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Role of ABC and Solute Carrier Transporters in the Placental Transport of Lamivudine

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Lamivudine is one of the antiretroviral drugs of choice for the prevention of mother-to-child transmission (MTCT) in HIV-positive women. In this study, we investigated the relevance of drug efflux transporters P-glycoprotein (P-gp) (MDR1 [ABCB1]), BCRP (ABCG2), MRP2 (ABCC2), and MATE1 (SLC47A1) for the transmembrane transport and transplacental transfer of lamivudine. We employed *in vitro* accumulation and transport experiments on MDCK cells overexpressing drug efflux transporters, *in situ*-perfused rat term placenta, and vesicular uptake in microvillous plasma membrane (MVM) vesicles isolated from human term placenta. MATE1 significantly accelerated lamivudine transport in MATE1-expressing MDCK cells, whereas no transporter-driven efflux of lamivudine was observed in MDCK-MDR1, MDCK-MRP2, and MDCK-BCRP monolayers. MATE1-mediated efflux of lamivudine appeared to be a low-affinity process (apparent K_m of 4.21 mM and V_{max} of 5.18 nmol/mg protein/min in MDCK-MATE1 cells). Consistent with *in vitro* transport studies, the transplacental clearance of lamivudine was not affected by P-gp, BCRP, or MRP2. However, lamivudine transfer across dually perfused rat placenta and the uptake of lamivudine into human placental MVM vesicles revealed pH dependency, indicating possible involvement of MATE1 in the fetal-to-maternal efflux of the drug. To conclude, placental transport of lamivudine does not seem to be affected by P-gp, MRP2, or BCRP, but a pH-dependent mechanism mediates transport of lamivudine in the fetal-to-maternal direction. We suggest that MATE1 might be, at least partly, responsible for this transport.

More than 36 million people are infected with HIV worldwide today (1). Half of them are women who, if pregnant, carry the risk of transferring the infection to their child *in utero*, at delivery, or during breastfeeding. Progress in preventing new HIV infections among children has been dramatic in recent years as the number of children becoming infected with HIV each year dropped from 520,000 to less than 240,000 between 2000 and 2014 (1), clearly demonstrating that well-timed antiretroviral prophylaxis can reduce the risk of mother-to-child transmission (MTCT) of HIV. Current guidelines (2, 3) recommend lamivudine ([−]-β-L-2′,3′-dideoxy-3′-thiacytidine) as one of the antiretroviral drugs of choice in first-line therapy of HIV-positive pregnant women, including first trimester pregnancies. In addition to diminishing the MTCT of HIV-1, lamivudine is also used to decrease the vertical transmission of hepatitis B virus in pregnancy (4, 5). Lamivudine is known to cross the placenta, with the predicted fetal-to-maternal area under the concentration-time curve (AUC) reaching 86% in humans (6). However, detailed knowledge of mechanisms affecting lamivudine transplacental transfer is still lacking.

The transplacental permeability of drugs predominantly depends on their physical-chemical characteristics, which determine the rate of passive diffusion. However, it can also be extensively influenced by transporter proteins expressed in the apical microvillous plasma membrane (MVM) of polarized trophoblasts (7). Among them the ATP-dependent (ABC) efflux transporters P-glycoprotein (P-gp; MDR1 [ABCB1]), breast cancer resistance protein (BCRP [ABCG2]), and multidrug resistance-associated protein 2 (MRP2 [ABCC2]) are well-confirmed active components of the placental barrier providing fetal protection against potentially toxic compounds, including drugs (8–10). Besides ABC transport proteins, some members of the SLC (solute carrier) transporter family can further modulate transplacental drug transfer (7). MATE1 (SLC47A1), typically expressed in the apical membrane of polarized cells, is an H^+ exchanger known to ensure the efflux of substrates that enter the cells via organic cation transporters (OCTs [SLC22A]) located in the basolateral membrane (11). This OCT-MATE1 excretory pathway is typical for the kidneys and liver. Nevertheless, recent studies indicate that this vectorial transport mechanism might also be relevant for the placenta (12, 13). Expression of multidrug and toxin extrusion proteins MATE1/Mate1 (SLC47A1/Slc47a1) and MATE2/Mate2 (SLC47A2/Slc47a2) has been recently studied in human and rat first trimester and term placentas (13–15), and Mate1 was suggested as an efflux component of vectorial fetal-to-maternal drug

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transfer of metformin and cationic neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) in the dually perfused rat term placenta model (12, 16).

Lamivudine was recently found to interact with MATE1 and its kidney variant, MATE2-K (17). Thus, we hypothesized that MATE1 could also affect the transplacental transport of lamivudine by mediating the efflux from polarized trophoblast cells back to the maternal blood. All three subtypes of human organic cation uptake transporters, OCT1 (SLC22A1), OCT2 (SLC22A2), and OCT3 (SLC22A3), were shown to contribute to lamivudine uptake into the cells, with decreasing efficacy in the following order: OCT1 > OCT2 > OCT3 (18, 19). OCT3 is considered the predominant placental OCT transporter (12–14, 20): nevertheless, significant mRNA expression of OCT1 and OCT2 was also shown in the first trimester as well as term human placenta (13, 14).

To the best of our knowledge, the possible impact of drug efflux transporters on transplacental pharmacokinetics of lamivudine has not previously been systematically evaluated. In the present work, we investigated the affinity of lamivudine to efflux transporters MDR1, BCRP, MRP2, and MATE1 and evaluated whether the maternal-to-fetal transfer of lamivudine could be affected by any of these efflux transporters. To address these aims, we performed *in vitro* transport and accumulation assays on cellular monolayers and the *in situ* method of dually perfused rat term placenta. Additionally, we verified the results in human placenta by uptake assay in microvillous placental membrane (MVM) vesicles.

MATERIALS AND METHODS

Materials and reagents. Lamivudine was kindly provided by the NIH as a part of the NIH AIDS Reagent Program. Radiolabeled lamivudine ([^{3}H]lamivudine at 21.3 or 5.2 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Mitoxantrone, ASP^+, and fluorescein isothiocyanate-labeled dextran (molecular mass, 40 kDa) were obtained from Sigma-Aldrich (St. Louis, MO). BCRP inhibitor Ko143 was purchased from Enzo Life Sciences AG (Lausen, Switzerland), and pentobarbital (Nembutal) was purchased from Abbott Laboratories (Abbott Park, IL). Cell culture media and sera were obtained from Sigma-Aldrich (St. Louis, MO) and Gibco BRL Life Technologies (Rockville, MD). All other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO). The bicinchoninic acid (BCA) assay kit was purchased from Thermo Scientific (Rockford, IL).

