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Equilibrative nucleoside transporter 1 (ENT1, *SLC29A1*) facilitates transfer of the antiretroviral drug abacavir across the placenta

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Running Title: Abacavir is a substrate for placental ENT1

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Abbreviations: ABCB1: P-glycoprotein; **ABCG2**: breast cancer resistance protein; **ANOVA**: one-way analysis of variance; **BeWo**: choriocarcinoma-derived cell line; **CNT**: concentrative nucleoside transporters; **DMEM**: Dulbecco's modified eagle medium; **DMSO**: dimethyl sulfoxide, **ENT**: equilibrative nucleoside transporter; **HEPES**: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; **MTCT**: mother-to-child transmission; **MVM**: microvillous plasma membrane; **NBMPR**: S^6-(4-nitrobenzyl)mercaptopurine riboside; **NIH**: National Institute of Health

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Abstract

Abacavir is a preferred antiretroviral drug for preventing mother-to-child HIV transmission; however, mechanisms of its placental transfer have not been satisfactorily described to date. Because abacavir is a nucleoside-derived drug, we hypothesized that the nucleoside transporters, ENTs (*SLC29A*) and/or Na^+-dependent CNTs (*SLC28A*), may play a role in its passage across the placenta. To test this hypothesis, we performed uptake experiments using BeWo cell line, human fresh villous fragments and microvillous plasma membrane (MVM) vesicles. Employing endogenous substrates of nucleoside transporters, [^{3}H]-adenosine (ENTs, CNT2, and CNT3) and [^{3}H]-thymidine (ENTs, CNT1, and CNT3), we showed significant activity of ENT1 and CNT2 in BeWo cells while experiments in the villous fragments and MVM vesicles, representing model of apical membrane of syncytiotrophoblast, revealed only ENT1 activity. When testing [^{3}H]-abacavir uptakes, we showed that of nucleoside transporters, ENT1 plays the dominant role in abacavir uptake into placental tissues, whereas contribution of Na^+-dependent transport, most likely mediated by CNTs, was observed only in BeWo cells. Subsequent experiments with dually perfused rat term placentas showed that Ent1 contributes significantly to overall [^{3}H]-abacavir placental transport. Finally, we quantified the expression of *SLC29A* in first- and third-trimester placentas, revealing that *SLC29A1* is the dominant isoform. Neither *SLC29A1* nor *SLC29A2* expression changed over the course of placental development, but there was considerable inter-individual variability in their expression. Therefore, drug-drug interactions and the effect of inter-individual variability in placental ENT1 expression on abacavir disposition into fetal circulation should be further investigated to guarantee safe and effective abacavir-based combination therapies in pregnancy.

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Introduction

Mother-to-child transmission (MTCT) is the most common route of HIV infection in children. The risk of vertical HIV transmission can be minimized by perinatal administration of a combination antiretroviral therapy (cART) that suppresses viral replication in maternal blood and genital secretions. It has recently been emphasized that cART should include an antiretroviral drug with high placental transfer for pre-exposure prophylaxis of the fetus (AIDSinfo, 2017).

The functional part of the placental barrier is the polarized multinucleated syncytiotrophoblast, which expresses various carriers including equilibrative nucleoside transporters (ENTs) (Staud et al., 2012). ENTs are ubiquitously occurring proteins belonging to the solute carrier (SLC) transporter superfamily that mediate bidirectional facilitated diffusion of nucleosides in tissues to maintain nucleoside homeostasis (Molina-Arcas et al., 2009). Beyond their physiological role, ENTs, especially the two important isoforms ENT1 and ENT2 (Griffiths et al., 1997), affect the pharmacokinetics of a broad array of nucleoside-derived drugs, including nucleoside reverse transcriptase inhibitors (didanosine and zalcitabine), anti-hepatitis therapeutics (ribavirin and entecavir) or antineoplastic drugs (gemcitabine and cladribine) (Yamamoto et al., 2007; Molina-Arcas et al., 2009; Ma et al., 2017). Both ENT1 (encoded by *SLC29A1*) and ENT2 (encoded by *SLC29A2*) are transcribed in the human syncytiotrophoblast (Govindarajan et al., 2007; Yamamoto et al., 2007; Errasti-Murugarren et al., 2011). ENT1 has been detected in the maternal-facing (apical) microvillous plasma membrane of the syncytiotrophoblast, whereas ENT2 is localized on both the apical and fetal-facing (basal) plasma membrane (Barros et al., 1995; Govindarajan et al., 2007; Errasti-Murugarren et al., 2011). ENT1-like activity has also been observed on the syncytiotrophoblast basal plasma membrane despite undetectable ENT1 expression at this locus (Errasti-Murugarren et al., 2011).

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Besides ENTs, placental expression of concentrative nucleoside transporters (CNTs, *SLC28A*) that mediate unidirectional Na^+ dependent influx of nucleosides and nucleoside analogues (Molina-Arcas et al., 2009) has also been suggested, however, with conflicting results. While Errasti-Murugarren et al. observed protein expression of CNT1 despite very low mRNA expression (Errasti-Murugarren et al., 2011), Govindarajan et al. did not detect protein expression of CNTs in term placenta (Govindarajan et al., 2007). In addition, Barros et al. demonstrated that there is no effect of Na^+ depletion on nucleosides uptake into MVM vesicles (Barros et al., 1991).

The expression of drug transporters in the placenta frequently varies during gestation and differs among individuals (Gil et al., 2005; Mao, 2008; Ahmadimoghaddam et al., 2013) . This holds true also for *SLC28A*, exhibiting significant inter-individual variability and higher expression in term placenta compared to the first trimester (Jiraskova et al., 2018); however, such data are lacking for *SLC29A*.

Abacavir is a nucleoside analogue belonging to the family of nucleoside reverse transcriptase inhibitors (NRTIs). It is currently a preferred anti-HIV compound for MTCT prevention (AIDSinfo, 2017). Transplacental transfer of abacavir in humans has been investigated only sparsely and its cord-to-maternal blood concentration ratio is variable, ranging from 62 to 163% at term (Chappuy et al., 2004; Best et al., 2006; Fauchet et al., 2014). The pharmacokinetics of antiviral drugs are frequently affected by the activity of drug transporters (Kis et al., 2010; Neumanova et al., 2014; Neumanova et al., 2015; Ceckova et al., 2016; Neumanova et al., 2016). Abacavir is a known substrate of P-glycoprotein (ABCB1) and Breast Cancer Resistance Protein (ABCG2) (Shaik et al., 2007b; Giri et al., 2008; Neumanova et al., 2015) and we have recently demonstrated that these transporters may limit abacavir maternal-to-fetal transfer (Neumanova et al., 2015). Importantly, abacavir was reported to reduce nucleoside uptake *in*

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vitro, raising the possibility that abacavir is also a substrate for ENTs (Hong et al., 2000; Li et al., 2015).

Because a detailed understanding of drug interactions with placental transporters is required to guarantee safe and effective therapy during pregnancy (Staud et al., 2012; Thomas and Yates, 2012; Staud and Ceckova, 2015), we sought to determine whether ENTs play a role in the transplacental transfer of abacavir. We analyzed abacavir uptake into the placental choriocarcinoma-derived cell line BeWo and fresh villous placental fragments and microvillous plasma membrane (MVM) vesicles, both derived from the human term placenta. We then performed *in situ* dual perfusion studies in the rat term placenta to quantify the role of ENTs in total transplacental abacavir clearance. In all experimental models we also considered potential contribution of CNTs to abacavir placental kinetics. Finally, using quantitative PCR method, we investigated the expression of *SLC29A1* and *SLC29A2* and their inter- and intra-individual variability in the first- and third-trimester human placenta.

Material and Methods

Chemicals and reagents

The radiolabeled compounds [^{3}H]-abacavir (0.05 Ci/mM or 0.1 Ci/mM), [^{3}H]-adenosine (23 Ci/mM), and [^{3}H]-thymidine (74 Ci/mM) were purchased from Moravek Biochemicals (Brea, CA, USA); adenosine represents a model substrate of ENT1, ENT2, CNT2, and CNT3 while thymidine is transported by ENT1, ENT2, CNT1, and CNT3 (Molina-Arcas et al., 2009). The specific ENT inhibitor S^6-(4-nitrobenzyl)mercaptopurine riboside (NBMPR) and the competitive inhibitors of ENTs and CNTs, uridine and adenosine (Molina-Arcas et al., 2009; Errasti-Murugarren et al., 2011), were purchased from Sigma-Aldrich (St. Louis, MO, USA); NBMPR (0.1 μM) selectively inhibits ENT1/Ent1 while concentration 100 μM abolishes the

activity of both human and rat ENT1/Ent1 and ENT2/Ent2 (Chishu et al., 2008; Sai et al., 2008; Molina-Arcas et al., 2009; Nishimura et al., 2011; Nishimura et al., 2012; Karbanova et al., 2017). Pentobarbital (Nembutal) was purchased from Abbott Laboratories (Abbott Park, IL, USA). Solvent DMSO was obtained from Sigma-Aldrich (St. Louis, MO, USA) and its volume/volume concentrations was 0.1 % in all experiments. All other chemicals were of analytical grade. Bicinchoninic acid assay (BCA assay) reagents were purchased from Thermo Scientific (Rockford, USA) and Bradford Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines

The human choriocarcinoma-derived BeWo cell line was purchased from the European Cell Culture Collection (ECACC; Salisbury, Wiltshire, UK). Cells were cultured in Ham's F12 medium supplemented with 10% FBS (Karbanova et al., 2017). The cells were cultured at 37 °C under an atmosphere containing 5% CO_2.

Animals

Pregnant Wistar rats were purchased from MediTox s.r.o. (Konarovice, Czech Republic) and maintained under 12/12 h day/night standard conditions with water and chow pellets *ad libitum*. Experiments were performed on day 21 of gestation (counted from the day when copulation plug was found). Overnight-fasted rats were anesthetized by administering a dose of 40 mg pentobarbital/kg bodyweight (Nembutal; Abbott Laboratories, Abbott Park, IL) 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 carried out in accordance with the Guide for the Care and Use of Laboratory Animals (2011) and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

Sample collection of human and rat placentas

Third-trimester placentas were obtained from uncomplicated pregnancies (n = 14) following elective cesarean section at term (between weeks 38 and 41 of gestation). First-trimester placentas (n = 7) were acquired from voluntarily interruption of physiologically ongoing pregnancies between weeks 9 and 13 of gestation as described previously (Ahmadimoghaddam et al., 2013). All participants provided written informed consent. Rat term placentas were collected from five rats on day 21 of pregnancy (n = 5). The samples were frozen in liquid nitrogen immediately after surgery and then stored at -80 °C until analysis.

