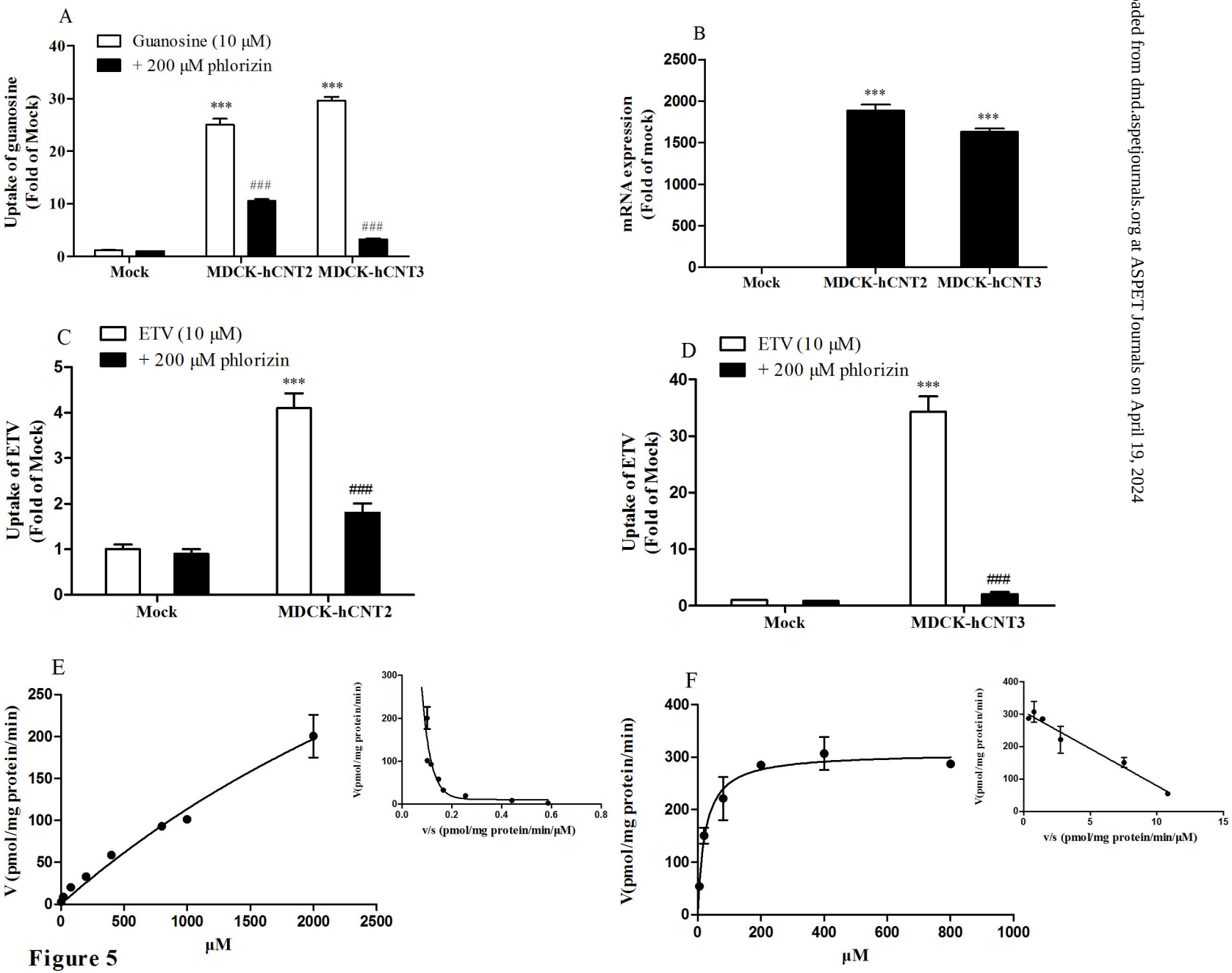


Figure 5

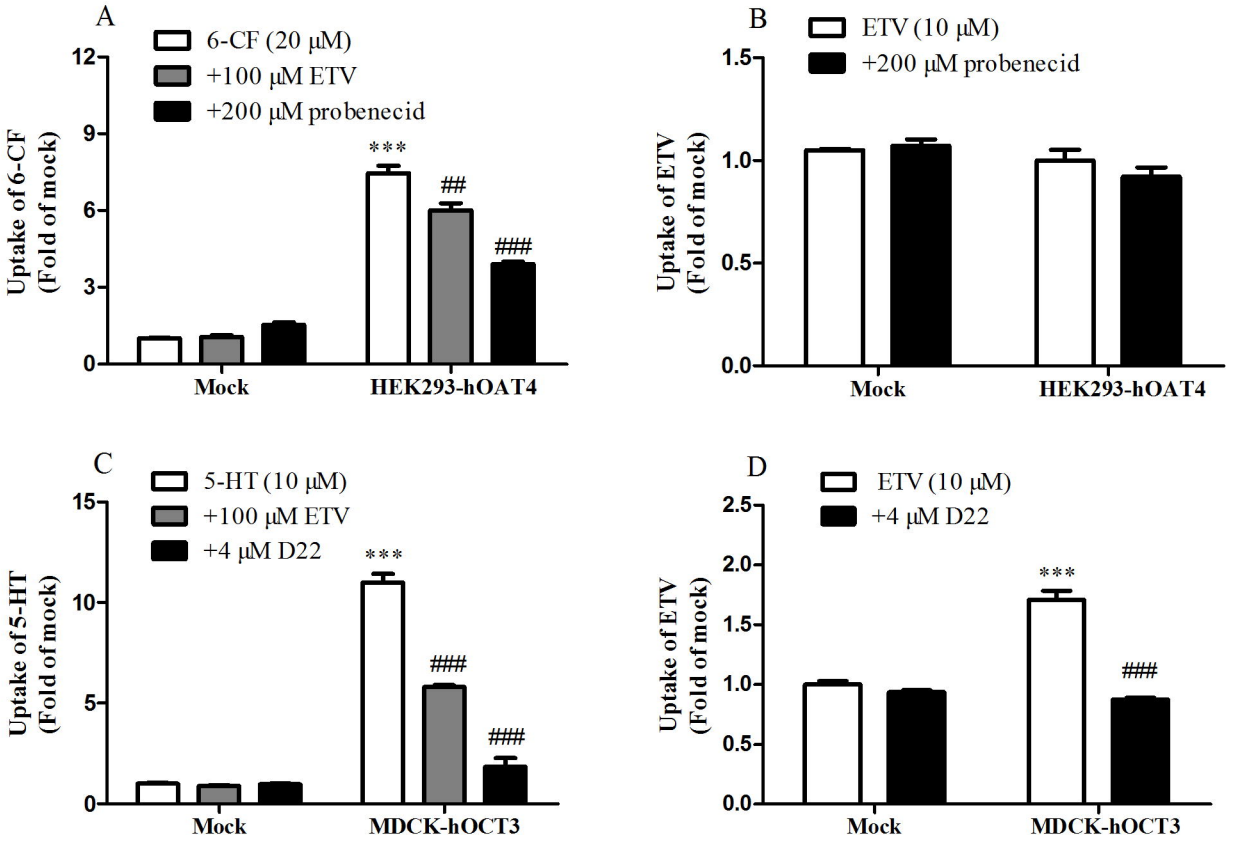

A
6-CF (20 μM)
+100 μM ETV
+200 μM probenecid
Uptake of 6-CF
(Fold of mock)
12
9
6
3
0
***
##
###
Mock
HEK293-hOAT4
B
ETV (10 μM)
+200 μM probenecid
Uptake of ETV
(Fold of mock)
1.5
1.0
0.5
0.0
Mock
HEK293-hOAT4
C
5-HT (10 μM)
+100 μM ETV
+4 μM D22
Uptake of 5-HT
(Fold of mock)
15
10
5
0
***
###
###
Mock
MDCK-hOCT3
D
ETV (10 μM)
+4 μM D22
Uptake of ETV
(Fold of mock)
2.5
2.0
1.5
1.0
0.5
0.0
***
###
Mock
MDCK-hOCT3