Cell lines and cell culture. The Madin-Darby canine kidney II (MDCKII) parental cell line and MDCKII cells stably transduced for expression of human transporters P-gp, BCRP, and MRP2, designated MDCK-MDR1, MDCK-BCRP, and MDCK-MRP2, respectively, were provided by Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). All of the MDCK cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Double-transfected MDCKII cell lines stably expressing human OCT1 or OCT2 and MATE1 transporters (MDCK-OCT1-MATE1 and MDCK-OCT2-MATE1), as well as the respective monotransfected cells (MDCK-OCT1, MDCK-OCT2, and MDCK-MATE1) and control vector cells (MDCK-Co), were established and characterized as described previously (17, 21, 22). Cells were cultured in MEM containing 10% heat-inactivated FBS. All cells used in our experiments were routinely cultivated in antibiotic-free medium and periodically tested for mycoplasma contamination. Stable expression of all inserted human transporters was verified by quantitative reverse transcription-PCR (qRT-PCR) and uptake assays with appropriate fluorescence substrates. Cells from passages 10 to 25 were used in all *in vitro* studies.

Animals. Pregnant Wistar rats were purchased from MediTox s.r.o. (Konarovice, Czech Republic) and maintained under 12-h/12-h day/night standard conditions with pellets and water provided *ad libitum*. Experiments were carried out on day 21 of gestation. Overnight-fasted rats were anesthetized with pentobarbital (40 mg/kg body weight) administered into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (23) and the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (24).

Human tissue samples. All human tissue samples were obtained following written informed consent with the approval of the Faculty Hospital Research Ethics Committee. Placentas were collected from uncomplicated pregnancies at term (38 to 40 weeks of gestation) delivered by Caesarean section in the Department of Obstetrics and Gynaecology, University Hospital in Hradec Kralove, Czech Republic. Human normal kidney samples were obtained after written informed consent from human biopsy specimens in the Hemodialysis Center, University Hospital in Hradec Kralove, Czech Republic.

Transcellular transport assays in the ABC transporter-overexpressing MCDK cells. Transport assays employing MDCK parental and ABC transporter-expressing MDCK cells were performed on microporous polycarbonate membrane filters (3.0-μm pore size, 24-mm diameter, Transwell 3414; Costar, Corning, NY) as described previously (25, 26). MDCK-MDR1, MDCK-BCRP, MDCK-MRP2, or MDCK parent cells were seeded at a density of 1.5×10^6 per insert and cultured 3 to 4 days until reaching confluence, with daily replacement of cell culture medium. Before starting the transport experiment, cells were washed with prewarmed phosphate-buffered saline (PBS) on both the apical and basal sides of monolayers, and Opti-MEM with radiolabeled lamivudine was added to either the apical or basolateral compartments. The lowest [^{3}H]lamivudine concentration used was 8 nM, as this concentration achieved the minimal specific activity required for analysis (0.04 μCi/ml). The experiments were run at 37°C in 5% CO_2, aliquots of 50 μl were collected at 2, 4, and 6 h from the opposite compartment, and radioactivity was measured by liquid scintillation counting (Tri-Carb 2900TR; PerkinElmer). At the end of the experiment, leakage of fluorescein isothiocyanate-labeled dextran (molecular mass, 40 kDa) was analyzed and accepted at up to a rate of 1% per hour. The percentage of radioactivity appearing in the acceptor compartment relative to the stock solution initially added to the donor compartment was calculated. Transport ratios (r_t) between basal-to-apical (BA) and apical-to-basal (AB) translocation after 6 h of incubation were calculated as described earlier (25) by dividing the percentage of drug transported in the BA direction by the percentage of drug crossing the monolayer in the AB direction.

Transcellular transport assays in SLC transporter-overexpressing MCDK cells. Transport experiments employing monotransfected MDCK-MATE1, MDCK-OCT1, and MDCK-OCT2 cells, double-transfected MDCK-OCT1-MATE1 and MDCK-OCT2-MATE1 cells, and the empty vector-transfected control MDCK-Co cells were performed on Transwell 3402 cell culture inserts (3.0-μm-pore size, 24-mm-diameter; Costar, Corning, NY). For all experiments, 0.5×10^6 cells per well were used and incubated for 3 days to confluence in the standard cultivation medium MEM (Gibco) plus 10% FBS. On the day of experiment, medium was removed from both sides, and the cellular monolayer was washed on both sides with prewarmed PBS. The experiment was started by addition of 0.8-ml Hanks' balanced salt solution (HBSS) buffer (pH 7.4) to the apical compartment and 0.8 ml of HBSS buffer (pH 7.4) with [^{3}H]lamivudine to the basolateral compartment. The [^{3}H]lamivudine concentration used was 100 nM, as this concentration achieved the maximal sensitivity required for detection of interaction with drug transporters and minimal specific activity required for final analysis in cell lysates. In the inhibition experiments with mitoxantrone, the cellular monolayers were preincubated with HBSS medium (pH 7.4) containing 2 μM mitoxan-

trone in both compartments 10 min prior to initiation of the transport experiment. The experiments were run at 37°C in 5% CO_2 for 2 h. Aliquots of 50 μl were sampled from the apical side at times 0.5, 1, and 2 h. At the end of the incubation period, the medium was immediately removed, the cells were washed twice with ice-cold PBS, and then the inserts were excised and the cellular monolayer was dissolved in 0.02% SDS solution. The radioactivity of the collected samples and lysed monolayers was measured by liquid scintillation counting (Tri-Carb 2900TR; PerkinElmer). The protein concentration in the cell lysates was quantified using a BCA assay. Net transport was obtained by subtraction of the transport by MDCK-Co cells from that by drug transporter-overexpressing cells. The kinetics graph (transport velocity of lamivudine versus substrate concentration) was fitted using the classic Michaelis-Menten equation, with V_{max} representing the maximal transport velocity (in nanomoles per milligram of protein per minute) and K_m representing the substrate concentration at half-maximal transport velocity (micromolar), in GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA).

qRT-PCR. The mRNA expression of MATE1 was quantified in the monotransfected MDCK-MATE1 cells and double-transfected MDCK-OCT1-MATE1 and MDCK-OCT2-MATE1 cells at passages 10 to 25, in which the cells were used for transport experiments, in the human placental villous tissue and in human kidney medulla used as the comparator sample due to the well-confirmed expression of MATE1 (27). Total RNA was isolated from confluent monolayers of the cells or small pieces of fresh tissue samples using the Tri reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Isolated RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water, and the concentration and purity of each sample were determined spectrophotometrically from A_{260}/A_{280} measurements (NanoDrop, Thermo Scientific, Wilmington, DE). Integrity of RNA was checked by agarose gel electrophoresis. cDNA was prepared from 1 μg extracted total RNA by Moloney murine leukemia virus (MMLV) reverse transcriptase using oligo(dT) VN nucleotides (gb reverse transcription kit; Generi Biotech, Hradec Kralove, Czech Republic). PCR analysis was performed on QuantStudio 6 (Life Technologies). cDNA (40 ng) was amplified using 2× probe master mix (Generi Biotech, Hradec Kralove, Czech Republic) and a predesigned PCR assay (hSLC47A1_Q2) for SLC transporter MATE1 (Generi Biotech). All samples were analyzed three times in triplicate, and threshold cycle (C_T) values were noted and averaged for each sample type. Since there was no other gene besides the human *MATE1* (*hMATE1*) gene shared among the analyzed samples, we show the relevant C_T values and limit the comparison of the MATE1 expression to estimation based on the ΔC_T to the comparator (kidney) and calculating the $2^{-\Delta CT}$ value.