RNA isolation, and Reverse Transcription

Total RNA was isolated from weighed tissue samples or directly from BeWo cells using Tri-Reagent solution purchased from Molecular Research Centre (Cincinnati, OH, USA) according to the manufacturer's instructions. The purity of the isolated RNA was checked by the A260/A280 ratio and RNA integrity was confirmed by electrophoresis on a 1% agarose gel. The concentration of RNA was calculated by A260 measurement. RNA was converted into cDNA using the gb Reverse Transcription Kit from Generi Biotech s.r.o. (Hradec Kralove, Czech Republic) on a Bio-Rad T100^{TM} Thermal Cycler (Hercules, CA, USA).

Qualitative end-point PCR analysis

End-point PCR was carried out in BeWo cells and samples of rat/human term placentas to verify expression of target genes in our experimental models. cDNA (25 ng) was amplified in a 20 µl reaction volume using MyTaq Red DNA Polymerase (Bioline, Cat. No. BIO-21108) according to the manufacturer's instructions using a Bio-Rad T100^{TM} Thermal Cycler (Hercules, CA, USA). For amplification of human *SLC29A1* and *SLC29A2* in human placentas and BeWo cells, we employed primers designed by Yamamoto et al. (2007) that provide amplicons of 512 and

470 bp, respectively (Yamamoto et al., 2007). PCR analysis of rat placental samples was performed using 5'-CCAAGAGGAGGAAGAGAGGAATC-3' and 5'-TAAAGAGGGAGGGCAGGTAGTG-3' as the forward and reverse primers, respectively, for *SLC29A1*, and 5'-CCCACAGACACCTTCAACTTCA-3' and 5'-GTGCTGTAGGTAGAAGGCATGGT-3' as the forward and reverse primers for *SLC29A2*, providing amplicons of 400 and 302 bp, respectively. The PCR cycling conditions used were 95 °C for 3 min followed by 50 cycles of 95 °C for 30 s, 56 °C for 30s, and 72 °C for 45 s, followed by 10 minutes at 72 °C. Amplicons were analyzed on a 1.5% agarose gel labeled with GelRed Nucleic Acid Stain (Biotium, Hayward, CA, USA) using the HyperLadderTM 100 bp DNA length marker (Bioline, Taunton, MA, USA).

Quantitative PCR analysis

Quantitative PCR (qPCR) analysis of *SLC29A1* and *SLC29A2* expression in rat term placentas, BeWo cells, first- and third-trimester human placentas was performed using QuantStudio™ 6 (Thermo Fisher Scientific, Waltham, MA, USA). cDNA (25 ng) prepared from rat tissue and BeWo cells was analyzed in 20 µl reaction volumes in a 96-well plate. Human placental cDNA (10 ng) was amplified in a 384-well plate, with total reaction volumes of 10 µl per well. PCR was performed using the TaqMan® Universal Master Mix II without UNG (Thermo Fisher Scientific, Waltham, MA, USA) and predesigned TaqMan® Real Time Expression PCR assays for human (*h*) or rat (*r*) *SLC29A1* (Hs01085704_g1c, Rn01648953_m1) and *SLC29A2* (Hs00155426_m1, Rn01479421_m1). For greater precision during the mRNA quantification of target genes, data were normalized against the geometric mean of expression of two previously identified TaqMan® housekeeping genes: *B2M* (Hs00187842_m1) and *GAPDH* (Hs02758991_g1) for human samples, and *Ywhaz* (Rn00755072_m1) and *Gapdh* (Rn01775763_g1) for rat samples (Ahmadimoghaddam et al., 2013; Cerveny et al., 2016). Stable expression of both reference genes was verified before beginning the quantitative

analysis. Each sample was amplified in triplicate, using the following PCR cycling profile: 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Analyses of amplification efficiency (Pfaffl, 2001) for *SLC29A1* and *SLC29A2* yielded values of ≈ 2-fold increase/PCR cycle (data not shown), making it possible to compare the expression data for the two genes. Expression levels are reported in arbitrary units (a.u.) and were derived by normalizing the expression of the target gene against that of the reference genes (Radilova et al., 2009).

In vitro accumulation studies in BeWo cells

For uptake experiments, BeWo cells were seeded at a density of 3.5×10^5 on 24-well culture plates (TPP, Trasadingen, Switzerland) and cultured for three days until confluence with daily medium replacement. Uptake experiments were performed as previously described with modifications (Yamamoto et al., 2007; Karbanova et al., 2017). Briefly, experiments were performed in 0.25 ml of control (Na^+ containing) buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 25 mM Tris) or Na^+ free buffer (Na^+ was replaced by N-methyl-D-glucamine). Two model substrates of NTs, [^{3}H]-adenosine (17.4 nM) and [^{3}H]-thymidine (5 nM), were used as positive controls (Molina-Arcas et al., 2009) at a final activity of 0.4 μCi/ml. Time dependent uptakes of [^{3}H]-adenosine (17.4 nM), [^{3}H]-thymidine (5 nM), and [^{3}H]-abacavir (10 μM) in both Na^+ containing and Na^+ free buffers were assessed. Based on time course data, effect of NBMPR (0.1 μM or 100 μM) or uridine (5 mM) as control inhibitors on [^{3}H]-adenosine (17.4 nM) and [^{3}H]-abacavir (10 μM) accumulation was subsequently evaluated over 5 minutes and 1 minute interval, respectively. Before the start of the experiment, cells were pre-incubated for 10 minutes in the respective buffer with or without the above-mentioned inhibitors. Accumulation was stopped by quick aspiration of the radioactivity-containing buffer. Then cells were washed twice with 0.75 ml buffer containing the appropriate inhibitor, after which the cells were lysed in 0.02% SDS. The intracellular

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concentrations of radioisotopes were determined and normalized against the protein content (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA).

Ex vivo accumulation studies in fresh villous fragments from human placenta

Ex vivo analysis of [^{3}H]-abacavir uptake by the human placenta was performed using the method of accumulation in fresh villous fragments of human placentas at term (Atkinson et al., 2006; Greenwood and Sibley, 2006; Neumanova et al., 2015; Karbanova et al., 2017). Briefly, placentas were collected at term after uncomplicated pregnancies (after 38–40 weeks of gestation) from women at St. Mary's Hospital, Manchester or from the University Hospital, Hradec Kralove after receiving the women's written informed consent as approved by the Local Research Ethics Committees (REC 12/NW/0574 and 201006S15P, respectively). Small fragments of villous tissue were dissected within 30 minutes of delivery, appropriately washed in either Na^+ containing Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5.6 mM glucose, pH 7.4) or Na^+ free Tyrode's buffer (Na^+ was replaced by choline chloride) and then tied to hooks. Before the experiments, the fragments were stabilized for 30 minutes in a mixture of respective Tyrode's buffer and DMEM 1:1 (4 ml). Initially we determined the time courses of [^{3}H]-adenosine (0.5 μCi/ml; 21.8 nM), [^{3}H]-thymidine (0.5 μCi/ml; 6 nM), and [^{3}H]-abacavir (0.5 μCi/ml, 5 μM) uptakes in control buffer with/without NBMPR (100 μM) or in absence of Na^+ to confirm ENTs and CNTs activity in placental fresh villous fragments. Based on the time course data, we next investigated the effect of selected inhibitors (NBMPR at 100 μM and 0.1 μM, or uridine at 5 mM) on [^{3}H]-adenosine and [^{3}H]-abacavir uptake after five minutes of incubation. To terminate accumulation and remove extracellularly bound isotope, fragments were vigorously washed twice in an excess of ice-cold Tyrode's buffer containing the appropriate inhibitor for 15 s. Villous fragments were then stored in distilled water for 18 hours, and the quantity of

radioisotope released was determined. The fragments were then removed from the water and dissolved in 0.3 M NaOH for 10 h at 37 °C, after which fragment protein content was quantified using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Finally, the NaOH lysate was analyzed for tissue-bound radioisotopes; samples were excluded from further analysis if the lysate's radioisotope content was above 1% of the amount of tracer added.

Preparation of microvillous plasma membrane (MVM) vesicles and uptake assays

MVM vesicles were employed to directly analyze the role of ENTs in [^{3}H]-abacavir transport across the apical plasma membrane of the human syncytiotrophoblast layer. Human placentas were obtained as described in previous paragraph. MVM vesicles were isolated by Mg^{2+} precipitation and differential centrifugation (Glazier et al., 1988; Ceckova et al., 2016). The final MVM pellet was resuspended in intravesicular buffer (IVB; 290 mM sucrose, 5 mM HEPES and 5 mM Tris, pH 7.4), vesiculated by passing 15 times through a 25-gauge needle, stored at 4°C and used within three days of isolation or frozen at -80°C and equilibrated to room temperature on the day of experiment. Comparable uptake rate in fresh and thawed vesicles was verified before initiation of uptake experiments. The MVM protein concentration was determined using the BCA assay. Optimal purity was confirmed by measuring the enrichment of MVM alkaline phosphatase activity related to the placental homogenate, whereas vesicle orientation was evaluated by comparing specific alkaline phosphatase activity upon addition of detergent, as described previously (Glazier et al., 1988). The alkaline phosphatase enrichment factor was 25.2 ± 6.39 (mean ± SD, n = 8) and percentage of physiologically orientated vesicles was 87.2 ± 8.79 % documenting that potential contamination of the MVM vesicles with basal plasma membrane and/or intracellular membranes was negligible (Glazier et al., 1988; Mahendran et al., 1994; Godfrey et al., 1998; Harrington et al., 1999) and thus quality of MVM vesicles was sufficient for functional study of apically localized placental ENTs and CNTs. Uptake of [^{3}H]-adenosine (3.3 μCi/ml; 0.145 μM), [^{3}H]-thymidine (3.3 μCi/ml; 1 μM) and

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[^{3}H]-abacavir (0.5 μCi/ml; 5 μM) into MVM vesicles was measured at room temperature using rapid vacuum filtration approach (Glazier and Sibley, 2006). MVM vesicles (10-20 mg protein/ml) were equilibrated to room temperature (21–25 °C) prior to uptake. 10 μl MVM were pre-incubated for 10 minutes with or without inhibitor (0.1 μM, 100 μM NBMPR, 1 mM uridine or 1 mM adenosine) in extravesicular buffer (EVB: 145 mM NaCl, 5 mM HEPES and 5 mM Tris, pH 7.4; for Na^+-free buffer KCl was used instead of NaCl). Uptake of [^{3}H]-adenosine, [^{3}H]-thymidine, or [^{3}H]-abacavir was initiated by adding the substrate diluted in EVB to the pre-incubated MVM vesicles. Uptake was halted after defined time-points by adding 2 ml ice-cold stopping buffer (130 mM NaCl, 10 mM Na_2HPO_4, 4.2 mM KCl, 1.2 mM $MgSO_4$, 0.75 mM $CaCl_2$, pH 7.4) with 100 μM NBMPR where appropriate and subsequent filtering through a 0.45 μm mixed cellulose ester filter (MF-Millipore membrane filter HAWP02500) under vacuum. Filters were washed with 10 ml stopping buffer, and the filter-associated radioactivity was determined. No protein controls (in which the MVM vesicle protein was replaced with IVB) were analysed in parallel to determine tracer binding to the filter, which was subtracted from the total vesicle count. Unspecific binding of all three compounds to the plasma membrane was excluded by measuring time zero uptakes, showing comparable values to the no protein controls.

