



Original article

Interplay of drug transporters P-glycoprotein (MDR1), MRP1, OATP1A2 and OATP1B3 in passage of maraviroc across human placenta



Lenka Tupova^a, Birgit Hirschmugl^b, Simona Sucha^a, Veronika Pilarova^c, Virág Székely^d,
Éva Bakos^d, Lucie Novakova^c, Csilla Özvegy-Laczka^d, Christian Wadsack^b, Martina Ceckova^{a,*}

^a Charles University, Faculty of Pharmacy in Hradec Kralove, Department of Pharmacology and Toxicology, Akademika Heyrovskeho 1203, Hradec Kralove, Czech Republic

^b Medical University of Graz, Department of Obstetrics and Gynecology, 8036, Graz, Austria

^c Charles University, Faculty of Pharmacy in Hradec Kralove, Department of Analytical Chemistry, Akademika Heyrovskeho 1203, Hradec Kralove, Czech Republic

^d Membrane Protein Research Group, Institute of Enzymology, Research Centre for Natural Sciences, Magyar tudósok krt. 2., H-1117, Budapest, Hungary

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ABSTRACT

Special attention is required when pharmacological treatment is indicated for a pregnant woman. P-glycoprotein (MDR1) is a well-known transporter localized in the maternal blood-facing apical membrane of placental syncytiotrophoblast and is considered to play an important role in protecting the developing fetus. Maraviroc, a MDR1 substrate that is registered for treatment of HIV infection, shows a low toxicity profile, suggesting favorable tolerability also if administered to pregnant women. Nevertheless, there is only poor understanding to date regarding the extent to which it permeates across the placental barrier and what are the transport mechanisms involved. Endeavoring to clarify the passage of maraviroc across placenta, we used in this study the method of closed-circuit perfusion of maraviroc across human placental cotyledon. The data obtained confirmed slight involvement of MDR1, but they also suggest possible interaction with other transport system(s) working in the opposite direction from that of MDR1. Complementary *in vitro* studies, including cellular experiments on choriocarcinoma BeWo cells as well as transporter-overexpressing MDCKII and A431 cell lines and accumulation in placental fresh villous fragments, revealed maraviroc transport by MRP1, OATP1A2, and OATP1B3 transporters. Based on mRNA expression data in the placental tissue, isolated trophoblasts, and fetal endothelial cells, especially MRP1 and OATP1A2 seem to play a crucial role in cooperatively driving maraviroc into placental tissue. By the example of maraviroc, we show here the important interplay of transporters in placental drug handling and its possibility to overcome the MDR1-mediated efflux.

1. Introduction

Research over the past few decades has made it clear that placenta should no longer be viewed as a mechanistic barrier between mother and fetus. Considerable progress has been made in understanding the transport role of this essential organ for maintaining pregnancy. In parallel with ensuring that nutrition is supplied to the developing tissues, the placenta needs to handle waste products and protect the fetus against potentially toxic xenobiotics, including drugs, which might be

present in maternal blood. Several transport proteins and carriers have been described in the placenta that mediate active and facilitated transfer of nutrients and other essential compounds in the maternal-to-fetal direction [1–3]. On the other hand, most of the xenobiotics are lipophilic in their structures and to some extent able to permeate the placenta by passive diffusion [4]. ATP-dependent transporters P-glycoprotein (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) present in the maternal blood-facing apical microvillous membrane of the trophoblast layer have the capability to efflux

Abbreviations: [³H]-MVC, radiolabeled maraviroc; A-B, apical-to-basolateral direction; B-A, basolateral-to-apical direction; BCRP or ABCG2, breast cancer resistant protein; CCR5, chemokine receptor type 5; DMEM, Dulbecco's modified Eagle's medium; ER, efflux ratio; FDA, Food and Drug Administration; GF or GF120918, elacridar; LY or LY335979, zosuquidar; MDCKII, Madin-Darby Canine Kidney II cells; MDR1 or ABCB1, P-glycoprotein; MRPs, multidrug resistant proteins; OATPs, organic anion transporters; OCTNs, organic carnitine transporters; Papp, permeable coefficient; PBS, phosphate buffered saline; Rr, relative ratio; SDS, sodium dodecyl sulphate; SLC, solute carriers

* Corresponding author at: Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Kralove, Charles University, Akademika Heyrovskeho 1203, 500 05, Hradec Kralove, Czech Republic.

E-mail address: martina.ceckova@faf.cuni.cz (M. Ceckova).

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potentially harmful compounds to the maternal blood and thereby protect the fetus [5–7]. These membrane transporters also constitute possible sites for drug–drug interactions. Therefore, the ability of any therapeutic compound to be transported by membrane proteins or to cause their inhibition needs to be assessed and drug–drug interactions considered, as emphasized by the International Transporter Consortium [8,9] and such global drug regulatory authorities as the U.S. Food and Drug Administration (FDA) and European Medicines Agency [10,11]. In the placenta, MDR1 and BCRP have been indicated to protect the fetus against various lipophilic drugs, including, for example, glyburide, digoxin, and vinca alkaloids [12–14]. In general, most of the drugs that are identified as MDR1 or BCRP substrates *in vitro* are assumed to be compromised in their transfer to fetus. Compared to MDR1 or BCRP, less is known about the placental multidrug resistant proteins (MRPs) transporters, among which MRP2 (ABCC2) and MRP1 (ABCC1) seem to be the most-expressed members of this subfamily. While MRP2 shows the same localization as do MDR1 and BCRP, and, similarly to these two transporters, it is believed to ensure efflux in the fetal-to-maternal direction [15], MRP1 has been detected mainly on the basal membrane of trophoblast and fetal endothelial cells [16–19].

In addition to the ABC drug efflux transporter family, the solute carriers (SLC) also are able to interact with drugs and their inhibition may lead to clinically relevant drug–drug interactions [20–22]. Several members of the organic anion-transporting polypeptide (OATP) and organic carnitine (OCTNs) uptake transporters are recognized for their roles in placental handling of various endogenous compounds, including L-carnitine [23,24]. Among these, mainly OATP2A1, OATP4A1 [25,26], and OCTN2 [27] have been described in the apical microvillous membrane of human placenta, while OATP2B1 seems to ensure influx through the opposite, basal membrane of trophoblast cells [28]. In addition to these carriers, lower expression of several other OATP members, e.g., OATP1A2, OATP1B3, and OATP3A1 [29–31], and OCTN1 [32] also has been reported in human placenta.

Many antiretroviral compounds have recently been identified as substrates of MDR1 and/or BCRP *in vitro* [33–36], thus demonstrating that placental ABC transporters-mediated drug–drug interactions might affect drug levels in fetal circulation [37]. In particular, administration of antiretroviral therapy to ideally all HIV-positive pregnant women is a much-discussed topic, because it decreases the risk of mother-to-child viral transfer from 45 % down to 1–2% or less [38,39]. Although the approach to the treatment is itself becoming more modern and effective, the introduction of newer drugs for use during pregnancy is scarcely advancing, and traditional therapeutics with well-known toxicity and safety profiles are preferred in order to avoid potential harmful effects to the fetus. Interestingly, however, a recent study comparing a recommended combination of three common antiretroviral drugs with an alternative variant showed no significant difference in incidence of adverse birth outcomes [40].

The chemokine receptor 5 (CCR5) inhibitor maraviroc blocks HIV from binding to the host cells and, due to its low toxicity, it has been approved not only for use in adults infected by the R5-tropic virus but also for the treatment of children [41]. Because sufficiently conclusive results are still lacking, however, maraviroc is not yet recommended in antiretroviral pharmacotherapy schedules for pregnant women [39]. Nevertheless, several case reports seem to be optimistic as they describe births of healthy, uninfected neonates of women exposed to maraviroc during pregnancy [42]. Importantly, the chemokine system plays a key role in the control of inflammatory and immune processes. Therefore, in addition to controlling HIV, maraviroc seems to bring therapeutic benefit also in non-AIDS related comorbidities – e.g. atherosclerosis [43] – and is considered as well in several off-label indications, including multiple sclerosis, rheumatoid arthritis, and some cancer indications [44–46].