ASP^+ uptake experiments. Uptake experiments were performed using control MDCK-Co and OCT1-, OCT2-, and MATE1-monotransfected MDCK cells in order to quantify the inhibitory effect of mitoxantrone to the particular SLC transporters. The aim of these experiments was to show that mitoxantrone could be used as a model inhibitor that selectively inhibits MATE1 in the subsequent transport studies using OCT-MATE double-transfected cells. Mitoxantrone was chosen as the model inhibitor because it has been shown to preferentially inhibit MATE1 over OCT1 and OCT2 according to the half-maximal inhibitory concentration (IC_{50}) values in other cellular models (28). MATE1 is a pH-dependent carrier that is able to act as an uptake transporter in experimental settings (29) but mediates efflux in physiological ones. Hence, extracellular alkalinization was used to promote MATE1-mediated uptake (30, 31). The single SLC transporter-transfected MDCK cells were seeded on a 96-well plate at a density of 45×10^3 cells per well and cultivated in standard cultivation medium (MEM plus 10% FBS). Twenty-four hours after seeding, uptake experiments with ASP^+, a common fluorescence substrate of OCT1, OCT2, and MATE1, were performed. Cells were washed twice with 100 μl prewarmed HBSS buffer (pH 7.4). Cell lines containing MATE1 were preincubated with 100 μl 20 mM NH_4Cl (pH 7.4) for 30 min. After washing the cells twice with 100 μl prewarmed HBSS buffer (pH 8.0), solutions of mitoxantrone with 1 μM ASP^+ were added, and the cells were incubated for 20 min. After washing the cells twice with 100 μl prewarmed HBSS buffer (pH 8.0), solutions of mitoxantrone with 1 μM ASP^+ were added for 20 min. At the end of the incubation period, the medium was removed, and cells were rinsed three times with ice-cold HBSS buffer (pH 7.4). Then, fluorescence was measured at a wavelength of 485 nm for excitation and 585 nm for emission. Substrate uptake was normalized to the protein concentration of the cell lysate measured by BCA assay.

Dual perfusion of rat term placenta. The method for dual perfusion of rat term placenta was used as described previously (32).

Open-circuit perfusion system. An open-circuit perfusion system was employed to study fetal-to-maternal (F→M) and maternal-to-fetal (M→F) lamivudine clearance. [^{3}H]lamivudine at a concentration of 12 nM was added to either the maternal (M→F studies) or fetal (F→M studies) reservoirs immediately after successful surgery. M→F transplacental clearance (Cl_{mf}) normalized to placenta weight was calculated according to equation 1:

$$Cl_{mf} = (C_{fv} \cdot Q_f)/(C_{ma} \cdot W_p) \quad (1)$$

where C_{fv} is the drug concentration in the umbilical vein effluent (nanomoles per liter), Q_f is the umbilical flow rate (milliliters per minute), C_{ma} is the concentration in the maternal reservoir (nanomoles per liter), and W_p is the wet weight of the placenta (grams). F→M transplacental clearance (Cl_{fm}) was calculated according to equation 2:

$$Cl_{fm} = [(C_{fa} - C_{fv})Q_f]/(C_{fa} \cdot W_p) \quad (2)$$

where C_{fa} is the drug concentration (nanomoles per liter) in the fetal reservoir entering the perfused placenta via the umbilical artery.

Closed-circuit (recirculation) perfusion system. A closed-circuit (recirculation) perfusion system was employed to study the effect of pH on the fetal/maternal lamivudine concentration ratio at equilibrium. Both the maternal and fetal sides of the placenta were infused with 9 nM [^{3}H]lamivudine and after a short-time stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. In the fetal recirculating reservoir, a pH of 7.4 was maintained throughout the experiments, whereas the pH in the maternal reservoir was adjusted by HCl/NaOH to 6.5, 7.4, or 8.5. Samples (250 μl) were collected every 10 min from the maternal and fetal reservoirs and the [^{3}H]lamivudine concentration was measured. This experimental setup ensured a steady concentration on the maternal side of the placenta and enabled investigation of the fetal/maternal ratio; any net transfer of the substrate would imply transfer against a concentration gradient and provide evidence of active transport.

Uptake assay in human placental microvillous plasma membrane vesicles. MVM vesicles were isolated from human term placentas using Mg^{2+} precipitation and differential centrifugation as described previously (33). The final MVM pellet was resuspended in an intravesicular buffer (IVB) at two different pH values adjusted by changing the HEPES/Tris ratio (IVB 7.4, containing 290 mM sucrose, 5 mM HEPES, and 5 mM Tris [pH 7.4]; or IVB 6.2, containing 290 mM sucrose, 9.5 mM HEPES, and 0.5 mM Tris [pH 6.2]), vesiculated by passing 15 times through a 25-gauge needle, and stored at −70°C until use in the uptake experiments. The MVM protein concentration was determined using the BCA assay, and purity was confirmed by measuring the enrichment of MVM alkaline phosphatase activity compared with the placental homogenate. The alkaline phosphatase enrichment factor was 21.8 ± 5.6 (mean ± standard deviation [SD]; $n = 7$).

Uptake of [^{3}H]lamivudine into MVM vesicles was measured at room temperature using rapid vacuum filtration (34). MVM vesicles (10 mg protein/ml) were equilibrated to room temperature (21 to 25°C) prior to uptake. Uptake of [^{3}H]lamivudine was initiated by mixing 10 μl MVM vesicles with 10 μl 100 nM [^{3}H]lamivudine in extravesicular buffer (EVB) at pH 7.4 or 8.4 (145 mM KCl, 10 mM Na^+-HEPES–HCl-Tris [pH 7.4] or IVB 8.4). After 1 min, uptake was stopped by addition of 2 ml ice-cold stop buffer (130 mM NaCl, 10 mM Na_2HPO_4, 4.2 mM KCl, 1.2 mM $MgSO_4$, and 0.75 mM $CaCl_2$ [pH 7.4]) and filtered through a 0.45-μm-pore mixed cellulose ester filter (MF-Millipore membrane filter HAWP02500) under vacuum. Filters were washed with 10 ml of stop buffer, and the filter-

TABLE 1 Lamivudine transport across monolayers of MDCK parent cells and human MDR1-, BCRP-, and MRP2 ABC transporter-expressing MDCK cells[a]

Cell line	BA/AB r_t
MDCK parent	0.923 ± 0.195
MDCK-MDR1	0.930 ± 0.140
MDCK-BCRP	1.25 ± 0.0277*
MDCK-MRP2	1.03 ± 0.131

[a] Cells were seeded on Transwell semipermeable supports and grown as monolayers, dividing, similarly to a polarized trophoblast layer, the basal compartment (corresponding to fetal side) and apical compartments (corresponding to maternal compartment). The basal-to-apical/apical-to-basal transport ratios (BA/AB r_t) for translocation of lamivudine (8 nM) across monolayers of ABC transporter-expressing cells and parent MDCK cells after 6 h are shown. Data are presented as means ± SD of ratios obtained in at least four independent experiments ($n \geq 4$). Student's *t* test was employed to compare the ratios of particular ABC transporter-expressing cells to the respective ratio in MDCK parent control cells, and results were considered statistically significant at $P \leq 0.05$ (*).