In situ dual perfusion of the rat term placenta

Transplacental transport of [^{3}H]-abacavir was measured using dually perfused rat term placentas in open- and closed-circuit setups as described previously (Neumanova et al., 2014; Neumanova et al., 2015; Ceckova et al., 2016; Neumanova et al., 2016; Karbanova et al., 2017; Cerveny et al., 2018). At the end of each experiment, the placenta was perfused with radioactivity-free buffer for 10 min, then excised from the uterine tissue and dissolved in

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Solvable tissue solubilizer (PerkinElmer, Waltham, MA, USA), after which tissue-bound [^{3}H]-abacavir was determined.

An open-circuit perfusion system was employed to assess the effect of NBMPR (0.1 μM and 100 μM) or uridine (5 mM) on maternal-to-fetal (M→F) and fetal-to-maternal (F→M) clearances of [^{3}H]-abacavir at an activity of 0.06 μCi/ml, corresponding to a concentration of 300 nM. The appropriate inhibitor was added to both reservoirs immediately after successful surgery, and [^{3}H]-abacavir was added to either the maternal (M→F studies) or the fetal (F→M studies) reservoir. After a five minutes stabilization period, sample collection was started (this experimental time point was designated 0 minutes). Fetal effluent samples were collected at five minutes intervals and placed in pre-weighed vials, after which [^{3}H]-abacavir concentration was determined and the transplacental clearance was calculated as described previously (Neumanova et al., 2014; Neumanova et al., 2015).

A closed-circuit (recirculation) perfusion system was employed to further study the involvement of ENTs in transplacental abacavir transport. The maternal and fetal sides of the placenta were infused with equal concentration of [^{3}H]-abacavir (0.06 μCi/ml, 300 nM). NBMPR (0.1 μM) was then added into both compartments and after a five-minute stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (250 μl) were then collected every 10 minutes from the maternal and fetal reservoirs.

Radioisotope analysis

The concentrations of [^{3}H]-adenosine, [^{3}H]-thymidine, and [^{3}H]-abacavir in experimental samples were measured by liquid scintillation counting (Tri-Carb 2910 TR; Perkin Elmer, Waltham, MA, USA). Tested concentrations of [^{3}H]-adenosine, [^{3}H]-thymidine, and [^{3}H]-abacavir differed among experimental procedures as the lowest possible concentrations providing sufficient activity measurable in respective experimental system were used; low

concentrations prevented transporter saturation guaranteeing method sensitivity. The radioisotopes have been previously used to investigate their interactions with membrane transporters (Pan et al., 2007; Shaik et al., 2007a; Giri et al., 2008; Chishu et al., 2008; Neumanova et al., 2015; Karbanova et al., 2017). The radioisotope quantitation of [^{3}H]-adenosine and [^{3}H]-thymidine is complicated by its extensive placental metabolism (Dancis et al., 1993; Acevedo et al., 1995). Since the major metabolites are not transported by ENTs (Osses et al., 1996), it can be assumed that these had negligible impact on the uptake studies.

Statistical analyses

Quantitative PCR data were processed using the non-parametric unpaired Mann-Whitney test or the parametric unpaired two-tailed Student's t test where appropriate. Results collected from accumulation studies in BeWo cells and dual perfusion studies on rat placentas were processed by parametric unpaired two-tailed Student's t test and one-way ANOVA after a *post hoc* Dunnett's multiple comparison test or . Uptake studies on fresh villous fragments and vesicles prepared from human placentas were analyzed using the non-parametric unpaired Kruskal-Wallis test following Dunn's multiple comparison test and multiple and non-parametric Wilcoxon matched-pairs signed rank tests, respectively. All statistical calculations were performed with Graph Pad Prism 6.0; *($p \leq 0.05$); ** $p \leq 0.01$); ***($p \leq 0.001$).

Results

End-point PCR analysis of *SLC*29A1 and *SLC29A2* mRNA expression in BeWo cells human and rat term placentas

We first confirmed the expression of *SLC29A1* and *SLC29A2* mRNA (encoding ENT1 and ENT2, respectively) in BeWo cells, human and rat term placentas. Amplicons specific for the

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primer pairs used (512 bp for *hSLC29A1* and 470 bp for *hSLC29A2*; 400 bp for *rSlc29a1* and 302 bp for *rSlc29a2*) were detected in all tested samples (Fig. 1).

In vitro investigation of nucleoside transporters' role in [^{3}H]-abacavir uptake using BeWo cells

BeWo cells were used as a simple *in vitro* model of the placental barrier to investigate potential roles of ENTs and CNTs in the transplacental kinetics of abacavir. We first analyzed time-dependent uptake of [^{3}H]-adenosine (17.4 nM), [^{3}H]-thymidine (5 nM), and [^{3}H]-abacavir (10 μM) (Fig. 2A, B, C) showing significant effect of Na^+ depletion on [^{3}H]-adenosine and [^{3}H]-abacavir. Subsequently, NBMPR (0.1 μM and 100 μM), uridine (5 mM) and Na^+ depletion were shown to reduce [^{3}H]-adenosine uptake over five minutes of incubation by more than 70% relative to an uninhibited control (Fig. 2D). These results confirmed the activity of ENT1 and CNTs in BeWo cells. All tested inhibitors affected one minute accumulation of [^{3}H]-abacavir in a similar but less pronounced fashion, reducing its uptake by around 30% (Fig. 2E) suggesting contribution of ENT1 and other Na^+ dependent transporters, most likely CNTs, to [^{3}H]-abacavir membrane transfer.

Ex vivo analysis of nucleoside transporters' role [^{3}H]-abacavir uptake by human fresh villous placental fragments

We performed *ex vivo* accumulation studies with fresh villous fragments isolated from the human placenta to evaluate the role of ENTs and CNTs in abacavir uptake. We observed that the placental fragments exhibited time-dependent increase in [^{3}H]-adenosine (21.8 nM) and [^{3}H]-thymidine (6 nM) accumulation without any significant effect of Na^+ depletion (Fig. 3A, B). It suggests none or negligible activity of CNTs in the apical membrane of the term placenta. NBMPR (100 μM) reduced time dependent accumulation of both model substrates confirming

ENT activity (Fig. 3A, B). Over a five minutes [^{3}H]-adenosine accumulation period the effect of treatment with NBMPR at 0.1 μM was similar to that for treatment at 100 μM (Fig. 3D), indicating that of ENTs, ENT1 is a dominant adenosine placental transporter; in contrast uridine (5 mM) had no significant effect (Fig. 3D).

The accumulation of [^{3}H]-abacavir was also time-dependent, however, insensitive to either the presence of NBMPR (100 μM) or depletion of Na^+ (Fig. 3C). Moreover, upon five minutes incubation period, none of the tested inhibitors revealed significant effect on [^{3}H]-abacavir uptake into fresh villous fragments after five minutes of incubation (Fig. 3E).

Investigation of nucleoside transporters' effects on [^{3}H]-abacavir uptake into human placental MVM vesicles

Because of the discrepancies between our *in vitro* and *ex vivo* results, we evaluated the role of ENTs and CNTs in [^{3}H]-abacavir uptake directly in MVM vesicles. [^{3}H]-adenosine (0.145 μM), [^{3}H]-thymidine (1 μM), and [^{3}H]-abacavir (5 μM) showed time-dependent accumulation into MVM vesicles (Fig. 4A, B, C). As observed in villous fragments, the uptakes were insensitive to Na^+ depletion confirming none or negligible functional expression of CNTs in the apical membrane of the term placenta. Addition of NMBPR (0.1 μM or 100 μM) or uridine (1 mM), caused significant reduction in the one-minute accumulation of [^{3}H]-adenosine (Fig. 4D); indicating the functional activity of ENTs in MVM vesicles. Similar inhibition pattern was observed in the case of [^{3}H]-abacavir (Fig. 4E) over one-minute uptake. Furthermore, we tested the inhibitory potential of adenosine (1 mM) to [^{3}H]-abacavir accumulation, revealing comparable effect to uridine (1 mM). Significant effect of 100 μM NBMPR on [^{3}H]-abacavir uptake into MVM vesicles was also observed over 5 and 10 minutes incubation (Fig. 4C).

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In situ open circuit perfusion of the rat term placenta; effect of ENTs on transplacental [^{3}H]-abacavir clearance

Because uptake studies performed *in vitro* and using MVM vesicles had indicated that ENTs may drive placental [^{3}H]-abacavir uptake, we investigated the ability of rat Ent(s) to facilitate abacavir transfer across the placental barrier. Treatment with NBMPR (0.1 μM, 100 μM), or uridine (5 mM) caused similar and significant changes in total [^{3}H]-abacavir transplacental clearance, reducing it by ≈ 50% in both the M→F (Fig. 5A) and F→M (Fig. 5B) directions. This strongly suggests that Ent1 does participate in abacavir transport across the rat term placenta into fetal circulation. In all cases, the proportion of placental tissue-bound [^{3}H]-abacavir was below 1% (data not shown).