In a very recent study, we identified maraviroc as a substrate of human MDR1 *in vitro*. Employing *in situ* dually perfused rat term placenta, significant rat Mdr1-mediated efflux of the drug to the maternal

compartment was observed, although this was subsequently abolished in the presence of MDR1/Mdr1 inhibitors [47]. The primary aim of this study was to address the protective role of MDR1 in the transfer of maraviroc across human placenta using the well-established model of closed-circuit perfusion of maraviroc across a single intact placental cotyledon. Based on the results indicating possible interplay of several other transport systems in the placenta that compromise the fetal-to-maternal efflux mediated by MDR1, we subsequently employed several *ex vivo* and *in vitro* studies in human placental tissue and relevant cell lines in order to identify the contributing transport mechanisms.

2. Material and methods

2.1. Human placentas

Placentas from normal pregnancies were collected at the Department of Obstetrics and Gynecology, Medical University of Graz and used immediately after delivery or caesarean section. All women were medication-free and negative for HIV and hepatitis B or C. The study was approved by the institutional ethical committee (no. 24-529 ex 11/12) and informed consent was provided by all participants involved in the study. The placentas for experiments in villous fragments were collected at the Faculty Hospital Hradec Kralove after delivery following uncomplicated pregnancies. Written informed consent was obtained as approved by the local research Ethics Committee (approval no. 201006S15 P).

2.2. Cell lines and generation of cell-based carrier systems

The human choriocarcinoma BeWo cell line, clone b30 was kindly provided by Prof. Christiane Albrecht (University of Bern, Switzerland) with kind permission from Dr. Alan Schwartz (Washington University, USA). Madin-Darby Canine Kidney II (MDCKII) cell lines, both parental and overexpressing human MRP1 transporter, were obtained from Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, Netherlands). Human epidermal cell lines A431, both control and overexpressing human OATP1A2, -1B3, -2A1, -2B1, or -3A1 transporters, were generated in the laboratories of the Institute of Enzymology (RCNS, Hungary) according to well-established protocols. Plasmid constructs of *SLCO2B1* and -1B3 were prepared and introduced in order to create the A431-OATP2B1 and -1B3 cell lines as described previously [48]. OATP1A2 overexpression was achieved as described by Bakos et al. [49]. A431 cell lines overexpressing OATP2A1, OATP3A1, or OATP4A1 were constructed as part of the current study. Briefly, the cDNA of human *SLCO2A1* (BC041140, plasmid ID: HsCD00338568, Harvard Plasmid Repository) was PCR-amplified using Phusion® High-Fidelity PCR Kit (NEB, Ipswich, MA, US) and the following primers: forward 5' GTAA ATGCGGCCGCAAGAATTGCGCCACCATGGGACCCAGGATAGG and reverse 5' GTACATGCGGCCGCTAAGCTTTACACTCGGGAATCCTC. The PCR fragment was cloned between the NotI HindIII sites of the pSB vector [48]. The cDNAs of *SLCO3A1* and *SLCO4A1* were amplified from the pAcUW-21-L/OATP3A1 and pAcUW-21-L/OATP4A1 vectors [50] with the following primers: 3A1 forward 5' TAAAGGATCCGCGGCCG CGCCACCATGCAGGGGAAGAAGCCG, 3A1 reverse 5' CATGTCTCGAG ACTAGTAAGCTTCTATAAAACGGACTCCATG, 4A1 forward 5' TAAAG GATCCGCCACCATGAAGAGCGCCAAAGGT, and 4A1 reverse 5' CATG TAACTAGTTACACCTTCTTTTACTATTTTG. PCR fragments were digested with BamHI and SpeI enzymes (NEB) and were cloned into the corresponding sites of the pRRL-CMV-MCS-IRES-ΔCD4 vector [48]. The base order of the cDNAs in all constructs was verified by sequencing. Empty pRRLΔCD4 or pSB vectors were used as negative controls. In the case of OATP2A1, A431 cells (ATCC) were transfected using a Fugene HD reagent (Promega, Madison, WI, USA.) according to the supplier's protocol and as described by [48]. *SLCO3A1* or *SLCO4A1* overexpression in A431 cells was achieved by lentiviral transduction [48]. Protein expression of all cloned OATPs in the particular cell lines was

confirmed by western blotting analysis (data not shown).

All cell lines were cultivated in antibiotic-free, complete high-glucose Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine, supplemented with 10 % fetal bovine serum at 37 °C with 5% CO₂ and regularly tested for *Mycoplasma* contamination. Cells from passages 5–20 for the MDCKII and A431 cell lines and passages from 5 to 15 for the BeWo cells were used in the experiments. Dimethyl sulfoxide concentrations in all the assays did not exceed 0.5 %.

2.3. Chemicals and reagents

Radiolabeled maraviroc ([³H]-MVC) was purchased from Moravex Biochemicals (Brea, CA, USA). DMEM, fetal bovine serum, sodium dodecyl sulfate (SDS), calcein-AM, dextran from *Leuconostoc* spp. (M_r ≈ 40,000), phosphate buffered saline (PBS), and scintillation cocktail were bought from Sigma-Aldrich (St. Louis, Missouri, USA). Opti-MEM®, reduced serum medium, Cascade Blue hydrazide, and the biconinonic acid (BCA) assay were obtained from Thermo Fisher Scientific. Maraviroc and ritonavir were provided by the NIH AIDS Reagent Program or purchased from MedChemExpress (Monmouth Junction, NJ, USA), which was also the source of control inhibitors elacridar (GF120918), zosuquidar (LY335979), verapamil, sulfasalazine, L-carnitine, and MK-571. All the salts necessary for preparation of perfusion and Thyrode's buffers were purchased from Merck (MerckGroup, Germany), and fluorescein-methotrexate triammonium salt was from Biotium (Hayward, CA, USA).

2.4. Ex vivo perfusion of human placenta

The *ex vivo* placental perfusion setup used herein has been described previously [51–53] and was adapted for the current study. Briefly, within 30 min after delivery, a single unimpaired cotyledon was selected and the supplying chorionic artery and vein pair was cannulated and immediately rinsed with perfusion medium (DMEM:Earls buffer 3:1; containing 15 g/L dextran, 2 g/L glucose, and 250 mg/mL amoxicillin; Sigma-Aldrich). Prepared cotyledon was placed in the middle of a perfusion chamber and the fetal arterial cannula was connected to a perfusion medium reservoir (37 °C). The maternal circulation was established by insertion of three rounded needles into the intervillous space of the cotyledon. Constant fetal (3 mL/min) and maternal (9 mL/min) artery inflows were generated using a magnetic pump (Codan, Salzburg, Austria). Flow recovery of the fetal circulation was ≥ 95 % in all experiments. In order to approximate *in utero* oxygen levels, fetal perfusion medium was pretreated with a gas mixture (95 % N₂, 5% CO₂) through a gas-exchange device (Living Systems, St. Albans, VT, USA) during the experiment. The back-flow pressure in the fetal vascular system was recorded in line during the experiment by a micro catheter pressure sensor (Millar, Houston, TX, USA) inserted into the fetal arterial cannula.