TABLE 2 qRT-PCR for *hMATE1* (*SLC47A1*) mRNA expression in MATE1-transfected MDCK cell lines, placenta, and kidney[a]

Cell line or tissue	C_T
MDCK-MATE1	17.4 ± 0.277
MDCK-OCT1/MATE1	21.3 ± 0.364
MDCK-OCT2/MATE1	17.3 ± 0.294
MDCK-Co	ND[b]
Human placenta	33.4 ± 5.86
Human kidney	23.6 ± 1.00

[a] RNA isolated from cells and tissues was reverse transcribed, and the resulting cDNA (40 ng) was quantified for expression of *hMATE1* (*SLC47A1*) using the QuantStudio 6 detector. Mean C_T values from two independent experiments performed in triplicate for each sample are shown for cell lines, human term placenta villous tissue ($n = 7$), and human kidney medulla samples ($n = 2$).
[b] ND, no expression detected.

associated radioactivity was determined by liquid scintillation counting. No-protein controls (replacement of MVM vesicle protein by relevant IVB) were included in parallel to determine tracer binding to the filter, which was subtracted from the total vesicle count.

Statistical analysis. All data were assessed and statistically analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA). Statistical significance was investigated using Student's *t* test, the Kruskal-Wallis test, or two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison posttest as applicable and described in the figure legends. A *P* value of ≤0.05 was taken to be statistically significant. Data are presented as the mean ± standard deviation (SD) or with the 95% confidence interval (CI) where appropriate. Half-maximal inhibitory concentration (IC_{50}) values for mitoxantrone were calculated by nonlinear regression using sigmoidal Hill kinetics with GraphPad Prism 6.0 software.

RESULTS

Transcellular transport of lamivudine across ABC transporter-expressing MDCK monolayers. Transcellular transport of [^{3}H]lamivudine (8 nM) across the polarized monolayers of MDCK parental and MDR1-, BCRP-, and MRP2-overexpressing cells was studied using the conventional bidirectional (concentration gradient) transport assay. No difference between basal-to-apical (BA) and apical-to-basal (AB) transfer of the drug was observed at time points 2 and 4 h, and transport ratios (r_t) close to 1 were observed after 6 h of transport (Table 1). The r_t value obtained in MDCK-BCRP cells (1.25 ± 0.0277) was significantly higher than that measured in MDCK parent cells (0.923 ± 0.195). However, the cutoff value for BCRP substrates (r_t of ≥2) set by the International Transporter Consortium was not reached (35). These data indicate that lamivudine is not a substrate of P-gp or MRP2, whereas BCRP might to some extent be responsible for acceleration of lamivudine transport in the BA direction.

Expression of *MATE1* mRNA in the relevant MDCK cell lines and in human placentas. In order to confirm the expression of *MATE1* mRNA in the cell lines and human placentas used in the subsequent functional studies, an RT-PCR approach was employed. Since there was no other gene shared among the analyzed samples, we limited the comparison of the expression to showing the mean C_T values (Table 2). The *MATE1* expression was confirmed in the MDCK-MATE1 and MDCK-OCT2-MATE1 cell lines with the same level of *MATE1* transcripts (C_T values), while a lower level (higher average C_T) was detected in MDCK-OCT1-MATE1 cells (Table 2). Nevertheless, the levels of expression in all cell lines exceeded significantly that of kidney mainly due to the lack of non-MATE1-expressing cells that are present in the whole-tissue samples. MATE1 expression in human placental villous tissue was highly variable and was found only in 7 out of 10 analyzed human placentas. The average C_T for the MATE1-expressing placentas was about 10 cycles higher (Table 2) than that in kidney medulla tissue, indicating that a result of approximately 2^{-10} = 1,000 times lower *MATE1* expression.

MATE1-mediated transfer of lamivudine across cellular monolayers. After addition of 100 nM lamivudine to the basolateral compartment, all three MATE1-expressing cell lines (i.e., MDCK-MATE1, MDCK-OCT1-MATE1, and MDCK-OCT2-MATE1) showed significantly higher transcellular transfer of [^{3}H]lamivudine from the basal to the apical compartment compared with MDCK-Co cells (increases to 262.4%, 196.8%, and 250.9%, respectively; $P \leq 0.01$, Student's *t* test) and their respective non-MATE1-expressing control cells (MDCK-Co, MDCK-OCT1, and MDCK-OCT2, respectively) (Fig. 1A, B, and C). The rate of lamivudine transcellular transport was lowest in the MDCK-OCT1/MATE1 monolayers among MATE1-expressing cells, in agreement with the lowest gene expression of *hMATE1* in this cell line shown by qRT-PCR (Table 2).

Intracellular concentrations of lamivudine in the monolayers of MDCK-MATE1, MDCK-OCT1-MATE1, and MDCK-OCT2-MATE1 cells were significantly lower than in the respective MATE1-free control cell lines (decreasing to 7.31%, 62.1%, and 24.6% compared to MDCK-Co, MDCK-OCT1, and MDCK-OCT2, respectively; $P < 0.001$, Student's *t* test) (Fig. 1D, E, and F). As expected, monolayers of MDCK-OCT1 and MDCK-OCT2 cells in the transport experiment accumulated significantly larger amounts of lamivudine than MDCK-Co cells ($P < 0.05$, Student's *t* test). Interestingly, the transcellular transfer of lamivudine across MDCK-MATE1 cells was not significantly different from that in double-transfected MDCK-OCT2-MATE1 cells that express the same level of *hMATE1* mRNA (Table 2) but accumulated a larger amount of lamivudine due to OCT2-mediated uptake (Fig. 1D and F).