In situ closed circuit perfusion of the rat term placenta; effect of NMBPR (0.1 μM) on fetal-to-maternal transport of [^{3}H]-abacavir at equilibrium

To further study transplacental abacavir transport, both sides of the placenta were perfused with the concentration of [^{3}H]-abacavir (300 nM) used in the closed circuit experimental setup and we analyzed the time course of [^{3}H]-abacavir fetal concentration (Fig. 6A). The fetal-to-maternal concentration ratio after 60 minutes of recirculation was subsequently quantified (Fig. 6B). NBMPR at a concentration of 0.1 μM significantly slowed F→M transport of [^{3}H]-abacavir (Fig. 6A), increasing the fetal-to-maternal concentration ratio (Fig 6B). This suggests that NBMPR (0.1 μM) might inhibit Ent1 or Ent1-like uptake of [^{3}H]-abacavir on the basal membrane of the syncytiotrophoblast in the rat term placenta, thus abolishing F→M transfer of [^{3}H]-abacavir against concentration gradient in rats.

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Quantitative RT-PCR analysis of *hSLC29A1/rSlc29a1* and *hSLC29A2/rSlc29a2* expression in first- and third-trimester human placentas, rat term placentas, and BeWo cells

To date, mRNA expression of placental *SLC29A/Slc29a* transporters has been quantitatively assessed only in term placentas of rats (Leazer and Klaassen, 2003; Nishimura et al., 2012), but not in BeWo cells or human first-/third-trimester placenta. The normalized expression of *hSLC29A1* in first- and third-trimester human placentas was significantly greater than that of *hSLC29A2* (by factor of around 30); the level of *hSLC29A2* mRNA was below the limit of detection in two samples of both first- and third- trimester placentas (Fig. 7A). For both *hSLC29A1* and *hSLC29A2*, the level of mRNA in the first-trimester placenta was similar to that in the third. However, both genes exhibited considerable inter-individual variability; in the case of *hSLC29A2*, the observed levels varied over two orders of magnitude (Fig. 7A). The expression of *rSlc29a1* in the rat term placentas was 15-fold stronger than that of *rSlc29a2* (Fig. 7B) further confirming the published data, and the expression of *hSLC29A1* in BeWo cells was 40-fold higher than that of *hSLC29A2* (Fig. 7C).

Discussion

To fully assess safety profile of pharmacotherapy in pregnant women, it is important to understand factors potentially affecting transplacental pharmacokinetics (Brownbill et al., 2016). As abacavir is a nucleoside analogue, we attempted for the first time to investigate the role of NTs in its transplacental transfer.

We first confirmed the presence of *SLC29A1* and *SLC29A2* mRNA in the BeWo cell line and human/rat term placentas (Fig. 1). Subsequently, we used the accumulation method in BeWo cells, a well-established *in vitro* model for studying drug interactions with placental ABCB1 and ABCG2 transporters (Utoguchi et al., 2000; Ceckova et al., 2006) as well as with SLC

transporters (Boumah et al., 1992; Mani et al., 1998; Yamamoto et al., 2007; Nabekura et al., 2015; Karbanova et al., 2017; Ma et al., 2017). The presence of functional ENTs in BeWo cells has previously been confirmed by binding assays with NBMPR and by observing the effect of NMBPR on the accumulation of thymidine (Boumah et al., 1992; Mani et al., 1998; Karbanova et al., 2017). Recently, we have shown that our clone of BeWo expresses *SLC28A2* and lower levels of *SLC28A3* mRNA (Jiraskova et al., 2018). Here, we observed the effect of Na^+ depletion on [^{3}H]-adenosine (CNT2 and CNT3 substrate), but not [^{3}H]-thymidine (CNT1 and CNT3 substrate) (Molina-Arcas et al., 2009) (Fig. 2A, B). Therefore, we suggest functional expression of only CNT2 in BeWo cells. As [^{3}H]-abacavir uptake into BeWo cells was sensitive to Na^+ depletion (Fig. 2C, E), we conclude that CNT2 may contribute to this phenomenon. Subsequently, we used [^{3}H]-adenosine to study the effects of particular inhibitors because its structure resembles abacavir more closely than does that of thymidine. NBMPR at both tested concentrations (0.1 μM, 100 μM) and uridine (5 mM) induced comparable reductions in [^{3}H]-adenosine uptake into BeWo cells (Fig. 2D), indicating that the observed effect was solely due to ENT1. Time-dependent uptake and a similar pattern of NBMPR effects was observed with [^{3}H]-abacavir , suggesting that abacavir uptake into BeWo cells is driven by ENT1 without any detectable contribution of ENT2 (Fig. 2C, E).

To further study the NTs-mediated placental uptake of abacavir, we investigated its accumulation into fresh villous fragments that have been previously used to study human placental amino acid transport (Greenwood and Sibley, 2006), the interactions of abacavir with ABCB1 and ABCG2 (Neumanova et al., 2015) and zidovudine/emtricitabine with ENTs (Karbanova et al., 2017). We validated this experimental model using [^{3}H]-adenosine and [^{3}H]-thymidine, observing time-dependent uptakes; significant sensitivity to treatment with 100 μM NBMPR confirmed functional ENT expression, however, we did not see any effect of Na^+ depletion (Fig. 3A, B). Five minutes experiments with [^{3}H]-adenosine in presence of

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NBMPR (0.1 μM) reinforced the conclusion that placental [^{3}H]-adenosine uptake is substantially driven by ENT1 (Fig. 3D). The overall influence of the tested inhibitors on [^{3}H]-abacavir accumulation was negligible (Fig. 3E). Lack of significant role of ENTs on [^{3}H]-abacavir uptake in villous fragments might be caused by lower affinity of abacavir to ENT1 compared to adenosine and/or higher lipophilicity (abacavir logP = 1.2 vs. adenosine logP = -1.05), potentially leading to increased uptake into other cell types (e.g. macrophages, endothelium, and/or smooth muscle cells) (Greenwood and Sibley, 2006).

Given the discrepancy between results in villous fragments and BeWo cells, we performed accumulation studies directly in human placenta-derived MVM vesicles (Glazier and Sibley, 2006). This method has previously been used to investigate the role of NTs in placental nucleoside uptake (Barros et al., 1991; Errasti-Murugarren et al., 2011). We observed time-dependent [^{3}H]-adenosine, [^{3}H]-thymidine, and [^{3}H]-abacavir uptakes and by comparing the inhibitory potencies of two NBMPR concentrations (0.1 μM and 100 μM), we confirmed ENT1-mediated uptake of [^{3}H]-adenosine and [^{3}H]-abacavir by the MVM vesicles (Fig. 4). On the other hand, the uptakes were insensitive to Na^+ depletion (Fig. 4), which is in line with previous findings drawn from studies in human (Barros et al., 1991) and rat MVM vesicles (Nishimura et al., 2012) and our observations in villous fragments (Fig. 3).

The different effects of Na^+ depletion in BeWo cells and *ex vivo* human placental models can be explained by three orders of magnitude higher expression of *SLC28A2* (coding for CNT2) in BeWo cells when compared with the human placenta (Jiraskova et al., 2018). Moreover, lack of CNTs effect seems to be in accordance with observation by Govindarajan et al., who did not detect any protein expression of CNTs in trophoblast layer of the term placenta (Govindarajan et al., 2007). As the levels of CNTs increase in the course of gestation (Jiraskova et al., 2018), we do not hypothesize that CNTs are involved in maternal-placental transfer in earlier phases of gestation either.

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In the next step, we employed an *in situ* model based on dually perfused rat placentas in open- and closed-circuit setups to evaluate the overall contribution of Ent(s) to transplacental abacavir kinetics at the organ level. Dual perfusion of the rat term placenta is an established and well-justified method that has been used to investigate the interactions of placental ABC and SLC transporters with various drugs including abacavir (Ahmadimoghaddam et al., 2012; Ahmadimoghaddam and Staud, 2013; Neumanova et al., 2014; Neumanova et al., 2015; Ceckova et al., 2016; Neumanova et al., 2016). ENTs in the placenta of Wistar rats have been functionally characterized by analyzing adenosine uptake from the maternal blood circulation into the feto-placental unit and into MVM vesicles prepared from the rat placenta (Nishimura et al., 2012). In the open-circuit setup, all tested inhibitors substantially reduced the total clearance of [^{3}H]-abacavir in the M→F and F→M directions, in a comparable manner (Fig. 5). These results suggest that ENT1 is an important transporter for more than just placental uptake, and that it also mediates the transport of abacavir into fetal circulation. Effect of Na^+ depletion could not be investigated in this model, as this condition deleteriously affected the rat term placenta causing edema and high pressure during the experiment. In closed circuit dual perfusion experiments, NBMPR (0.1 μM) significantly reduced fetal-to-maternal transport of [^{3}H]-abacavir (Fig. 6A, B). This was surprising because inhibiting apically localized Ent1 should have the opposite effect, further reducing the abacavir concentration in fetal circulation. However, our observation seems to be consistent with previous reports describing Ent1-like activity on the basal membrane of the syncytiotrophoblast (Barros et al., 1995; Govindarajan et al., 2007; Errasti-Murugarren et al., 2011). When considering our previously published data (Neumanova et al., 2015) and the data presented here, we hypothesize cross-talk among placental ABCB1, ABCG2, and ENT1 that might differ at concentration equilibrium and when the maternal abacavir concentration is higher than the fetal concentration (see Fig. 8).

In the next step, we quantified the mRNA expression in the placental models/tissues showing that *hSLC29A1* is the dominantly transcribed ENT in BeWo cells and in the first- and third-trimester human placenta (Fig. 7) and we also confirmed previously reported expression profile of *rSlc29a* expression in rat term placenta (Leazer and Klaassen, 2003; Nishimura et al., 2012). Low *hSLC29A2* placental expression may thus hamper capability of our experimental models to detect abacavir interactions with ENT2. In contrast to findings for other placental transporters (e.g. ABCB1, ABCG2, and CNTs) (Gil et al., 2005; Meyer zu Schwabedissen et al., 2006; Jiraskova et al., 2018), the expression of *hSLC29A1* and *hSLC29A2* do not change in the course of gestation (Fig. 7A). On the other hand, considerable inter-individual variability was observed for both genes (Fig. 7A). As the mRNA seems to correlate with ENT1 function as evidenced in pharmacoresistance studies (Giovannetti et al., 2006; Marce et al., 2006; Tsujie et al., 2007; Eto et al., 2013), we hypothesize that the observed placental mRNA variability might be reflected in protein/function level. Therefore, it may represent a potential reason for differences in reported cord-to-maternal blood concentration ratios (Chappuy et al., 2004; Best et al., 2006; Fauchet et al., 2014).