2.4.1. Settings of perfusion experiments

The experimental time slots were set based on the findings from other, similar experiments evaluating the contribution of ABC transporters to placental drug transfer [13]. A single perfusion experiment setup was initiated with a 90 min fetal and maternal closed-circuit period when maraviroc (600 ng/mL) was added to both circulations (minutes 0–90). This was followed by 30 min washout with maraviroc-free media (minutes 90–120), 30 min of inhibitor pre-incubation (minutes 120–150), and 90 min of maraviroc with MDR1 inhibitors elacridar (2 μM) or ritonavir (10 μM) (minutes 150–240), all applied to both compartments. Samples were taken every 30 min from fetal vein and maternal artery in volume of 1.5 mL. Samples were centrifuged to separate perfusate from the remaining blood elements or residual tissue and stable isotopically labelled internal standard (SIL-IS) of maraviroc (100 ng/mL, maraviroc D6, TRC, Canada) was added into 1 mL of clear supernatant. Completed samples were stored at –80 °C in a freezer

until analysis. After the experiment period, tissue integrity was tested by a 30 min open-circuit antipyrine period. Antipyrine concentration was determined by high-performance liquid chromatography (HPLC-UV) in accordance with the protocol by [54] in maternal and fetal perfusates. Only perfusion experiments reaching fetal-to-maternal antipyrine ratios ≥ 0.3 within 30 min in a fetal open circuit were considered as successful experiments and were further processed.

2.4.2. Analysis of maraviroc in perfusates

The determination of maraviroc in placental perfusion medium was carried out using the Acquity Ultra Performance LC (UPLC) system (Waters, Milford, MA, USA) coupled with a Micromass Quattro micro API benchtop triple quadrupole mass spectrometer (Waters, Manchester, UK). Samples of 2 μL were injected onto an Acquity BEH C18 analytical column (50 × 2.1 mm i.d.; particle size 1.7 μm). The analytes were separated using gradient elution with 0.1 % aqueous formic acid (eluent A) and acetonitrile (eluent B) at a flow rate of 0.35 mL/min. The gradient started with 5% of eluent B in A, and it was increased to 95 % B in 3 min. The percentage of eluent B was reduced to the original conditions of 5% at 3.1 min. The total time of chromatographic separation, including column equilibration, was 5 min. Quantification of analyte was achieved via selected reaction monitoring using the precursor ion [M + H]⁺ with *m/z* = 514.1 and two selected fragment ions including quantifier *m/z* = 388.9 and qualifier ion *m/z* = 280.0 to increase method selectivity. Ion source parameters were set up as follows: capillary voltage – 1.0 kV, extractor – 3.0 V, RF lens – 0.1 V, ion source temperature – 130 °C, cone voltage – 35 V. The desolvation gas (nitrogen) flow was 1,000 L/h and temperature 450 °C. Nitrogen was also used as a cone gas with a flow rate of 100 L/h. Collision energy was set individually for each selected reaction monitoring transition at 20 eV (514.1 → 388.9) and at 30 eV (514.1 → 280.0). The data was acquired using MassLynx 4.1 software and processed by QuanLynx. The sample concentrations of maraviroc reported in ng/mL were converted into percentages of the respective initial stock solution concentration (100 %).

The pretreatment of 200 μL perfusion samples involved liquid-liquid extraction using dichloromethane in a 1:2 (v/v) ratio as an extraction agent. The sample was agitated (1400 rpm, 30 °C, 10 min) and centrifuged (14,000 rpm, 4 °C, 5 min). Subsequently, 400 μL of extract was taken, evaporated to dryness using nitrogen, and reconstituted in 20 % acetonitrile. The samples containing maraviroc were quantified using a matrix calibration curve and SIL-IS.

2.4.3. Detection of maraviroc metabolites

To determine whether maraviroc may be metabolized by human placenta tissue during perfusion experiments, detailed screening for maraviroc metabolites was performed. Presence of metabolites was screened using a UHPLC-HRMS (high-resolution mass spectrometry) method. The Acquity UPLC I-class (Waters, USA) UHPLC system was coupled to a Synapt G2Si (Waters, UK) of q-TOF type. Chromatography was carried out using an Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm) column and gradient elution with acetonitrile and 0.1 % formic acid in water. Maraviroc is believed predominantly to be metabolized to various hydroxy metabolites by oxidation on phenyl or difluorocyclohexyl rings and to a few decomposition products [55,56]. The *m/z* value 530.3301 of protonated molecule and its possible adducts were searched using reconstructed ion chromatograms (mass tolerance 10 ppm) in electrospray ionization-positive mode corresponding to the summary formula of C₂₉H₄₁F₂N₅O₂. For the decomposition products, the chemical formulae C₁₃H₂₂N₄O and C₁₃H₂₂N₄ were expected [55] and the *m/z* of 251.1866 and 235.1917 for protonated molecules were searched together with possible adducts. The column temperature was kept at 40 °C and autosampler temperature at 10 °C. High-resolution, fullscan measurements were carried out in electrospray positive ion mode in the range of 50 to 1,200 *m/z*. The ion source conditions were set up as follows: capillary voltage – 0.8 kV, sampling cone voltage – 40 V, source

offset – 80 V, source temperature – 120 °C, desolvation temperature – 600 °C, desolvation gas flow – 500 L/h, cone gas flow – 50 L/h, and nebulizer gas flow – 6 bar. Leucine enkephalin was used as a lock mass reference for internal calibration and sodium formate for external calibration. MassLynx 4.1 software was used for data acquisition.

2.5. Isolation of trophoblast and fetoplacental endothelial cells from human term placenta

Endothelial cells were isolated from arterial vessels dissected from the apical surface of the chorionic plate of human healthy-term placenta following the protocol published by [57]. Arterial endothelial cells were cultivated in a 75 cm² cultivation flask until confluence (4–5 days), then harvested for RNA isolation.

The primary trophoblast cells were isolated from four human term placentas after uncomplicated pregnancy and delivery. Rinsed villous placental tissue was digested by a mixture of trypsin and DNase I and the released cells were captured at the top of the percoll gradient. After the centrifuging step, cells were purified using immunomagnetic beads conjugated with MCA-81 antibody against HLA-A, B, and C (Serotec, Puchheim, Germany) [58]. Trophoblast cells were seeded into 75 cm² flasks (23×10^6), cultivated for 48 h, then isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany).

2.6. RNA isolation, reverse transcription, and quantitative PCR analysis

A piece of tissue was sampled from each perfused placenta and processed for subsequent use in detecting gene expression of selected placental transporters. In order to evaluate the contribution of trophoblast cells and fetal endothelial cells to the expression, these cells were isolated from placental tissue as described above and subjected to the RT-qPCR analysis. Thereby, we verified the mRNA expression of relevant transporters in BeWo cells. TriReagent (Molecular Research Center, Cincinnati, OH, USA) and Gb Reverse Transcription Kit (Generi Biotech, Hradec Kralove, Czech Republic) were used for RNA isolation and reverse transcription following the manufacturer's instructions. The cDNA samples thusly obtained were evaluated by PCR using TaqMan® Universal Master Mix II without uracil-N-glycosylase (Thermo Fisher Scientific) and predesigned TaqMan® Real Time Expression PCR assays for human *ABCB1* (Hs00184500_m1), *ABCC1* (Hs01561483_m1), *SLCO1A2* (Hs00366488_m1), *SLCO1B3* (Hs00251986_m1), *SLCO2A1* (Hs01114926_m1), *SLCO2B1* (Hs01030343_m1), *SLCO3A1* (Hs00203184_m1), and *SLCO4A1* (Hs00249583_m1) (Applied Biosystems, Thermo Fisher Scientific USA) and then analyzed using the QuantStudio™ 6 system (Thermo Fisher Scientific). Each sample was amplified in triplicate using the following PCR cycling profile: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Expression levels are reported as number of gene copies per µg of transcribed RNA based on a five-point concentration calibration curve of a randomly chosen comparator placental sample. This sample was also analyzed for the number of gene copies using droplet digital PCR assay as described previously [59]. Briefly, the ddPCR™ Supermix for Probes (Bio-Rad, Hercules, CA, USA) and the predesigned TaqMan assays (Thermo Fisher Scientific) stated above were used. The analysis was performed using the QX200 Droplet Generator, T100™ Thermal Cycler, and QX200™ Droplet Reader, all from BioRad. According to the number of positive droplets compared to negative, the concentration of each of the target genes was calculated using the QuantaSoft™ Software. Only wells in which the number of droplets exceeded 13,000 were used. Knowing the number of copies of each target gene (expressed as number of transcripts/µL RNA) in our comparator placental sample, the numbers of gene transcripts in all the other cDNA samples were deduced by performing a qPCR analysis in which the five-point concentration curve of the calibrator cDNA was present.