The apparent affinity of lamivudine to the MATE1 transporter was further evaluated by transport assays in MDCK-MATE1 monolayers using lamivudine concentrations ranging from 100 nM to 10 mM. The K_m value for transfer of lamivudine across MDCK-MATE1 monolayers was 4,213 ± 558.2 μM (Fig. 2), in-

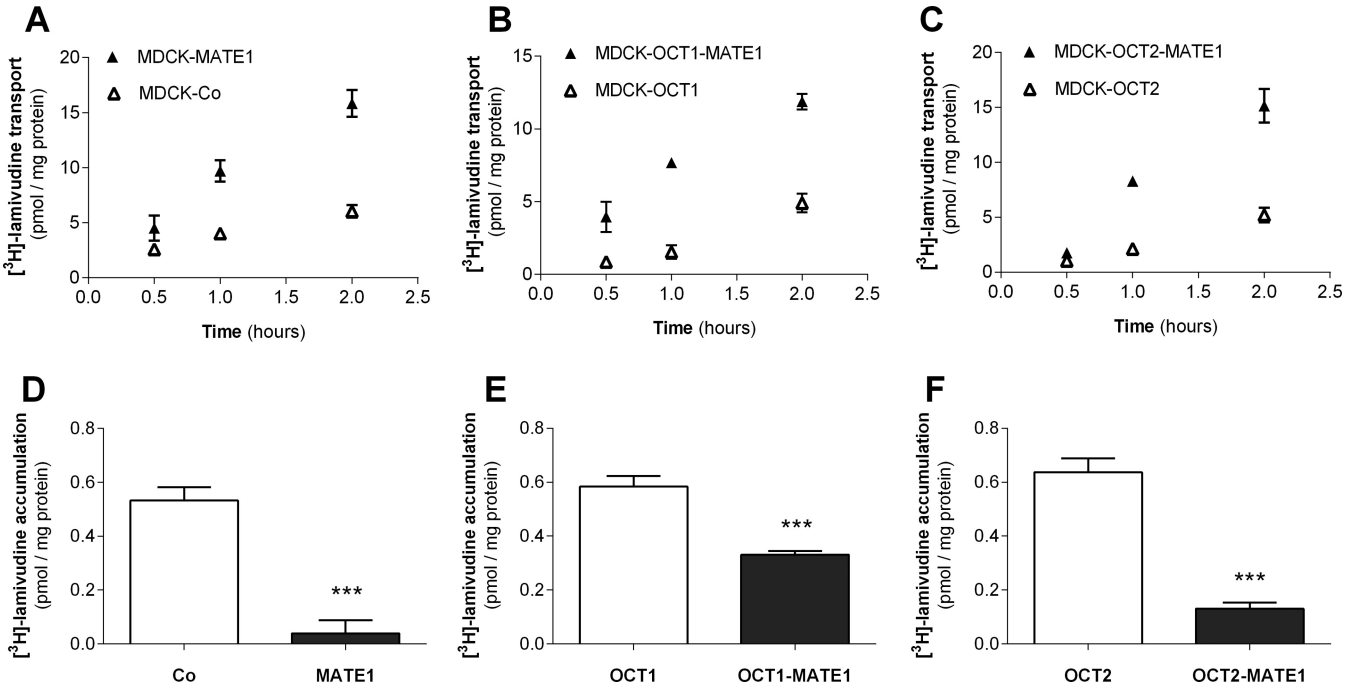

FIG 1 Transport of lamivudine in single-transfected MDCK cells overexpressing OCT1, OCT2, and MATE1, double-transfected MDCK-cells overexpressing OCT1 or OCT2 and MATE1 (MDCK-OCT1-MATE1, MDCK-OCT2-MATE1), and vector control cells (MDCK-Co). Cells were seeded on Transwell semipermeable supports dividing a basal compartment and an apical compartment. Lamivudine (100 nM) was added to the basal compartment and sampled at time points 0.5, 1, and 2 h from the apical side of monolayers (A, B, C). Intracellular accumulation of lamivudine in the monolayers was determined in cell lysates at the end of the experiment (D, E, F). Data were analyzed by Student's *t* test (***, $P < 0.001$ versus respective control [Co], OCT1, or OCT2) and are shown as means ± SD ($n \geq 3$).

dicating that MATE1-mediated transfer of lamivudine is a low-affinity process.

Mitoxantrone-mediated MATE1 inhibition of lamivudine transport. To further test the contribution of MATE1 to the transcellular transfer of lamivudine, a potent inhibitor of MATE1, mitoxantrone, was employed. First, ASP^+ uptake was investigated in MDCK-OCT1, MDCK-OCT2, and MDCK-MATE1 cells to assess the inhibitory potency of mitoxantrone to the different SLC transporters. (Accumulation buffer of pH 7.4 was used for both OCT-expressing cell lines, whereas pH 8.4 was used for the MDCK-MATE1 cells to reverse the direction of the ASP^+ transport to uptake.) When comparing IC_{50}s, the observed selectivities for MATE1 inhibition were 4.8 and 9.1 times higher than those for OCT1 and OCT2 ($P < 0.001$), respectively (Fig. 3A). Based on these results and the dose-dependency inhibition curve of ASP^+ uptake, 2 μM mitoxantrone was chosen for the subsequent transport assay to predominantly inhibit MATE1 over OCTs in MATE1/OCT transporters expressing cellular monolayers (Fig. 3B and C).

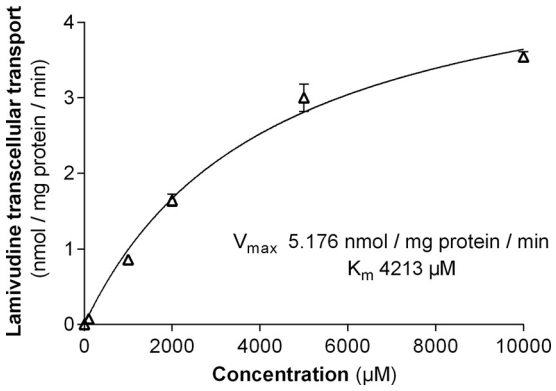

FIG 2 Concentration-dependent net transcellular transport of lamivudine by MATE1. Basolateral-to-apical transport of lamivudine across MDCK-MATE1 and MDCK-Co cells cultured as monolayers on Transwell membranes was investigated for increasing concentrations of nonradiolabeled lamivudine (1×10^{-4}, 1×10^{-3}, 0.01, 0.1, 1.0, 2.0, 5.0, and 10 mM) with addition of tracer [^{3}H]lamivudine (16.7 nM) applied to the basolateral compartment. The transcellular transport of lamivudine across MDCK-Co monolayers was subtracted from that in MDCK-MATE1 cells at each concentration point. Kinetic parameters (K_m and V_{max}) were estimated by fitting MATE-specific transport rates to a Michaelis-Menten nonlinear equation. Data (nanomoles per milligram of protein per minute) represent the mean ± SD from three independent experiments.

Addition of mitoxantrone (2 μM) to the cellular monolayers reduced the basolateral-to-apical transport of lamivudine in all of the MATE1-expressing cells (MDCK-MATE1, MDCK-OCT1-MATE1, and MDCK-OCT2-MATE1) to the level of the MDCK-Co control cell line ($P < 0.001$, two-way ANOVA) (Fig. 3B) and showed no statistically significant difference in lamivudine transport among the mitoxantrone-inhibited cell lines. No effect of mitoxantrone on the transcellular transport across MDCK-OCT1 and MDCK-OCT2 monolayers was observed (data not shown). Consistent with these data, addition of mitoxantrone significantly increased lamivudine accumulation in the monolayers of MATE1-, OCT1-MATE1-, and OCT2-MATE1-expressing cells ($P < 0.01$, two-way ANOVA), whereas no increase was observed in MDCK-Co cells (Fig. 3C).