This study provides the first evidence that ENT1 is the dominant placental ENT isoform showing significant uptake of nucleosides while ENT2 and CNTs do not exhibit any activity on the apical side of the syncytiotrophoblast in the term placenta. We also showed for the first time that the expression of *SLC29A1* and *SLC29A2* mRNA is comparable in the first- and third-trimester placenta, although there is substantial inter-individual variability in the expression of both genes. Drug-drug interactions (e.g. with suggested substrates of ENTs ribavirin) and role of inter-individual variability in placental ENT1 expression in drug disposition into fetal circulation of ENTs substrates should be further investigated to guarantee safe and effective abacavir-based combination therapies in pregnancy.

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Authorship Contributions

L.C. and Z.P. contributed equally to this work

Participated in research design: Cerveny, Ptackova, Ceckova, Glazier, Greenwood, Karahoda, Staud

Conducted experiments: Ptackova, Ceckova, Karbanova, Jiraskova, Karahoda, Cerveny

Performed data analysis: Cerveny, Ptackova, Ceckova, Karahoda, Glazier, Greenwood

Wrote or contributed to the writing of the manuscript: Cerveny, Ptackova, Ceckova, Glazier, Greenwood, Karahoda, Staud

References

Acevedo CG, Rojas S, Ramirez M, and Bravo I (1995) Transport and metabolism of adenosine in the perfused human placenta. *Placenta* **16:**611-622.

Ahmadimoghaddam D, Hofman J, Zemankova L, Nachtigal P, Dolezelova E, Cerveny L, Ceckova M, Micuda S, and Staud F (2012) Synchronized activity of organic cation transporter 3 (Oct3/Slc22a3) and multidrug and toxin extrusion 1 (Mate1/Slc47a1) transporter in transplacental passage of MPP+ in rat. *Toxicol Sci* **128:**471-481.

Ahmadimoghaddam D and Staud F (2013) Transfer of metformin across the rat placenta is mediated by organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) protein. *Reprod Toxicol* **39:**17-22.

Ahmadimoghaddam D, Zemankova L, Nachtigal P, Dolezelova E, Neumanova Z, Cerveny L, Ceckova M, Kacerovsky M, Micuda S, and Staud F (2013) Organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) transporter in the placenta and fetal tissues: expression profile and fetus protective role at different stages of gestation. *Biol Reprod* **88:**55.

AIDSinfo (2017) Panel on Treatment of HIV-Infected Pregnant Women and Prevention of Perinatal Transmission. Recommendations for Use of Antiretroviral Drugs in Pregnant HIV-1-Infected Women for Maternal Health and Interventions to Reduce Perinatal HIV Transmission in the United States. Department of Health and Human Services. Available at http://aidsinfo.nih.gov/contentfiles/lvguidelines/PerinatalGL.pdf. Accessed 29 November 2017.

Atkinson DE, Sibley CP, Fairbairn LJ, and Greenwood SL (2006) MDR1 P-gp expression and activity in intact human placental tissue; upregulation by retroviral transduction. *Placenta* **27:**707-714.

Barros LF, Bustamante JC, Yudilevich DL, and Jarvis SM (1991) Adenosine transport and nitrobenzylthioinosine binding in human placental membrane vesicles from brush-border and basal sides of the trophoblast. *J Membr Biol* **119:**151-161.

Barros LF, Yudilevich DL, Jarvis SM, Beaumont N, Young JD, and Baldwin SA (1995) Immunolocalisation of nucleoside transporters in human placental trophoblast and endothelial cells: evidence for multiple transporter isoforms. *Pflugers Arch* **429:**394-399.

Best BM, Mirochnick M, Capparelli EV, Stek A, Burchett SK, Holland DT, Read JS, Smith E, Hu C, Spector SA, Connor JD, and Team PPS (2006) Impact of pregnancy on abacavir pharmacokinetics. *AIDS* **20:**553-560.

Boumah CE, Hogue DL, and Cass CE (1992) Expression of high levels of nitrobenzylthioinosine-sensitive nucleoside transport in cultured human choriocarcinoma (BeWo) cells. *Biochem J* **288 (Pt 3):**987-996.

Brownbill P, Chernyavsky I, Bottalico B, Desoye G, Hansson S, Kenna G, Knudsen LE, Markert UR, Powles-Glover N, Schneider H, and Leach L (2016) An international network (PlaNet) to evaluate a human placental testing platform for chemicals safety testing in pregnancy. *Reprod Toxicol*.

Ceckova M, Libra A, Pavek P, Nachtigal P, Brabec M, Fuchs R, and Staud F (2006) Expression and functional activity of breast cancer resistance protein (BCRP, ABCG2) transporter in the human choriocarcinoma cell line BeWo. *Clin Exp Pharmacol Physiol* **33:**58-65.

Ceckova M, Reznicek J, Ptackova Z, Cerveny L, Muller F, Kacerovsky M, Fromm MF, Glazier JD, and Staud F (2016) Role of ABC and Solute Carrier Transporters in the Placental Transport of Lamivudine. *Antimicrob Agents Chemother* **60:**5563-5572.

Cerveny L, Neumanova Z, Karbanova S, Havlova I, and Staud F (2016) Long-term administration of tenofovir or emtricitabine to pregnant rats; effect on Abcb1a, Abcb1b

and Abcg2 expression in the placenta and in maternal and fetal organs. *J Pharm Pharmacol* **68:**84-92.

Cerveny L, Ptackova Z, Durisova M, and Staud F (2018) Interactions of protease inhibitors atazanavir and ritonavir with ABCB1, ABCG2, and ABCC2 transporters: Effect on transplacental disposition in rats. *Reprod Toxicol* **79:**57-65.

Dancis J, Lee J, Mendoza S, and Liebes L (1993) Nucleoside transport by perfused human placenta. *Placenta* **14:**547-554.

Errasti-Murugarren E, Diaz P, Godoy V, Riquelme G, and Pastor-Anglada M (2011) Expression and distribution of nucleoside transporter proteins in the human syncytiotrophoblast. *Mol Pharmacol* **80:**809-817.

Eto K, Kawakami H, Kuwatani M, Kudo T, Abe Y, Kawahata S, Takasawa A, Fukuoka M, Matsuno Y, Asaka M, and Sakamoto N (2013) Human equilibrative nucleoside transporter 1 and Notch3 can predict gemcitabine effects in patients with unresectable pancreatic cancer. *Br J Cancer* **108:**1488-1494.

Fauchet F, Treluyer JM, Preta LH, Valade E, Pannier E, Urien S, and Hirt D (2014) Population pharmacokinetics of abacavir in pregnant women. *Antimicrob Agents Chemother* **58:**6287-6289.

Gil S, Saura R, Forestier F, and Farinotti R (2005) P-glycoprotein expression of the human placenta during pregnancy. *Placenta* **26:**268-270.

Giovannetti E, Del Tacca M, Mey V, Funel N, Nannizzi S, Ricci S, Orlandini C, Boggi U, Campani D, Del Chiaro M, Iannopollo M, Bevilacqua G, Mosca F, and Danesi R (2006) Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. *Cancer Res* **66:**3928-3935.

Giri N, Shaik N, Pan G, Terasaki T, Mukai C, Kitagaki S, Miyakoshi N, and Elmquist WF (2008) Investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) on

pharmacokinetics and central nervous system penetration of abacavir and zidovudine in the mouse. *Drug Metab Dispos* **36:**1476-1484.

Glazier JD, Jones CJ, and Sibley CP (1988) Purification and Na+ uptake by human placental microvillus membrane vesicles prepared by three different methods. *Biochim Biophys Acta* **945:**127-134.

Glazier JD and Sibley CP (2006) In vitro methods for studying human placental amino acid transport: placental plasma membrane vesicles. *Methods Mol Med* **122:**241-252.

Godfrey KM, Matthews N, Glazier J, Jackson A, Wilman C, and Sibley CP (1998) Neutral amino acid uptake by the microvillous plasma membrane of the human placenta is inversely related to fetal size at birth in normal pregnancy. *J Clin Endocrinol Metab* **83:**3320-3326.

Govindarajan R, Bakken AH, Hudkins KL, Lai Y, Casado FJ, Pastor-Anglada M, Tse CM, Hayashi J, and Unadkat JD (2007) In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta. *Am J Physiol Regul Integr Comp Physiol* **293:**R1809-1822.

Greenwood SL and Sibley CP (2006) In vitro methods for studying human placental amino acid transport placental villous fragments. *Methods Mol Med* **122:**253-264.

Griffiths M, Yao SY, Abidi F, Phillips SE, Cass CE, Young JD, and Baldwin SA (1997) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (ei) equilibrative nucleoside transporter from human placenta. *Biochem J* **328 (Pt 3):**739-743.

Harrington B, Glazier J, D'Souza S, and Sibley C (1999) System A amino acid transporter activity in human placental microvillous membrane vesicles in relation to various anthropometric measurements in appropriate and small for gestational age babies. *Pediatr Res* **45:**810-814.

Hong M, Schlichter L, and Bendayan R (2000) A Na(+)-dependent nucleoside transporter in microglia. *J Pharmacol Exp Ther* **292:**366-374.

Chappuy H, Treluyer JM, Jullien V, Dimet J, Rey E, Fouche M, Firtion G, Pons G, and Mandelbrot L (2004) Maternal-fetal transfer and amniotic fluid accumulation of nucleoside analogue reverse transcriptase inhibitors in human immunodeficiency virus-infected pregnant women. *Antimicrob Agents Chemother* **48:**4332-4336.

Chishu T, Sai Y, Nishimura T, Sato K, Kose N, and Nakashima E (2008) Potential of various drugs to inhibit nucleoside uptake in rat syncytiotrophoblast cell line, TR-TBT 18d-1. *Placenta* **29:**461-467.

Jiraskova L, Cerveny L, Karbanova S, Ptackova Z, and Staud F (2018) Expression of Concentrative Nucleoside Transporters (SLC28A) in the Human Placenta; Effects of Gestation Age and Prototype Differentiation-affecting Agents. *Mol Pharm*.

Karbanova S, Cerveny L, Ceckova M, Ptackova Z, Jiraskova L, Greenwood S, and Staud F (2017) Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine. *Placenta* **60:**86-92.

Kis O, Robillard K, Chan GN, and Bendayan R (2010) The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters. *Trends Pharmacol Sci* **31:**22-35.

Leazer TM and Klaassen CD (2003) The presence of xenobiotic transporters in rat placenta. *Drug Metab Dispos* **31:**153-167.