2.7. Bidirectional transport assays across monolayers of MDCKII-MRP1, MDCKII-parental, and BeWo cell lines

Transport assays across monolayers of MDCKII-MRP1 and parental cells were carried out as described previously [60,61]. MRP1 cells were seeded at density 0.6×10^6 cells on 12-well, 3 µm microporous polycarbonate membrane filters (Transwell 3402; Costar Corning, New York, NY, USA) and cultured for 72 h to confluence.

Transport assays employing monolayers of BeWo cells were performed based on a modified protocol originated from Crowe et al. [62] using microporous polycarbonate membrane filters (0.4 µm pore size, 12 mm diameter; Transwell 3401; Costar Corning) coated previously with human placental collagen (Sigma-Aldrich). BeWo cells were seeded at density 0.1×10^6 per insert and cultured for 6 days (37 °C /5% CO₂) with daily replacement of cell culture medium. The transepithelial electrical resistance (TEER) was measured using a Millicell® ERS VoltOhmmeter (Millipore, Merck) prior to each experiment.

On the day of an experiment, cell monolayers were rinsed with PBS (37 °C), then pre-incubated for 15 min in Opti-MEM with/without the control inhibitors elacridar (2 µM), ritonavir (10 µM), or MK-571 (50 µM). Due to transport of maraviroc by endogenous canine Mdr1 [47], a certain part of the experiments in MDCKII cells were run in the presence of the Mdr1 inhibitor zosuquidar (2 µM), which does not interact with human MRP1 [63]. The assay was started by the addition of [³H]-MVC 0.2 µCi/mL (65 nM) into the apical or basal compartment with regular sampling from the opposite compartment.

Transport of [³H]-MVC was detected by liquid scintillation counting and expressed as percentages calculated from measured values relative to the stock solution. The efflux ratio (ER = $\text{Papp}_{\text{B-A}}/\text{Papp}_{\text{A-B}}$) was calculated within a linear period from values of permeable coefficients (Papp) reached after 2 h of incubation.

Semipermeable membranes were cut out from each well, and cell monolayer was lysed in 0.02 % SDS after the experiment. The accumulation of [³H]-MVC in cells was measured and standardized to total protein.

2.8. Accumulation assay in BeWo and A431 cell lines

BeWo and A431 cells overexpressed -OATP1A2, -1B3, -2A1, -2B1, -3A1, or -4A1 and their appropriate controls were seeded onto a 24-well plate (Techno Plastic Products, Trasadingen, Switzerland) at density 0.3×10^6 cells per well and cultured for 24 h (A431) or 48 h (BeWo) at 37 °C in 5% CO₂. On the day of an experiment, the culture medium was removed and cells were rinsed with PBS (37 °C). The BeWo cells were then incubated for 15 min with Opti-MEM with and without the inhibitors elacridar (2 µM), ritonavir (10 µM), verapamil (100 µM), L-carnitine (1 mM), MK-571 (50 µM), and cold maraviroc at a high concentration (100 µM). In the case of A431 cells, an uptake buffer consisting of 125 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 12 mM MgSO₄, 5.6 mM D-glucose, and 1 M HEPES (pH 7.4) was prepared and sulfasalazine (100 µM) was used as a control inhibitor. Accumulation was initiated by the addition of [³H]-MVC 0.2 µCi/mL (65 nM) and stopped after 30 min (A431) or 60 min (BeWo) by removing the supernatant. The cells were immediately washed two times with ice-cold PBS (4 °C) and subsequently lysed in 0.2 % SDS. Maraviroc accumulation was measured by liquid scintillation counting of the lysates and normalized to protein levels. None of the applied inhibitory drugs affected viability of the cells, as confirmed by monitoring the cellular confluency and protein content in each well at the end of experiment.

2.9. Inhibitory assays on MRP1-mediated efflux and OATP1B3 and OATP1A2 influx

In order to determine whether maraviroc, elacridar, or ritonavir inhibit MRP1, OATP1B3, or OATP1A2 transporters, the following

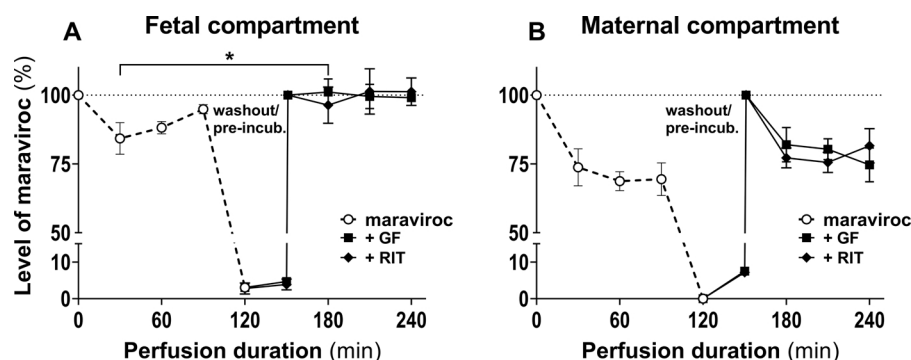


Fig. 1. Transport of maraviroc across human placental cotyledon. **A, B:** Maraviroc (600 ng/mL), shown here as 100 %, was perfused in a closed-circuit setup with the same initial concentration in both fetal (**A**) and maternal (**B**) compartments. Drop of maraviroc levels was observed in both compartments within the first 90 min of the perfusion experiment. Addition of MDR1 inhibitors elacridar (GF, 2 μ M) and ritonavir (RIT, 10 μ M) prevented decrease in maraviroc levels on the fetal but not maternal side (150–240 min) compared to the non-inhibited phase. Time periods 90–120 min and 120–150 min depict the washout and pre-incubation phases, respectively. Data are presented as means \pm SD ($n \geq 3$) and analyzed by Mann–Whitney test (* $p \leq 0.05$).

inhibitory assays were performed:

Inhibitory assay in MDCKII-MRP1 was run as described previously [47]. Cells were seeded on 96-well plates at density 5×10^4 and incubated to full confluence. A preincubation period (15 min) either in pure Opti-MEM or in the presence of a range of maraviroc, ritonavir, and elacridar concentrations (1–50 μ M) or control MRP1 inhibitor MK-571 (50 μ M) preceded an experimental period. The assay was initiated by addition of the fluorescent MRP1 substrate calcein-AM (10 μ M). Relative fluorescent units were measured in 1 min intervals for 30 min, at constant temperature 37 $^{\circ}$ C, using a Genios Plus microplate reader (Tecan, Salzburg, Austria). Efflux response was expressed as the percentage of the concentration at which full inhibition was reached or as a percentage inhibitory effect of model inhibitor. IC₅₀ values were determined by nonlinear regression analysis using data fitted onto data points by the GraphPad Prism 8.2.0 program (GraphPad Software Inc., La Jolla, CA, USA).

Similarly, inhibitory assays were performed on OATP-over-expressing A431 cells as described previously [48]. Briefly, cells grown on 96-well plates were pre-incubated with increasing amounts of inhibitors for 5 min at 37 $^{\circ}$ C. The assay was started by the addition of fluorescein-methotrexate (1 μ M) for 15 min in the case of OATP1A2 or of Cascade Blue (10 μ M) for 30 min in that of OATP1B3. After several washings with PBS, fluorescence was measured using an EnSpire[®] fluorescent plate reader (PerkinElmer, Waltham, MA, USA). Uptake rates were expressed as percentages of uptakes measured in the absence of any inhibitor. IC₅₀ values were determined by nonlinear regression analysis using data fitted on data points by the GraphPad Prism 8.2.0 program.