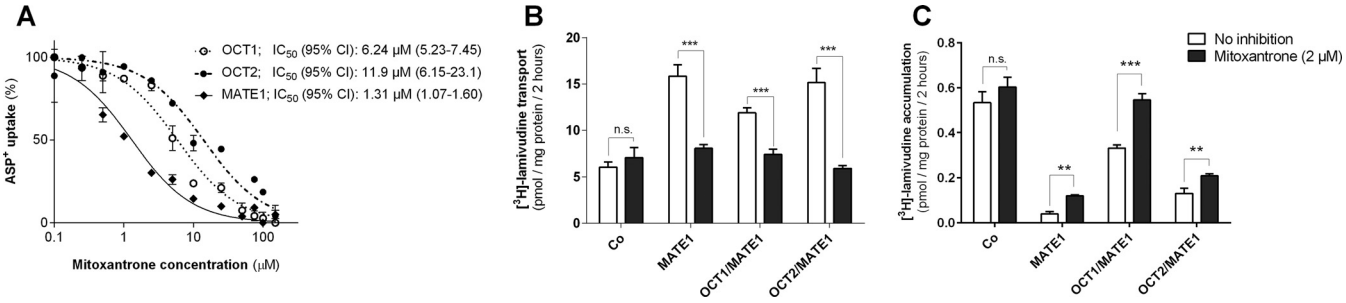

FIG 3 Inhibitory effect of mitoxantrone on lamivudine transport by MATE1. (A) IC_{50}s reflecting inhibition of ASP^+ uptake into OCT1-, OCT2-, and MATE1-expressing MDCK cells by mitoxantrone. The IC_{50}s with 95% confidential intervals were calculated from three independent measurements. (B and C) Effect of 2 μM mitoxantrone on transcellular transport (B) and intracellular accumulation (C) of lamivudine (100 nM) in monolayers of MATE1-expressing and control cells. Data were analyzed by two-way ANOVA with multiple comparisons (**, $P < 0.01$, ***, $P < 0.001$, versus respective noninhibited controls) and are shown as means ± SD ($n \geq 3$). n.s., not significant.

Lamivudine transport across the perfused rat placenta: effect of pH. The ratio between F⟶M and M⟶F clearances found in the perfusion studies, in which [^{3}H]lamivudine (12 nM) was applied from the maternal or fetal side of the placenta, was 1.8, indicating possible active transplacental transport of lamivudine from the fetal to maternal side. Nevertheless, the differences between M⟶F and F⟶M clearances did not reach statistical significance ($P = 0.096$, Student′s t test). Less than 1% of the lamivudine dose was detected in the placenta after the perfusion experiments, suggesting limited tissue binding or accumulation in trophoblasts and negligible effect on the clearance calculation. To further study lamivudine transplacental transport, both sides of the placenta were perfused with the same concentration of [^{3}H]lamivudine (12 nM) in a closed-circuit experimental setup. We observed a slight decrease of lamivudine concentration in the fetal perfusate, achieving 95.3% ± 2.46% of the initial concentrations over 60 min of perfusion. This decline might indicate a small contribution of active transport against the concentration gradient from the fetal to the maternal side of the placenta. To study the effect of pH on lamivudine fetal-to-maternal transport, which could reflect involvement of MATE1-mediated efflux, the pH in the maternal reservoir was set to 6.5 or 8.5, while the pH in the fetal reservoir was set to 7.4. The fetal lamivudine concentration changed significantly between the experiments with pH 6.5 and 8.5 (Fig. 4), suggesting involvement of proton-dependent transport of lamivudine across the placenta.

Uptake of [^{3}H]lamivudine into human placenta MVM vesicles: effect of pH. To determine the relevance of MATE1-mediated transport for the transplacental transfer of lamivudine in human placenta and, more specifically, MATE1 involvement in the transport of lamivudine across the MVM of human placenta, uptake of lamivudine into MVM vesicles isolated from human term placenta of uncomplicated pregnancies was measured. The lamivudine accumulation into the vesicles was stimulated by a higher extravesicular pH. However, the magnitude of the increase in response to the imposed outwardly directed proton gradient was rather variable between MVM vesicle isolates and reached statistical significance only with the pH gradient of 2.2 pH units (pH 6.2 to 8.4), but not with the 1-pH-unit gradient (pH 7.4 to 8.4) (Fig. 5).

DISCUSSION

Lamivudine is considered a first-line antiretroviral drug to prevent MTCT in HIV-positive pregnant women (3). The concentra-

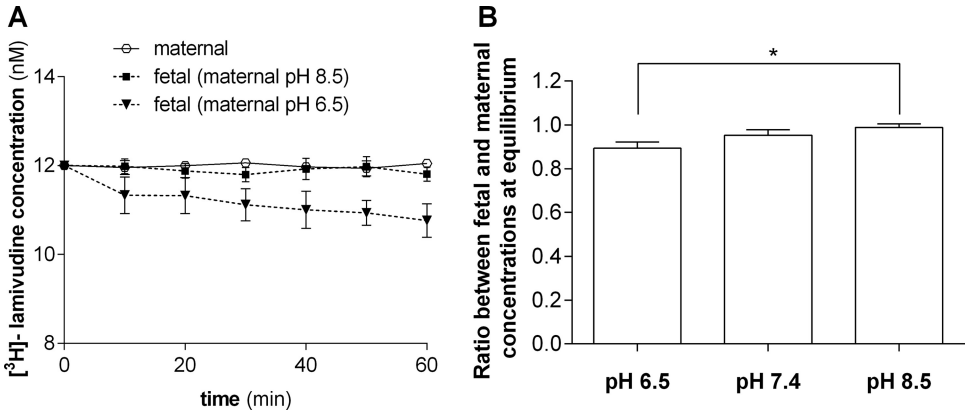

FIG 4 Effect of maternal pH on elimination of lamivudine from the fetal circulation. In the closed-circuit perfusion setup, both the fetal and maternal sides of the placenta were simultaneously infused with 12 nM [^{3}H]lamivudine. The fetal pH was set to 7.4, whereas the pH in the maternal reservoir was set to 6.5, 7.4, or 8.5. The fetal perfusate was recirculated for 60 min, and then fetal and maternal concentrations of lamivudine were compared. (A) Lamivudine fetal concentration over a 60-min perfusion at pH 6.5 and 8.5 applied on the maternal site. (B) Final ratio between fetal and maternal concentrations at equilibrium showing statistically significant difference between ratios calculated for perfusions at pH 6.5 and pH 8.5 (*, $P < 0.05$, Kruskal-Wallis test), suggesting involvement of a proton-cation antiporter system in lamivudine transplacental transport. Data are presented as means ± SD ($n \geq 3$).