Li RW, Yang C, Chan SW, Hoi MP, Lee SM, Kwan YW, and Leung GP (2015) Relaxation effect of abacavir on rat basilar arteries. *PLoS One* **10:**e0123043.

Ma Z, Yang X, Jiang T, Bai M, Zheng C, Zeng S, Sun D, and Jiang H (2017) Multiple SLC and ABC Transporters Contribute to the Placental Transfer of Entecavir. *Drug Metab Dispos* **45:**269-278.

Mahendran D, Byrne S, Donnai P, D'Souza SW, Glazier JD, Jones CJ, and Sibley CP (1994) Na+ transport, H+ concentration gradient dissipation, and system A amino acid transporter activity in purified microvillous plasma membrane isolated from first-trimester human placenta: comparison with the term microvillous membrane. *Am J Obstet Gynecol* **171:**1534-1540.

Mani RS, Hammond JR, Marjan JM, Graham KA, Young JD, Baldwin SA, and Cass CE (1998) Demonstration of equilibrative nucleoside transporters (hENT1 and hENT2) in nuclear envelopes of cultured human choriocarcinoma (BeWo) cells by functional reconstitution in proteoliposomes. *J Biol Chem* **273:**30818-30825.

Mao Q (2008) BCRP/ABCG2 in the placenta: expression, function and regulation. *Pharm Res* **25:**1244-1255.

Marce S, Molina-Arcas M, Villamor N, Casado FJ, Campo E, Pastor-Anglada M, and Colomer D (2006) Expression of human equilibrative nucleoside transporter 1 (hENT1) and its correlation with gemcitabine uptake and cytotoxicity in mantle cell lymphoma. *Haematologica* **91:**895-902.

Meyer zu Schwabedissen HE, Grube M, Dreisbach A, Jedlitschky G, Meissner K, Linnemann K, Fusch C, Ritter CA, Volker U, and Kroemer HK (2006) Epidermal growth factor-mediated activation of the map kinase cascade results in altered expression and function of ABCG2 (BCRP). *Drug Metab Dispos* **34:**524-533.

Molina-Arcas M, Casado FJ, and Pastor-Anglada M (2009) Nucleoside transporter proteins. *Curr Vasc Pharmacol* **7:**426-434.

Nabekura T, Kawasaki T, Kamiya Y, and Uwai Y (2015) Effects of Antiviral Drugs on Organic Anion Transport in Human Placental BeWo Cells. *Antimicrob Agents Chemother* **59:**7666-7670.

Neumanova Z, Cerveny L, Ceckova M, and Staud F (2014) Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta. *AIDS* **28:**9-17.

Neumanova Z, Cerveny L, Ceckova M, and Staud F (2016) Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine. *Biopharm Drug Dispos* **37:**28-38.

Neumanova Z, Cerveny L, Greenwood SL, Ceckova M, and Staud F (2015) Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir. *Reprod Toxicol* **57:**176-182.

Nishimura T, Chishu T, Tomi M, Nakamura R, Sato K, Kose N, Sai Y, and Nakashima E (2012) Mechanism of nucleoside uptake in rat placenta and induction of placental CNT2 in experimental diabetes. *Drug Metab Pharmacokinet* **27:**439-446.

Nishimura T, Tanaka J, Tomi M, Seki Y, Kose N, Sai Y, and Nakashima E (2011) Enhancement of zidovudine transfer to molt-4 cells, a human t-cell model, by dehydroepiandrosterone sulfate. *J Pharm Sci* **100:**3959-3967.

Osses N, Pearson JD, Yudilevich DL, and Jarvis SM (1996) Hypoxanthine enters human vascular endothelial cells (ECV 304) via the nitrobenzylthioinosine-insensitive equilibrative nucleoside transporter. *Biochem J* **317 (Pt 3):**843-848.

Pan GY, Giri N, and Elmquist WF (2007) Abcg2/Bcrp1 mediates the polarized transport of antiretroviral nucleosides abacavir and zidovudine. *Drug Metabolism and Disposition* **35:**1165-1173.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29:**e45.

Radilova H, Libra A, Holasova S, Safarova M, Viskova A, Kunc F, and Buncek M (2009) COX-1 is coupled with mPGES-1 and ABCC4 in human cervix cancer cells. *Mol Cell Biochem* **330:**131-140.

Sai Y, Nishimura T, Shimpo S, Chishu T, Sato K, Kose N, Terasaki T, Mukai C, Kitagaki S, Miyakoshi N, Kang YS, and Nakashima E (2008) Characterization of the mechanism of zidovudine uptake by rat conditionally immortalized syncytiotrophoblast cell line TR-TBT. *Pharm Res* **25:**1647-1653.

Shaik N, Giri N, Pan G, and Elmquist WF (2007a) P-glycoprotein-mediated active efflux of the anti-HIV1 nucleoside abacavir limits cellular accumulation and brain distribution. *Drug Metabolism and Disposition* **35:**2076-2085.

Shaik N, Giri N, Pan G, and Elmquist WF (2007b) P-glycoprotein-mediated active efflux of the anti-HIV1 nucleoside abacavir limits cellular accumulation and brain distribution. *Drug Metab Dispos* **35:**2076-2085.

Staud F and Ceckova M (2015) Regulation of drug transporter expression and function in the placenta. *Expert Opin Drug Metab Toxicol***:**1-23.

Staud F, Cerveny L, and Ceckova M (2012) Pharmacotherapy in pregnancy; effect of ABC and SLC transporters on drug transport across the placenta and fetal drug exposure. *J Drug Target* **20:**736-763.

Thomas SH and Yates LM (2012) Prescribing without evidence - pregnancy. *Br J Clin Pharmacol* **74:**691-697.

Tsujie M, Nakamori S, Nakahira S, Takahashi Y, Hayashi N, Okami J, Nagano H, Dono K, Umeshita K, Sakon M, and Monden M (2007) Human equilibrative nucleoside transporter 1, as a predictor of 5-fluorouracil resistance in human pancreatic cancer. *Anticancer Res* **27:**2241-2249.

Utoguchi N, Chandorkar GA, Avery M, and Audus KL (2000) Functional expression of P-glycoprotein in primary cultures of human cytotrophoblasts and BeWo cells. *Reproductive Toxicology* **14:**217-224.

Yamamoto T, Kuniki K, Takekuma Y, Hirano T, Iseki K, and Sugawara M (2007) Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and Xenopus laevis oocytes expressing recombinant plasma membrane human nucleoside transporters. *Eur J Pharmacol* **557:**1-8.

Footnotes

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Figure legends

Figure 1 End-point PCR analysis of *hSLC29A1/rSlc29a1* and *hSLC29A2/rSlc29a2* mRNA expression in BeWo cells and term human and rat placentas. The expected sizes of the PCR products were 512 bp for *hSLC29A1*, 470 bp for *hSLC29A2*, 400 bp for *rSlc29a1*, and 302 bp for *rSlc29a2*.

Figure 2 *In vitro* accumulation study in choriocarcinoma-derived BeWo cells. Time dependent uptakes of [^{3}H]-adenosine (17.4 nM) (A), [^{3}H]-thymidine (5 nM) (B), and [^{3}H]-abacavir (10 μM) (C) in control (Na^+ containing) buffer and under Na^+ depletion showed that [^{3}H]-adenosine and [^{3}H]-abacavir, but not [^{3}H]-thymidine, intracellular concentrations, were reduced by Na^+ depletion. NBMPR (0.1 μM and 100 μM), uridine (5 mM), and Na^+ depletion significantly reduced intracellular concentrations of [^{3}H]-adenosine (D) and [^{3}H]-abacavir (E) over 5 min and 1 min accumulation, respectively. Each value is reported in pM/μg protein as the mean ± SD; n = 4 for time-dependent uptakes and n = 5 for accumulation in selected time points. The parametric unpaired two-tailed Student's t test (A-C) and one-way ANOVA with Dunnett's *post hoc* test (D and E) were used to evaluate difference from control samples. Statistical significance is denoted * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$).

Figure 3 *Ex vivo* accumulation studies in human placental fresh villous fragments. Time dependent uptakes of [^{3}H]-adenosine (21.8 nM) (A), [^{3}H]-thymidine (6 nM) (B), and [^{3}H]-abacavir (5 μM) (C) in control (Na^+ containing) buffer, under Na^+ depletion, or in presence of NBMPR (100 μM) showed that exposure to NBMPR (100 μM) resulted in decreased intracellular accumulation of [^{3}H]-adenosine and [^{3}H]-thymidine, but not [^{3}H]-abacavir, while Na^+ depletion did not exhibit any effect. Over a five-minute accumulation, uptake of [^{3}H]-adenosine was significantly slowed by the inhibitor NBMPR (0.1 μM and 100 μM) (D). None of the tested inhibitors significantly affected accumulation of [^{3}H]-abacavir (E). Data are

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presented as median of pM/μg protein with interquartile range; $n \geq 3$ for time-dependent accumulation and n = 6 for evaluation of effects of selected NTs inhibitors in 5 min time point ("n" is equal to number of placenta donors). Statistical significance was evaluated using the non-parametric Kruskal-Wallis test followed by *post hoc* Dunn's multiple comparison test. Significant differences relative to the control are labeled * ($p \leq 0.05$) and ** ($p \leq 0.01$).

Figure 4 *Ex vivo* accumulation studies in human MVM vesicles isolated from human term placentas. Time dependent uptake studies with [^{3}H]-adenosine (0.145 μM) (A), [^{3}H]-thymidine (1 μM) (B), and [^{3}H]-abacavir (5 μM) (C) in control (Na^+ containing) buffer, under Na^+ depletion, or in presence of NBMPR (100 μM) showed that Na^+ depletion did not cause any significant effect on [^{3}H]-adenosine, [^{3}H]-thymidine, or [^{3}H]-abacavir and NBMPR (100 μM) significantly slowed [^{3}H]-abacavir accumulation. One-minute accumulation of [^{3}H]-adenosine (D) and 5 μM [^{3}H]-abacavir (E) was significantly decreased in the presence of NBMPR (0.1 or 100 μM) or uridine (1 mM). Additionally, adenosine (1 mM) showed significant effect on [^{3}H]-abacavir uptake. The data are presented as median with interquartile range ($n \geq 3$ for time-dependent uptakes and n = 9 for 1 min accumulation studies). Significance was evaluated by multiple, non-parametric Wilcoxon matched-pairs signed rank tests; significant differences relative to the control are denoted * ($p \leq 0.05$) and ** ($p \leq 0.01$).