2.10. Maraviroc accumulation assay in fresh villous fragments of human placenta

The study was conducted based on the method developed by SL Greenwood (University of Manchester, UK) and as described previously [35,64,65]. Briefly, 4–5 cubes (1 cm³) of fresh placental villous tissue were dissected from placenta and rinsed two times in a mixture (1:1) of DMEM and Tyrode's buffer (pH 7.4). Small, fresh villous fragments were cut out and fixed with thread on metal hooks, immersed into DMEM/Tyrode's solution to equilibrate (37 $^{\circ}$ C), and then transferred into pre-incubating solutions of elacridar (2 μ M), ritonavir (10 μ M), MK-571 (50 μ M), and sulfasalazine (100 μ M) for 10 min. Accumulation assay was initiated by transfer of fragments into Tyrode's buffer containing [³H]-MVC 0.2 μ Ci/mL (65 nM) with and without inhibitors. The assay was stopped after 10, 30, 90, 120, or 180 min by twice-washing fragments in non-radioactive solutions of Tyrode's buffer. The fragments were then soaked in distilled water for at least 15 h to lyse the surface trophoblast layer and release accumulated radioactivity. Finally, the placental tissue fragments were cut from the hooks and lysed in 0.3 M NaOH for 10 h at 37 $^{\circ}$ C. The released radioactive maraviroc was quantified by liquid scintillation counting and normalized to total protein levels using the BCA assay. Additional experiments addressing

the possible formation of maraviroc metabolites in the villous tissue were performed by exposing fragments to maraviroc (600 ng/mL), corresponding to the concentration used in placental perfusion study. UHPLC-HRMS analysis for maraviroc metabolites was then performed in the lysates comprising the water solution after 15 h lysis of fragments and in the lysates of the remaining fragmental tissue.

2.11. Statistical analysis

The data obtained are presented as means \pm standard deviations (SD) of at least three independent experiments. Statistical analysis was performed using GraphPad Prism software version 8.2.0 (GraphPad Software). The p -values were calculated by one-way ANOVA or non-parametric Mann–Whitney test and considered as statistically significant if $p \leq 0.05$.

3. Results

3.1. Dual perfusion of human placenta cotyledon

Dual perfusion of human placenta cotyledon in the closed-circuit setup was first performed to verify the contribution of MDR1-mediated efflux to transplacental permeability of maraviroc as the transporter's substrate. After the initial 30 min of perfusion, levels of maraviroc dropped to 86.7 % in the fetal compartment and 73.7 % on the maternal side. The maraviroc levels remained then rather stable on both placental sides, showing, however, slight tendency to increase in the fetal compartment up to 94.9 % at 90 min (Fig. 1A) while slowly decreasing to 69.4 % at 90 min on the maternal side (Fig. 1B). Both added MDR1 inhibitors, elacridar and ritonavir, prevented a drop of maraviroc levels in the fetal compartment (Fig. 1A) but did so only slightly in the maternal compartment (Fig. 1B).

Low concentrations of maraviroc were also found in perfusates at the end of washout (minutes 90–120) and during pre-incubation (minutes 120–150), with slightly higher levels in the maternal compartment. Therefore, faster washout of maraviroc retained in the placental tissue to the maternal circuit is suggested.

No maraviroc metabolites were detected in either the perfusion samples with/without inhibitors or the lysates from fresh villous fragments exposed to maraviroc solutions, thus indicating that the drug was not metabolized during experiments and therefore biotransformation is not responsible for observed changes in maraviroc concentrations in perfusion assays.

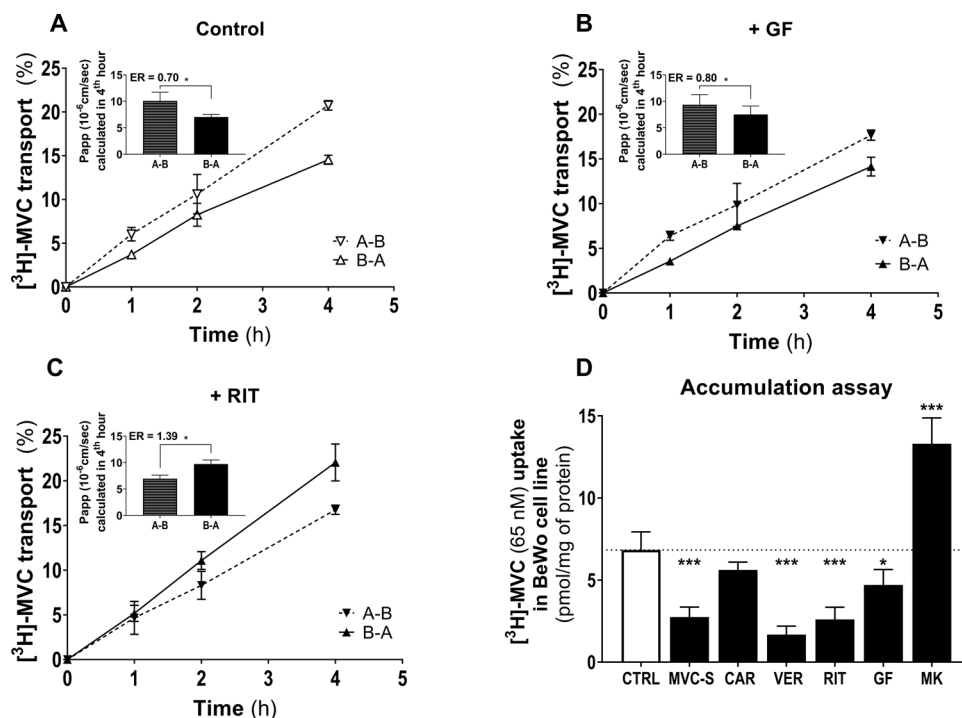
3.2. Bidirectional transport and accumulation of maraviroc in BeWo cells

Following the perfusion studies, transport of maraviroc across polarized monolayer of BeWo choriocarcinoma cell line lacking MDR1 expression (Table 1) was employed. Both MDR1 inhibitors, elacridar (2 μ M) and ritonavir (10 μ M), were added with the intent to evaluate possible contributions of placental transport systems other than MDR1

Table 1
mRNA gene expression of studied transporters.

Transporter gene	Placentas	Endothelial cells	Trophoblast cells	BeWo cells
<i>ABCB1</i>	8.5×10^3 (0.08– 1.7×10^4)	0.7×10^2 (0.3– 1.4×10^2)	2.3×10^3 (0.16– 6.4×10^3)	n.d.
<i>ABCC1</i>	4.1×10^3 (0.05– 1.1×10^4)	4.9×10^3 (4.2– 6.3×10^3)	5.3×10^3 (0.16– 1.1×10^4)	2.3×10^3 (1.8– 3.1×10^3)
<i>SLCO1A2</i>	6.3×10^2 (0.4– 6.9×10^3)	2.9×10^2 (2.4– 7.7×10^2)	1.2×10^3 (0.03– 3.4×10^3)	3.6×10^3 (1.5– 4.9×10^3)
<i>SLCO1B1</i>	n.d.	n.d.	n.d.	n.d.
<i>SLCO1B3</i>	0.4×10^2 (0.1– 0.8×10^2)	0.1×10^2 (0.02– 1.2×10^2)	1.2×10^2 (0.91– 1.4×10^2)	n.d.
<i>SLCO2A1</i>	9.0×10^3 (0.2– 2.4×10^4)	3.6×10^3 (1.6– 6.7×10^3)	7.6×10^3 (0.21– 2.6×10^4)	5.4×10^2 (3.5– 8.1×10^2)
<i>SLCO2B1</i>	1.1×10^4 (0.12– 2.8×10^4)	0.6×10^2 (0.4– 0.9×10^2)	1.5×10^3 (0.12– 1.7×10^4)	9.7×10^2 (8.3– 16.0×10^2)
<i>SLCO3A1</i>	1.0×10^3 (0.44– 2.1×10^3)	6.7×10^2 (1.1– 2.5×10^2)	3.3×10^3 (0.06– 3.5×10^3)	4.1×10^2 (2.5– 4.7×10^2)
<i>SLCO4A1</i>	3.1×10^3 (0.14– 1.2×10^4)	9.2×10^3 (0.32– 1.4×10^4)	1.6×10^3 (0.65– 4.2×10^3)	1.8×10^4 (1.7– 1.9×10^4)

Data is presented as median (inter-quartile range) of number of gene copies/ μ g of transcribed mRNA; n.d. = not detected.



in uptake and/or efflux of maraviroc. The data shows accelerated maraviroc transport in apical-to-basolateral (A-B) relative to basolateral-to-apical (B-A) direction and with ER value of 0.70 (Fig. 2A). Addition of elacridar significantly decreased A-B transport from 20.8%–17.7% ($p < 0.05$) while not affecting the B-A direction, thus resulting in ER value of 0.80 (Fig. 2B). Very interesting activity was observed in the case of ritonavir, which also reduced A-B transport to 16.7 % ($p < 0.05$) but increased also B-A transport from 14.5%–22.1%, resulting in ER = 1.39. The primarily preferred direction of maraviroc transfer across BeWo monolayer was thus reversed (Fig. 2C). These results strongly supported the theory of one or more other transporters being involved in maraviroc transport across placental barrier.