Figure 6

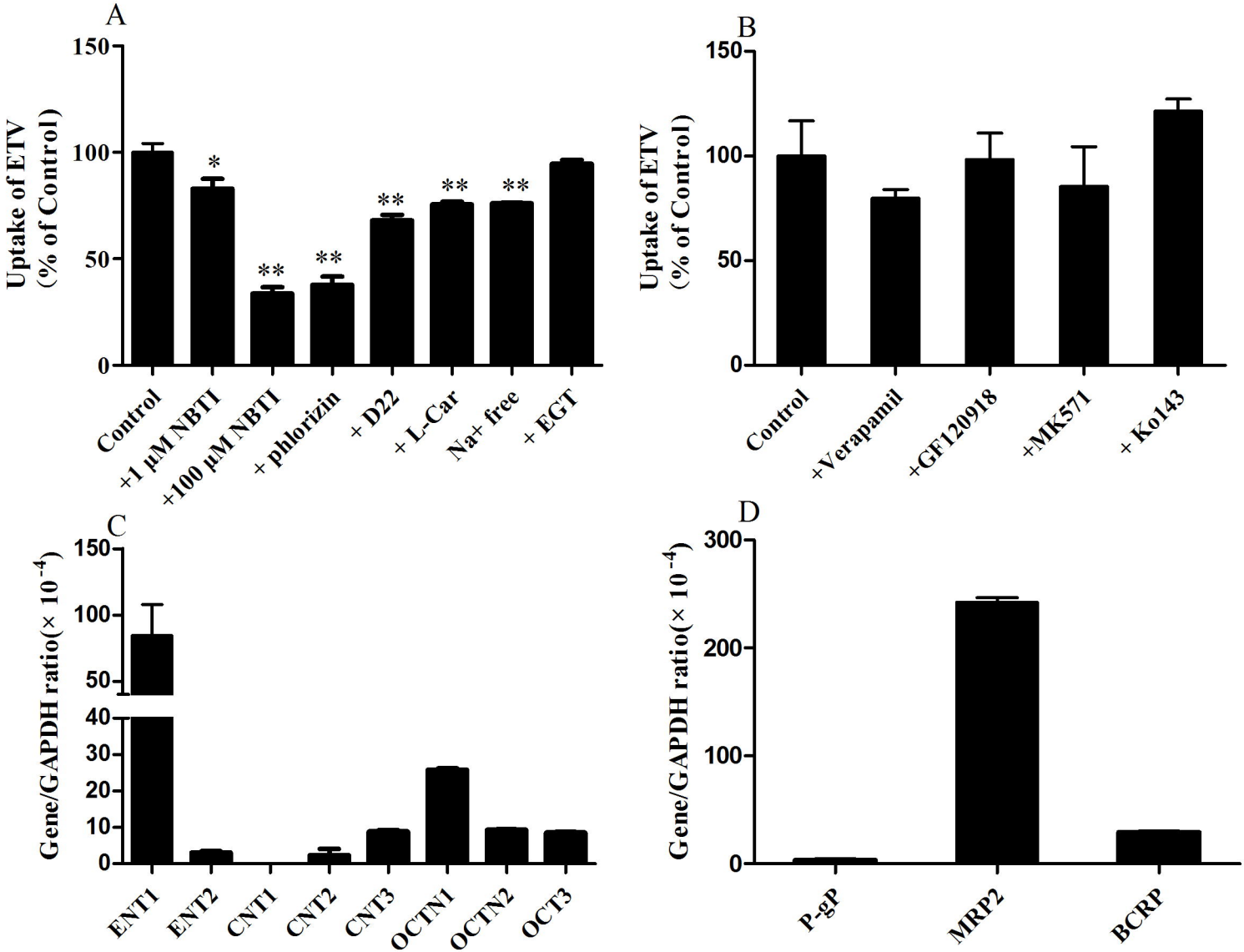