Figure 5 Open circuit perfusion experiments - effect of ENTs on transplacental clearance of [^{3}H]-abacavir (0.06 μCi/ml, 300 nM) in the M→F (A) and F→M (B) directions. Both NBMPR (at concentrations of 0.1 μM or 100 μM) and uridine (5 mM) significantly reduced transplacental [^{3}H]-abacavir clearance in both directions, implying Ent1 facilitates abacavir passage across the placenta. The proportion of [^{3}H]-abacavir bound to placental tissues was below 1% in all cases (data not shown). Data are presented as mean ± SD, n = 4. Significance was evaluated by one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test;

statistically significant differences from the control are denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$), or *** ($p \leq 0.001$).

Figure 6 Closed circuit perfusion experiments; effect of NMBPR (0.1 μM) on fetal-to-maternal transport of [^{3}H]-abacavir at equilibrium. We analyzed the time course of the fetal [^{3}H]-abacavir concentration (A), and used the fetal-to-maternal concentration ratio to quantify the drug's transport after 60 minutes of recirculation (B). Adding NBMPR (0.1 μM) to both compartments significantly slowed the overall [^{3}H]-abacavir transfer against the concentration gradient in the maternal-to-fetal direction, leading to a significant increase in the fetal-to-maternal concentration ratio after 60 minutes. Results are presented as mean ± SD. Significance was evaluated by one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test; significant differences relative to the fetal concentration (A) or results for control animals (B) are denoted by *** ($p \leq 0.001$), and are based on n = 3 measurements in all cases.

Figure 7 Quantitative RT-PCR analysis of *hSLC29A1/rSlc29a1* and *hSLC29A2/rSlc29a2* mRNA expression in first- and third trimester human placentas (A), rat term placentas (B), and BeWo cells (C). Gene expression levels were normalized against the geometric mean expression of two housekeeping genes - *GAPDH* and *B2M* for human samples, and *Gapdh* and *Ywhaz* for rat samples. Data are reported in arbitrary units (a.u.) as the median with interquartile range (A, B) or mean ± SD (C); *n* = 14 for third-trimester human placentas and BeWo samples, seven for first-trimester human placentas, and five for rat placentas. Statistical significance for human and rat placenta samples was evaluated using the non-parametric unpaired Mann-Whitney test; ** ($p \leq 0.01$), *** ($p \leq 0.001$). The parametric unpaired two-tailed Student's t test *** ($p \leq 0.001$) was used to evaluate significance for BeWo cells.

Figure 8 Hypothesized role of ENT1, ENT1-like, ABCB1, ABCG2 and passive diffusion in the transfer of abacavir across the syncytiotrophoblast when the maternal abacavir

concentration is higher than the fetal concentration (A) and at equilibrium (B). In the former case, ENT1 and passive diffusion transfer abacavir unidirectionally from the maternal to the fetal circulation, outweighing the efflux activity of ABCB1 and ABCG2. At equilibrium, ABCB1/ABCG2 activity probably creates transient concentration gradients on both poles of the syncytiotrophoblast that may drive passive abacavir uptake (via passive diffusion and diffusion facilitated by ENT1 on the apical membrane and ENT1-like on the basal membrane) back into the syncytiotrophoblast. However, when the intracellular concentration exceeds the fetal concentration, we assume that ENT1-like- and passive diffusion-mediated transport act in opposite directions. We thus speculate that passive transport of abacavir is unidirectional on the apical membrane but may be bidirectional on the basal membrane.

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Figure 1

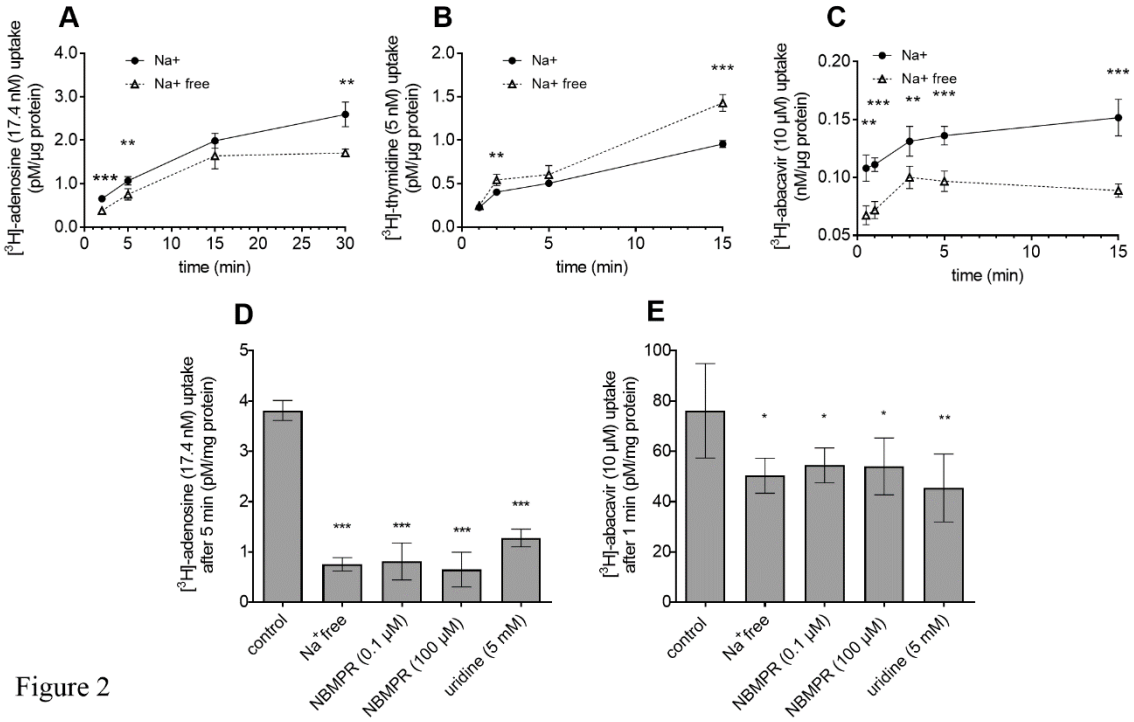

Figure 2

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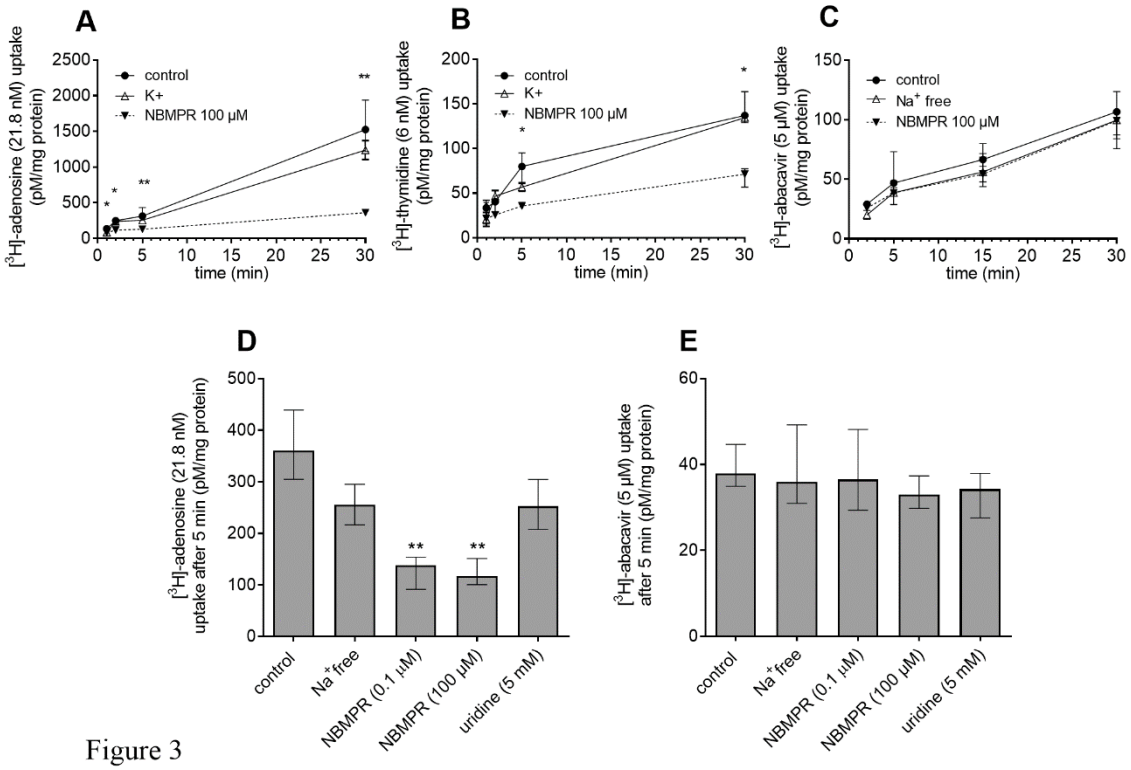