Following transport assay, the simple accumulation study in BeWo cells was carried out with the aim of helping to identify the mechanisms contributing to maraviroc permeability (Fig. 2D). Decrease in maraviroc accumulation (2.5-fold) was observed when a high concentration of unlabeled maraviroc (100 μ M) was applied, thus indicating saturation of a transporter-mediated uptake of maraviroc to the cells rather than its efflux. The accumulation of maraviroc in the cells also significantly decreased in the presence of 100 μ M verapamil (4.07-fold), 10 μ M ritonavir (2.63-fold), and 2 μ M elacridar (1.45-fold), suggesting inhibition of an uptake transporter. On the contrary, enhanced (1.95-fold) accumulation was detected in the presence of the ABCCs inhibitor

MK-571 (50 μ M), indicating inhibition of MRPs transporter-mediated maraviroc efflux. The fact that no effect of L-carnitine (1 mM) was observed on maraviroc accumulation excludes a contribution of OCTN1/2 transporters.

3.3. Gene expression of evaluated transporters

A detailed quantitative analysis of mRNA transporter expression for all perfused placentas and BeWo cells along with independent samples from isolated trophoblasts and fetal endothelial cells is summarized in Table 1. As expected, *ABCB1* was detected in all perfused placentas but not in BeWo cells, while *ABCC1* was markedly expressed in placentas and also in BeWo. *SLCO2A1*, *SLCO2B1*, *SLCO3A1*, and *SLCO4A1* were substantially expressed in all the perfused placentas, although a rather lower number of transcripts was detected for *SLCO1A2* and this was only negligible for *SLCO1B3*. BeWo cells showed high levels of *SLCO4A1* and *SLCO1A2* transcripts. Expression analysis of independently isolated trophoblast and fetal endothelial cells showed presence of all evaluated transporters with the exception of *SLCO1B1*, with transcript levels being about one order of magnitude higher for *ABCB1*, *SLCO1A2*, *SLCO1B3*, and *SLCO2B1* in trophoblast than in fetal endothelial cells. Similar substantial levels of *ABCC1*, *SLCO4A1*, and *SLCO2A1* mRNA transcripts were found in trophoblast and fetal endothelial cells.

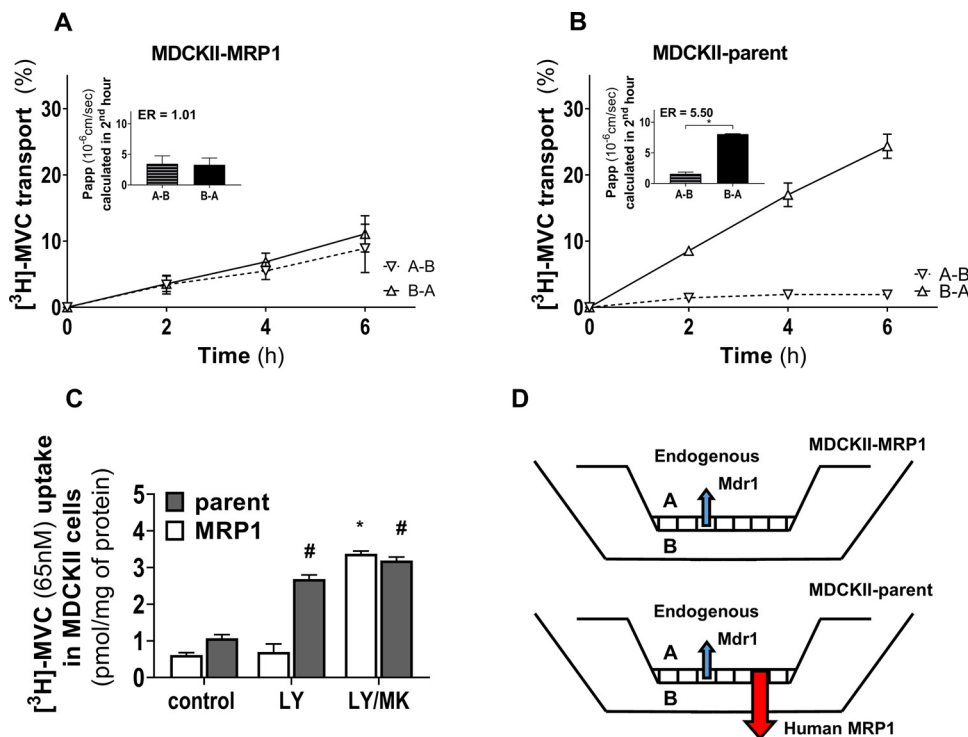


Fig. 3. Transport of [³H]-MVC (65 nM) across monolayer of MDCKII-MRP1 and -parent cells. **A, B:** Basolateral-to-apical (▲; B-A) transport and apical-to-basolateral (▼; A-B) transport of [³H]-MVC across MDCKII-MRP1 (**A**) and MDCKII-parental (**B**) cells was determined. Efflux ratio (ER) was calculated based on permeable coefficient (Papp) values reached after 2 h of the transport. **C:** Effect of Mdr1 inhibitor zosuquidar (LY, 2 μM) and control MRP1 inhibitor MK-571 (MK, 50 μM) on maraviroc accumulation in MDCKII-parental and MDCKII-MRP1 cells. **D:** Depiction of endogenous Mdr1 and human MRP1, both interacting with maraviroc in MDCKII and MDCKII-MRP1 cellular models.

3.4. Maraviroc is a substrate of human MRP1

Following through on the evidence from data obtained in previous experiments, interaction of [³H]-MVC (65 nM, 0.2 μCi/mL) with human MRP1 was studied in MDCKII cells. These cells show overexpression of human MRP1 transporter in the basolateral membrane [61], which accelerates the transfer of its substrates from the apical to the basolateral compartment. As shown in Fig. 3B, maraviroc is readily transported through MDCKII parental cells from the basolateral to the apical side (ER = 5.50) by the function of the endogenous Mdr1 transporter [47]. On the other hand, there is no significant basolateral-to-apical transport of maraviroc in MDCKII-MRP1 cells (ER = 1.01), as shown in Fig. 3A. This data results in the high relative ratio ($R_r = ER_{\text{parental}}/ER_{\text{MRP1}}$) of 5.44 when comparing transport in the two tested cell lines and reveals maraviroc to be a substrate of human MRP1. Addition of zosuquidar (2 μM) showed significant increase in accumulation of maraviroc within monolayers of MDCKII-parental cells, thus reflecting inhibition of endogenous Mdr1. This effect could not be observed in MDCKII-MRP1 due to the retained efflux activity of non-inhibited MRP1 pumping maraviroc out of the cells. Dual addition of zosuquidar and the MRP1 inhibitor MK-571 (50 μM) resulted in significant increase in maraviroc accumulation within both cell lines (Fig. 3C).