Figure 7

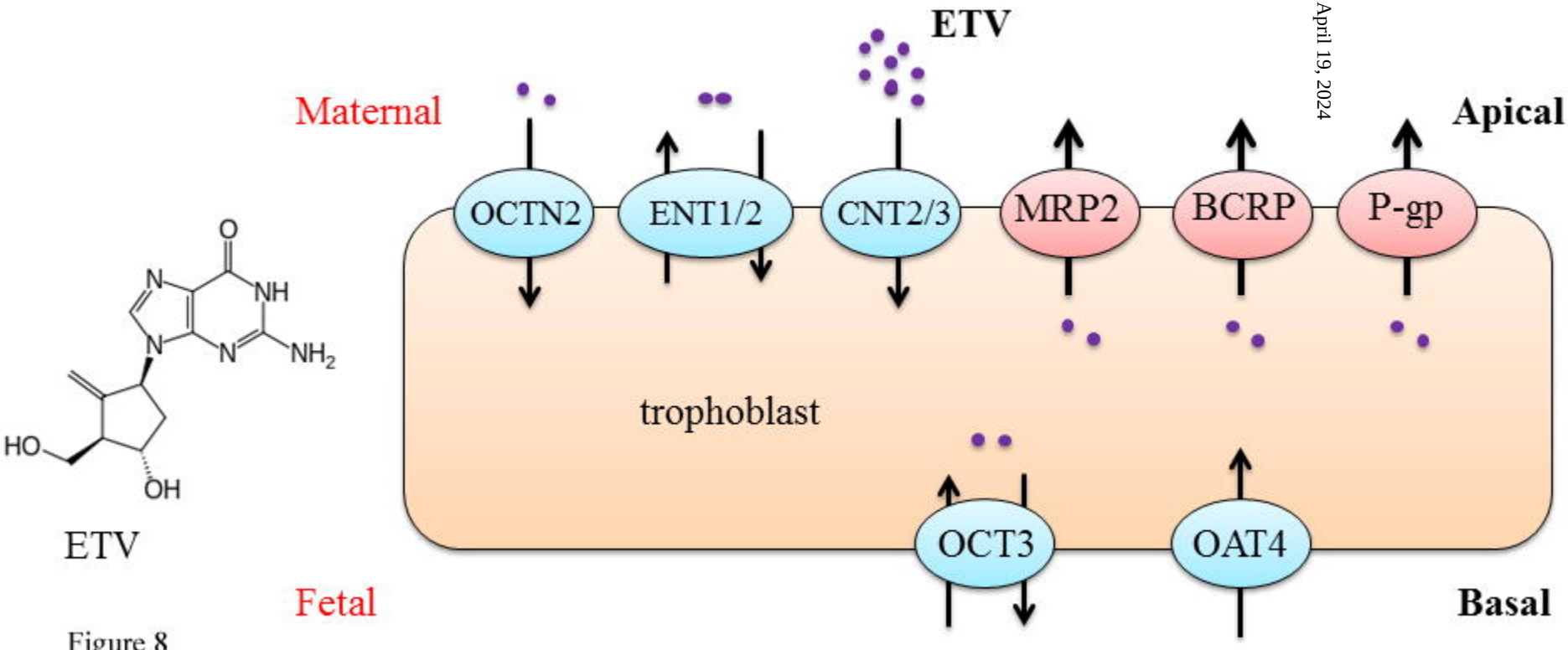


Figure 8