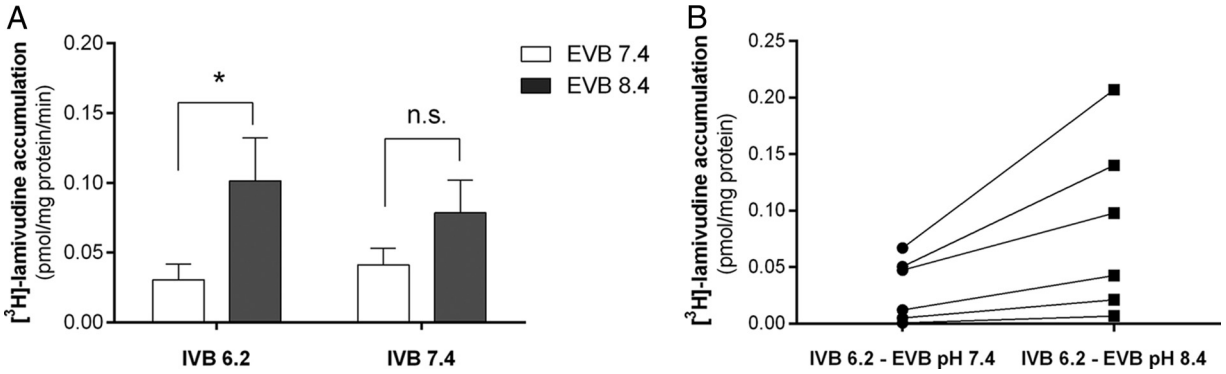

FIG 5 Effect of H^+ gradient on [^{3}H]lamivudine uptake by MVM vesicles from human term placentas. MVM vesicles were prepared in intravesicular buffer (IVB) at pH 6.2 or 7.4. (A) One-minute uptake of [^{3}H]lamivudine was examined in extravesicular buffer (EVB) containing 100 nM [^{3}H]lamivudine at pH 7.4 or 8.4. (B) Paired measurements for pH 6.2 IVB MVM vesicles showing stimulation of [^{3}H]lamivudine uptake in the presence of an increased pH gradient (change, 422% ± 276% [mean ± SD]; $n = 6$; $P = 0.044$, paired t test). Data are presented as means ± SD from experiments with 5 to 7 placentas. n.s., not significant.

tions of lamivudine found in cord blood at delivery have been reported to reach maternal levels, suggesting that the drug can freely cross the placenta by passive diffusion (36, 37). Nevertheless, a fetal-to-maternal area under the concentration-time curve (AUC) ratio of 0.86 (6) indicates that transplacental crossing of lamivudine is not entirely passive. One explanation is that a fetal-to-maternal directed transport mechanism is involved that diminishes the transplacental passage of lamivudine from mother to fetus. Such information would be of considerable importance for optimizing the pharmacotherapy of pregnant women because placental drug transporters could be involved in pharmacokinetic drug-drug interactions (DDI) affecting fetal drug exposure to concomitantly administered drugs and leading to impaired treatment outcome or adverse effects (7, 38). This issue is of particular importance in anti-HIV therapy, where combination of two or more antiretroviral drugs is recommended (3).

Several drug efflux transporters are functionally expressed in the apical microvillous plasma membrane of human syncytiotrophoblasts (8–10). In the present project, we aimed to assess whether lamivudine is a substrate of placental drug transport proteins and address whether transplacental transfer of lamivudine is affected by drug efflux transporters.

Interaction of lamivudine with P-gp was evaluated by de Souza et al. (39), using a transport assay with P-gp-expressing monolayers. Based on the lack of interaction of lamivudine with a potent P-gp inhibitor (GG 918), the authors concluded that P-gp does not significantly affect the transport of lamivudine (39). Correspondingly, we did not observe any P-gp-accelerated transport of lamivudine (8 nM) across the MDCK-MDR1 monolayers, confirming the lack of relevant P-gp involvement in lamivudine transport.

Transport of lamivudine by BCRP was suggested by Kim et al., who observed decreased lamivudine uptake in MDCK-BCRP cells and saturable lamivudine transport across BCRP-expressing monolayers in a 60-min experiment (40). Nevertheless, functionally relevant polymorphic variants of BCRP had no effect on lamivudine disposition in healthy volunteers, indicating that BCRP is unlikely to make a relevant contribution to lamivudine pharmacokinetics. To further address this issue, we performed a transport study with a low lamivudine concentration in MDCK-BCRP cells in a bidirectional concentration gradient setup. The lamivudine transport ratio (r_t) in MCDK-BCRP significantly exceeded that in the parental MDCK cells (Table 1); however, it did not reach the value of 2, which is considered by the International Transporter Consortium guidelines as a cutoff level for classifying a drug as a transporter substrate (35). We therefore hypothesize that lamivudine might be only a very-low-affinity BCRP substrate, questioning the transport of lamivudine by BCRP observed by Kim et al. in a short-time-setup assay (40). Similarly to the results in MDCK-MDR1 and MDCK-BCRP cells, no active transporter-driven transfer of lamivudine was observed in MDCK-MRP2 cells, showing that lamivudine is not significantly transported by any of the three main placental ABC transporters.

In situ dually perfused rat term placenta represents a valuable alternative model to study placental pharmacology and physiology. Using this approach, we have previously identified ABC transporter-mediated placental passage of several compounds, including antiretrovirals (25, 26, 32, 41). In the present study, we showed that the fetal-to-maternal clearance of lamivudine was not significantly higher than the maternal-to-fetal clearance and that the decrease of lamivudine concentration in fetal compartment during the recirculation experiment was insignificant (Fig. 4). These observations are in agreement with the above-discussed *in vitro* transport experiments and indicate no or only minor involvement of active transporter-mediated fetus-to-mother-directed efflux of lamivudine.