3.5. Maraviroc is a substrate of human OATP1A2 and OATP1B3 but not of OATP2A1, 2B1, 3A1, or 4A1

In vitro uptake of [³H]-MVC (65 nM, 0.2 μCi/mL) was measured in A431 cells overexpressing human placental OATP transporters OATP1A2, OATP1B3, OATP2A1, OATP2B1, OATP3A1, or OATP4A1 and compared to the respective mock control cells (Fig. 4A). Significantly increased levels of maraviroc were detected in A431-OATP1A2 (1.68-fold) and A431-OATP1B3 (2.21-fold) cells, thereby indicating that maraviroc is a substrate of these transporters. No difference in accumulation of maraviroc was observed in the OATP2A1-, OATP2B1-, OATP3A1-, or OATP4A1-expressing cells. Decrease of maraviroc accumulation in the presence of the OATP inhibitor sulfasalazine (SLF, 100 μM) in A431-OATP1B3 (Fig. 4B) or -OATP1A2 (Fig. 4C) cells further confirms that maraviroc is a transported substrate

of these two OATPs.

3.6. Accumulation inhibitory assay

Because earlier experiments had revealed maraviroc to be a substrate of MRP1, OATP1A2, and OATP1B3, we further aimed to verify whether the inhibitors elacridar and ritonavir that were used during placental perfusions (Fig. 1) and in BeWo transport and accumulation assays (Fig. 2) could (in addition to MDR1) also affect these transporters.

We found that ritonavir increases accumulation of calcein-AM in MDCKII-MRP1 cells with maximal inhibitory potency at 50 μM and $IC_{50} = 13.9 \mu\text{M}$ (Fig. 5A). No changes were observed in the calcein-AM accumulation when elacridar or maraviroc were present (tested concentration range was 1–50 μM).

Inhibitory effects of ritonavir, elacridar, and maraviroc toward OATP1A2 or OATP1B3 transporters appear as a decrease of fluorescein-methotrexate or Cascade-Blue hydrazide accumulation in A431-OATP1A2 and A431-OATP1B3 cells, respectively. Ritonavir and elacridar revealed inhibitory activity toward OATP1A2 (IC_{50} of 3.70 μM and 1.21 μM, respectively), while only weak OATP1A2 inhibition ($IC_{50} = 72.9 \mu\text{M}$) was calculated for maraviroc (Fig. 5B). Ritonavir revealed also potent inhibition of OATP1B3 ($IC_{50} = 0.82 \mu\text{M}$) in A431-OATP1B3 cells. The same effect was observed with elacridar ($IC_{50} = 3.17 \mu\text{M}$). Maraviroc exhibited no inhibition of OATP1B3 transport, but a low level of activation (37 %) could be observed at high maraviroc concentrations (Fig. 5C).

The inhibition of OATP1A2- and OATP1B3-mediated uptake and MRP1-mediated efflux of maraviroc by ritonavir and elacridar was subsequently confirmed in accumulation assays using inhibitory concentrations corresponding to those from the dually perfused human placenta experiments (Fig. 5D).

3.7. Uptake of maraviroc into fresh villous fragments of human term placenta

Fresh fragments prepared from human placental villous tissue reflect primarily the situation in the maternal-facing syncytiotrophoblast

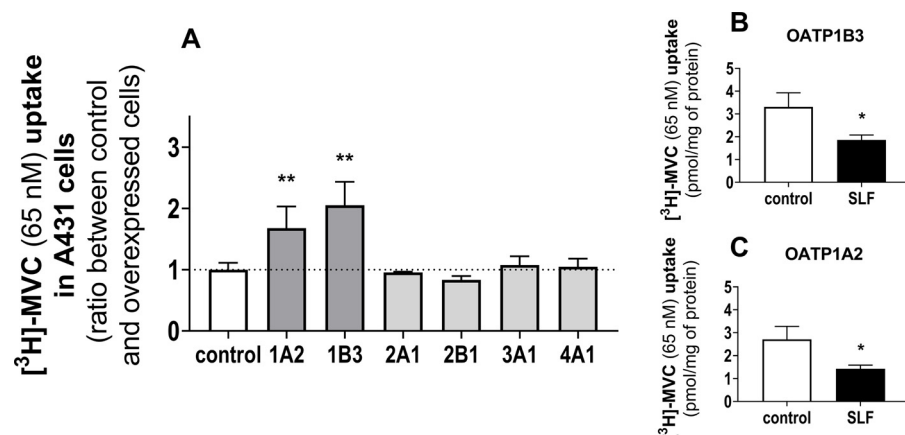


Fig. 4. [3 H]-MVC (65 nM) uptake in A431 cells over-expressing human OATP transporters -1A2, -1B3, -2A1, -2B1, -3A1, and -4A1. **A:** Tested cell lines were incubated in the presence of [3 H]-MVC for 30 min and maraviroc accumulation values reached were compared with results in control cell lines. **B, C:** Effect of OATP inhibitor sulfasalazine (SLF, 100 μ M) on maraviroc transport mediated by OATP1A2 or -1B3. Data are presented as means \pm SD ($n \geq 4$). Statistical significance was evaluated by Mann-Whitney test (* $p \leq 0.05$, ** $p \leq 0.01$).

layer (Fig. 6D). Accumulation of maraviroc measured in placental fragments bathed in [3 H]-MVC (65 nM) solution showed increase over time (up to 120 min; Fig. 6A). Elacridar (2 μ M) and ritonavir (10 μ M) were added for 30 min (Fig. 6B) and 90 min (Fig. 6C). Neither of these showed significant effect on accumulation of maraviroc. When more specific OATPs and MRP1 inhibitors, sulfasalazine (100 μ M) and MK-571 (50 μ M), respectively, were added, significant changes in maraviroc accumulation were observed (Fig. 6B, C)

4. Discussion

More than 10 years have passed since maraviroc was registered by FDA as the first selective blocker of CCR5 receptor for the treatment of R5-tropic HIV-infected adults. During this period, further study of maraviroc's safety and efficiency has produced favorable results and enabled expansion of the drug's usage also to HIV-infected children [41,66]. Due to the unique mechanism of action targeting CCR5, the intense placental expression of which is closely linked to the higher risk of mother-to-child transmission of HIV, and owing to its expected therapeutic benefit also in other non-HIV related indications, it seems probable that maraviroc will more frequently be administered to pregnant women [43–45,67]. Detailed knowledge is therefore needed regarding transplacental transfer of maraviroc and the mechanisms involved. Maraviroc is a quite lipophilic compound ($\log P = 5.80$) [68] and is presumed to cross biological membranes easily *via* passive diffusion. We have recently described this antiretroviral as a substrate of human MDR1 and, using a rat placental model, demonstrated the role for rat Mdr1 in preventing maraviroc's penetration from mother to fetus [47]. A possible contribution of ABC transporters in placental handling of maraviroc in humans has already been suggested by a study showing accelerated fetal-to-maternal clearance of maraviroc in an open-circuit perfusion setup, albeit without providing any direct proof, such as by pharmacological inhibition [69]. Here, we employed dual *ex vivo* perfusion of a human placental cotyledon in a closed-circuit setup, which limits transfer of drug *via* passive diffusion and emphasizes transporters activity. The contribution of placental MDR1 was demonstrated through rapid drop of maraviroc concentration on the fetal side after 30 min of perfusion (Fig. 1A), which was prevented by the MDR1 inhibitors ritonavir (10 μ M) and elacridar (2 μ M). To the best of our knowledge, this is the first time that functional involvement of human MDR1 has been shown in maraviroc distribution across placental barrier. Based on the known active efflux capability of the microvillous membrane, the amount of maraviroc transferred from the fetal compartment would be expected to appear on the maternal side. On the contrary, even more-pronounced decline in maraviroc levels was observed in the maternal circulation, and no significant increase was observed with the presence of inhibitor (Fig. 1B). The decline in maraviroc concentration on both sides of perfused placenta raised the

question as to where does the escaping maraviroc appear? Having excluded possible adherence of maraviroc to the perfusion tubes and other plastic lab ware, we checked also for the occurrence of known maraviroc metabolites in perfusates and homogenized placental tissue. Although maraviroc has been described as a subject of biotransformation through CYP3A4 and CYP3A5 isoenzymes in the liver [70,71], no maraviroc metabolites were detected in our perfusates or tissue lysates, reflecting probably the too-low biotransformation activity of placental tissue [72] and a too-short perfusion time of the tissue to enable significant formation of metabolites.