Figure 3

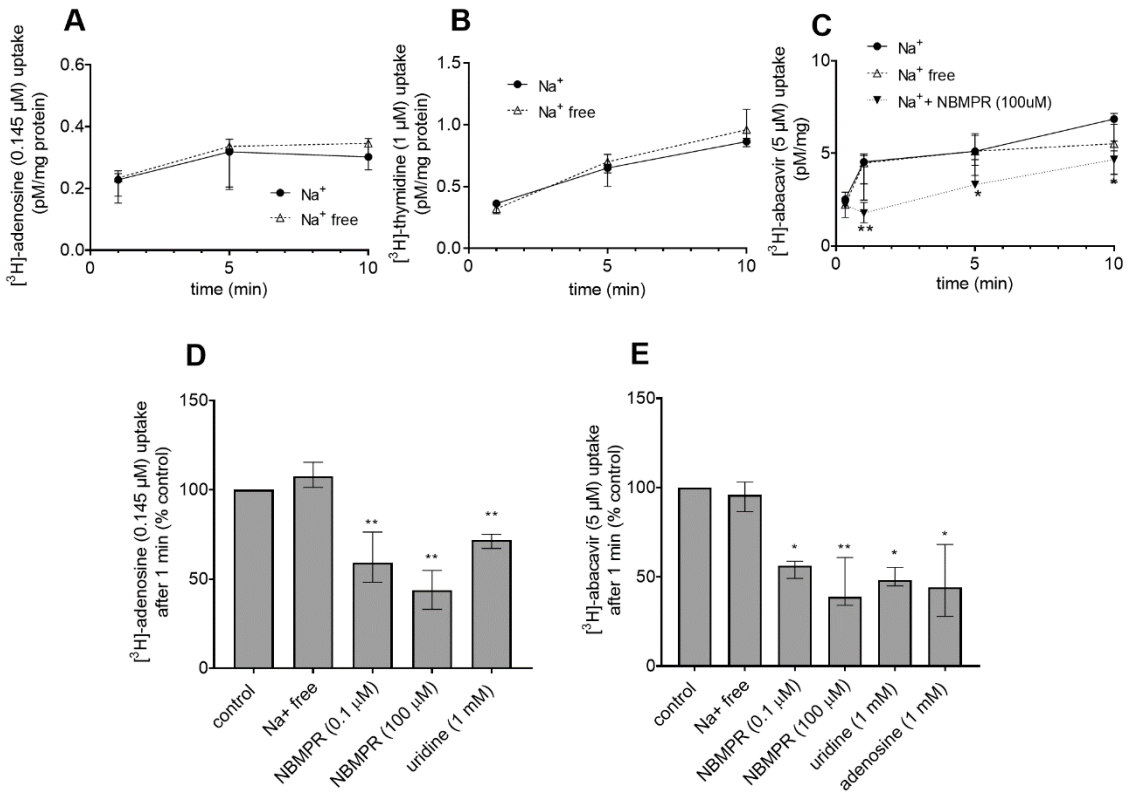

Figure 4

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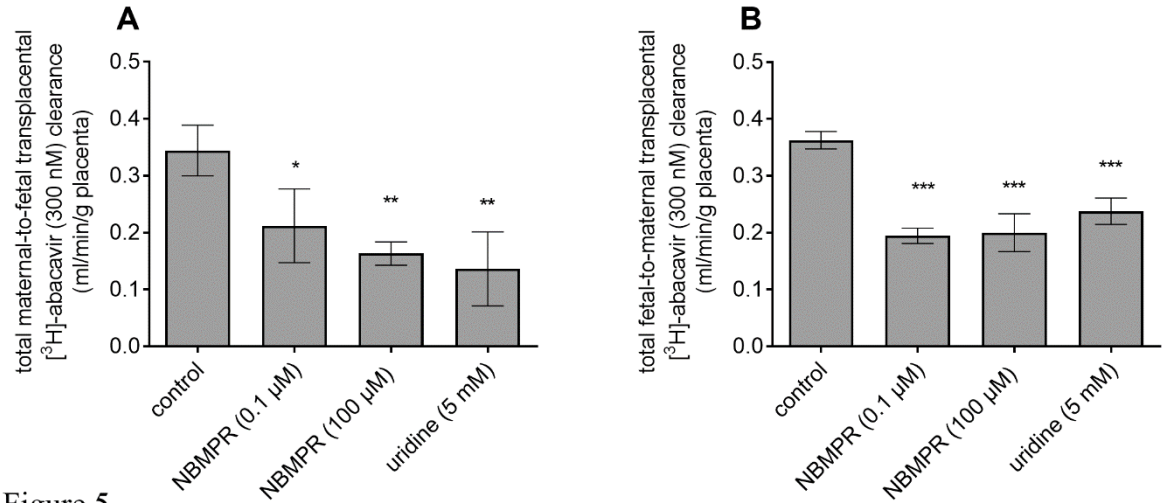

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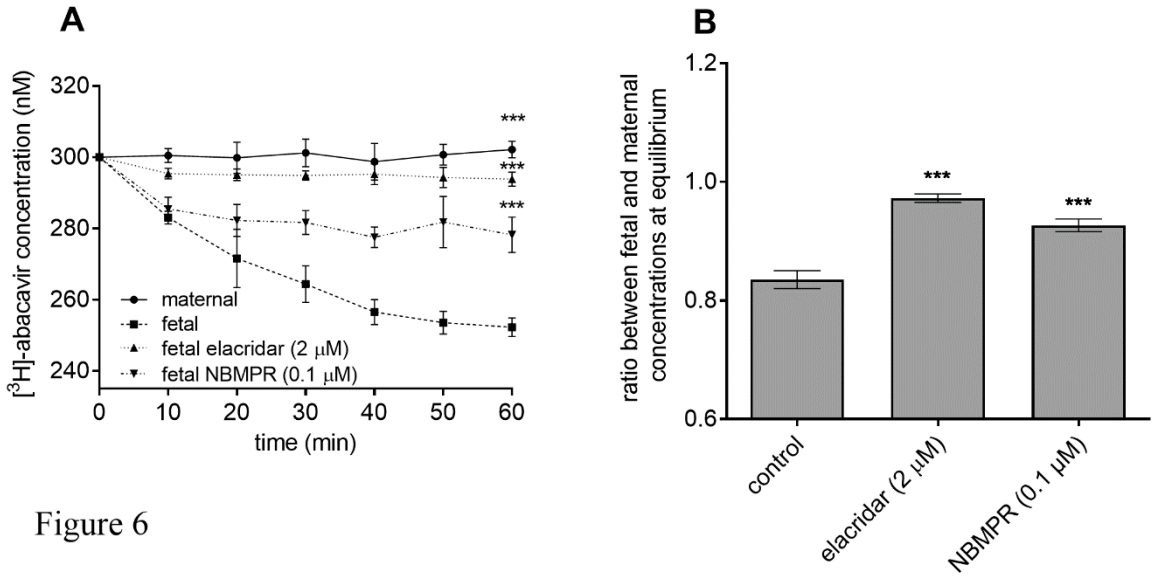

Figure 6

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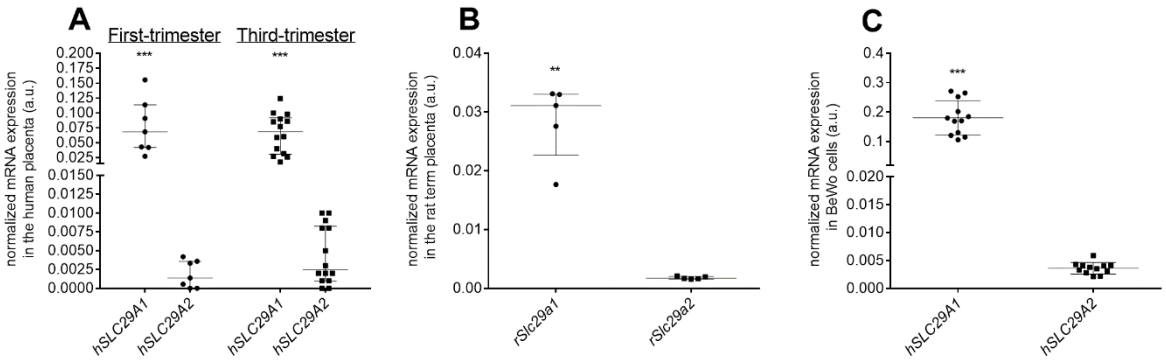

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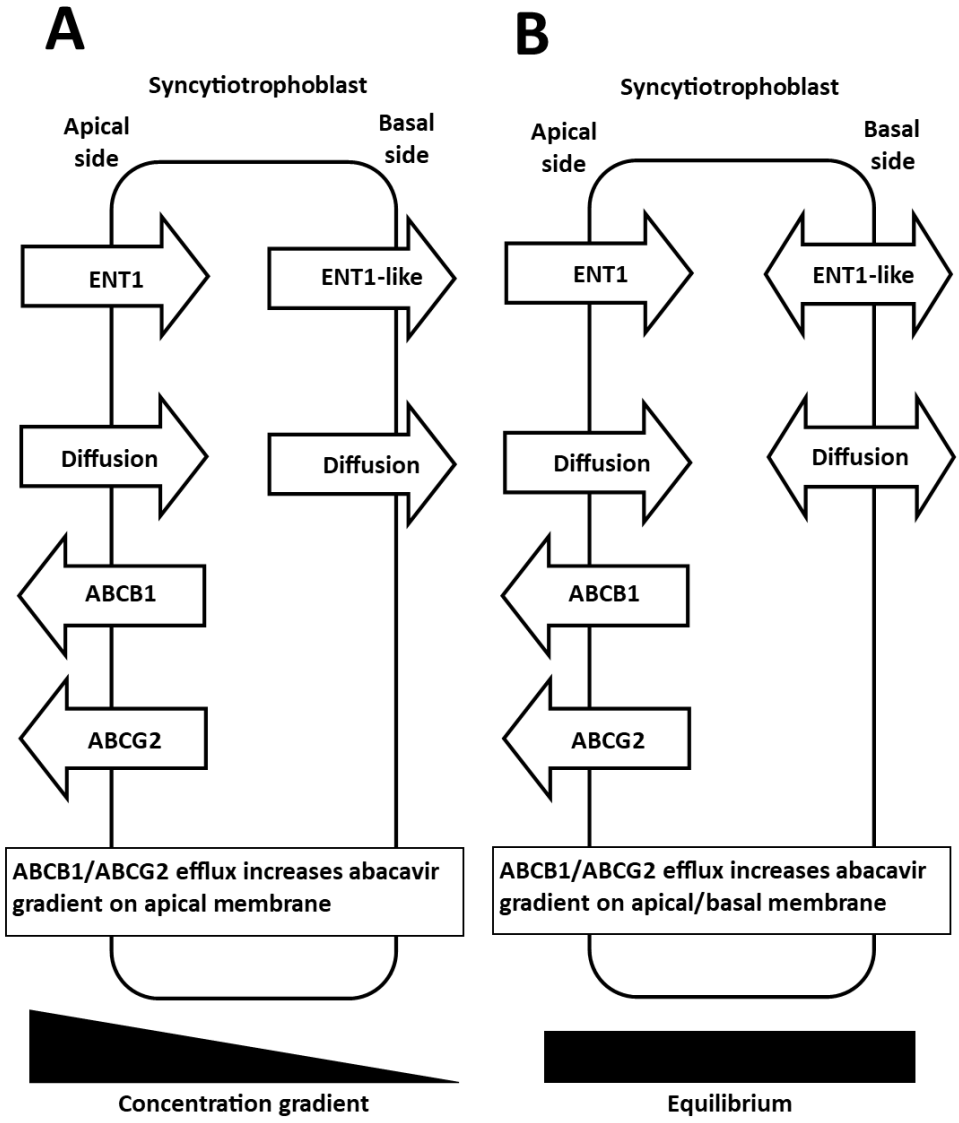

Figure 8