Lamivudine has recently been shown to be a substrate of human MATE1 and the kidney-specific MATE2-K transporters, and MATE-mediated pH-dependent efflux was suggested to contribute to renal tubular excretion of lamivudine (17). This antiretroviral has also been shown to be a substrate of all three subtypes of human organic cation transporter, with efficacy dependent on kinetic parameters and the V_{max}/K_m ratio decreasing in the following order: OCT1 > OCT2 > OCT3 (18, 19). In the present study, we evaluated the affinity of lamivudine to the MATE1 transporter and aimed to address in detail the role of OCTs in the transcellular transfer of lamivudine using double-transfected MDCK-OCT1-MATE1 and MDCK-OCT2/MATE2 cell lines and relevant mono-transfected and vector control MDCK cells. By applying low concentrations of lamivudine to increase the sensitivity of the assay, we confirmed that MATE1 significantly increases the transcellular passage of lamivudine while decreasing intracellular accumula-

tion of the drug (Fig. 1). Monolayers of OCT1- and OCT2-single-transfected cells accumulated higher levels of lamivudine but retained a similar transcellular transport efficacy to that in vector control cells, confirming that OCT transporters affect the influx of lamivudine into the cells. In agreement with previous observations with 10 μM lamivudine (17), lamivudine transfer across MDCK-OCT2-MATE1 monolayers did not differ significantly from that in MDCK-MATE1 cells expressing the same level of MATE1 mRNA (Table 2). Mitoxantrone used at a concentration preferentially inhibiting MATE1 over OCT1 and OCT2 (2 μM) significantly decreased the lamivudine transcellular transfer in all of the MATE1-expressing cells to the level of control cells but increased the cellular accumulation in all of the MATE1-expressing cell lines (Fig. 3). Our data thereby suggest that lamivudine transcellular transfer is controlled mainly by MATE1-mediated efflux and is not significantly affected by OCT-mediated uptake.

Kinetic analysis of the MATE1-mediated transport of lamivudine revealed a rather low-affinity and transport capacity of MATE1 to the antiretroviral (K_m, 4.21 mM; V_{max}, 5.18 nmol/mg protein/min). The transport efficacies of OCT1 and OCT2 transporters to lamivudine have been reported as a V_{max}/K_{mOCT1} of 8.03 (18) or 8.0 (19) μl/mg protein/min and V_{max}/K_{mOCT2} of 4.1 (18) or 4.4 (19) μl/mg protein/min, respectively, exceeding the efficacy of MATE1 in the transport of lamivudine found in the present study (V_{max}/K_m, 1.23 μl/mg protein/min). We therefore suggest that MATE1-mediated efflux might be the rate-limiting step in the transcellular transfer of lamivudine, which is consistent with the results of our transport studies, showing that the transport rate of lamivudine across MDCK-MATE1 cells did not differ from that across MDCK-OCT2-MATE1 cells. Nevertheless, the *in vitro*-determined K_m values do not necessary preclude the kinetic impacts of the transporter *in vivo*. For instance, some drugs of high K_m values determined *in vitro* play a significant role in MATE1 excretory pathways *in vivo* (42).

Moreover, the concentration of half-maximal-velocity lamivudine transport by MATE1 is about 3 orders of magnitude higher than the maximal therapeutic concentration (≈3 to 11 μM) achieved in the plasma of pregnant women (37, 43), thus making saturation of MATE1 *in vivo* unlikely.

The involvement of pH-dependent transport in the transplacental pharmacokinetics of lamivudine was further evaluated *in situ* by employing dually perfused rat term placenta. Whereas expression of *Mate1* appears to be absent in murine placenta (44, 45), abundant placental expression has been found in rats (12, 14, 15), and the transporter was found to mediate the pH-dependent fetal-to-maternal transfer of MPP^+ (12) and metformin (16) in the perfused rat placenta (12, 14). Using the same placental model, the ratio between F→M and M→F clearances of 12 nM lamivudine reached 1.8, indicating possible active transplacental transport of lamivudine from the fetal to maternal side. However, this value was much lower than that measured for 10 nM MPP^+ (value of 123) and 100 nM metformin (value of 7.3) in previous studies (12, 16) and did not reach statistical significance. We observed that decreasing the maternal pH increased the fetal-to-maternal transfer of lamivudine in the closed-circuit setup, indicating involvement of a pH-dependent transport mechanism on the maternal-facing side of trophoblasts in the transplacental transfer of lamivudine.

In contrast to rat, human placenta seems to express only low and highly variable levels of hMATE1 mRNA (12, 14). Accordingly, we observed hMATE1 mRNA expression only in 7 of 10 analyzed placentas, showing the level of MATE1 mRNA transcripts about 1,000 times lower than that with human kidney medulla tissue (Table 2). To address the relevance of MATE1 for transplacental transfer of lamivudine in humans, we additionally evaluated the pH-dependent transport of lamivudine directly on microvillous plasma membrane (MVM) vesicles isolated from the MATE1-expressing human term placental trophoblast. The uptake of lamivudine across MVM appears to be sensitive to the imposed H^+ gradient, suggesting that MATE1-mediated H^+/lamivudine exchange contributes to the transfer of lamivudine across human placenta. However, the results were variable and achieved statistical significance only with a higher pH gradient (2.2 pH units) but not with a lower pH gradient (1 pH unit). It is possible that temporal dissipation of the H^+ gradient across the MVM plasma membrane contributed to the variability in the capacity of MATE1 to accumulate lamivudine as the substrate (33). The imposition of a steeper transmembrane H^+ gradient across the MVM plasma membrane may help clarify this further.

The maternal-fetal interface does not offer such a steep pH gradient as proximal tubules in the kidney favoring the MATE1-mediated efflux, but it cannot be excluded that MATE1-mediated transport in the placenta is linked also to another H^+-transferring transporter(s) providing a sufficient H^+ ion gradient to drive the efflux, such as the Na^+/H^+ exchanger and ATP-dependent H^+ pump that were found functionally expressed in human placenta (46–48). It has also been suggested that MATE-driven organic cation excretion may occur independently of a pH gradient across the brush-border membrane (49), supporting the possible physiological role of MATE1 in the placenta. Nevertheless, the contribution of other pH-dependent transport mechanism, such as MATE2 (13), in transfer of lamivudine across the MVM cannot be excluded. In addition, a higher portion of the un-ionized form of lamivudine available for passive diffusion into the vesicles provided by the pH gradient should be also taken into account. We therefore suggest that MATE1 might represent one of the mechanisms mediating the maternal-to-fetal transfer of lamivudine in human placenta; however, its contribution to this transfer is probably rather limited. The low impact of the MATE1 efflux transporters in the transplacental transfer of lamivudine may also be caused by high transplacental passage of lamivudine, driven by passive diffusion or any concentration gradient-driven mechanisms, such nucleoside transport proteins (50–52). Nevertheless, since the human placenta tends to express higher levels of MATE1 in the first trimester compared with term (13) it is feasible to hypothesize that the MATE1-mediated transplacental transport of lamivudine might be of higher importance in earlier phases of pregnancy.

To conclude, we have shown here that P-gp, MRP2, and BCRP do not affect the transplacental transfer of lamivudine, making the risk of pharmacokinetic DDI between lamivudine and other antiretroviral substrates of the ABC transporters in the placenta unlikely. On the other hand, we demonstrated a low-affinity efflux of lamivudine by MATE1, which seems to act independently of the OCT-mediated cellular uptake of lamivudine. We further showed that a pH-dependent mechanism mediates transport of lamivudine in the fetal-to-maternal direction in rat as well as human placenta and concluded that MATE1 might be at least partly responsible for this transport. However, further research is needed to address the role MATE1 may play in human placenta during

pregnancy and to elucidate the risk of MATE1-mediated DDI in the transplacental pharmacokinetics of lamivudine in humans.

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