The unexpected behavior of maraviroc during the human placental perfusions and partly hidden effect of MDR1-mediated efflux differ considerably from observation in rat placenta, where we had described robust contribution of rat Mdr1 in transplacental transfer of maraviroc, correlating also with only low levels of maraviroc (less than 2%) retained in rat placental tissue [47]. Unlike the findings in rat, maraviroc seems to be retained within the human placenta, as up to 10 % of the maraviroc was released from the tissue to the perfusates (in particular in maternal compartment) after pre-incubation periods, when placenta was perfused by maraviroc-free buffer with inhibitors. These observations from human placenta perfusions thereby indicate the involvement of some other storage and/or transport mechanism(s) in placental handling of maraviroc.

In order to identify those membrane transporters possibly contributing to the placental handling of maraviroc and diminishing the impact of MDR1-mediated maraviroc efflux as shown in the *ex vivo* placenta perfusion study, several cell-based models were employed. Because the human choriocarcinoma BeWo cell line mimics placental trophoblast layer *in vitro* but shows no expression of ABCB1 [5,17 and Table 1], it served as an ideal model for fast, initial screening (Fig. 2). Among the applied inhibitors, MK-571, inhibiting efflux provided by MRP transporters, significantly increased maraviroc accumulation in BeWo cells. Based on the accelerated transport of maraviroc in the apical-to-basolateral direction across polarized monolayers of BeWo cells, the basolaterally located MRP1 was suggested as the MRP transporter most probably involved. Indeed, subsequent transport assays across the monolayers of MDCKII-MRP1 cells revealed maraviroc to be a MRP1 substrate (Fig. 3).

Complementary accumulation studies in MDCKII-MRP1 cells (Fig. 3C) further revealed high preference of maraviroc for MRP1- rather than endogenous canine Mdr1-mediated efflux, which drives the transport in the opposite direction. This observation leads to a very interesting conclusion in the context of our recent study, wherein we describe a much stronger tendency for maraviroc to interact with the canine endogenous Mdr1 variant than with human MDR1 transporter in MDCKII-MDR1 cells [47]. Considering these two findings and the fact that transport experiments in MDCKII-MDR1 cells in our previous study and in MDCKII-MRP1 here were carried out under the identical

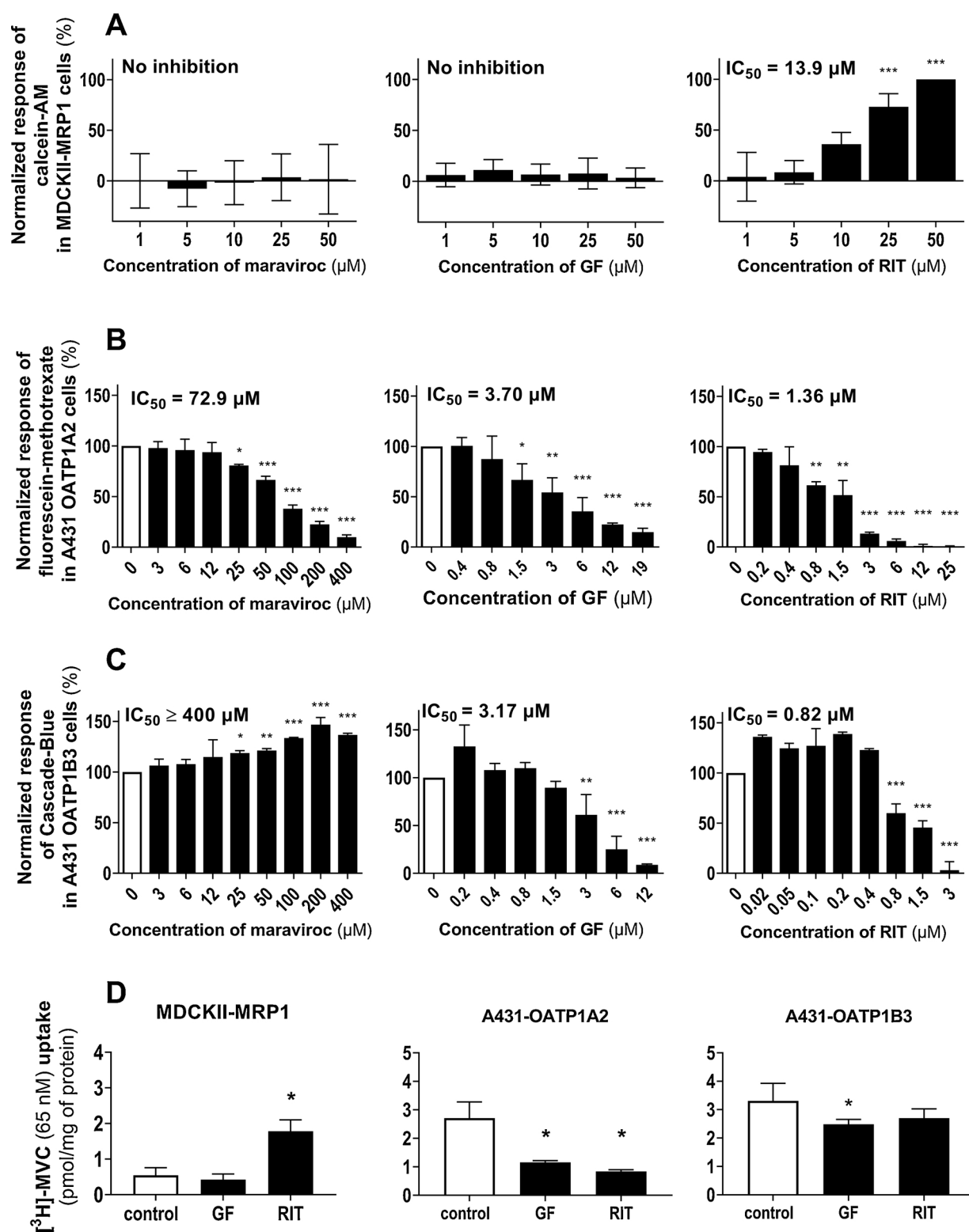


Fig. 5. Inhibitory assay in MDCKII-MRP1, A431-OATP1A2, and -1B3 cells. **A:** Changes in accumulation of fluorescent MRP1 substrate calcein-AM in MDCKII-MRP1 cells in the presence of tested inhibitors maraviroc, ritonavir, and elacridar. **B:** Effect of maraviroc, elacridar, and ritonavir on fluorescein-methotrexate uptake in A431-OATP1A2 cells. **C:** Uptake of Cascade Blue hydrazide in A431-OATP1B3 in presence of maraviroc, ritonavir, and elacridar. **D:** Effect of elacridar (GF, 2 μM) and ritonavir (RIT, 10 μM) on accumulation of [³H]-MVC (65 nM) in MDCKII-MRP1, A431-OATP1A2 and -1B3 cells. Data are presented as means \pm SD ($n \geq 3$). Statistical significance was evaluated by one-way ANOVA and Mann-Whitney test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

protocol, we can suggest rather higher affinity of maraviroc to human MRP1 than to human MDR1.

Interestingly, when a high concentration of maraviroc (100 μM) was applied in BeWo cells, it led to significant decrease in maraviroc

accumulation, thus indicating saturation of uptake rather than an efflux transport mechanism. Similar decrease of maraviroc uptake was observed also with the application of nonspecific inhibitors ritonavir and verapamil. Published data showing maraviroc affinity to human

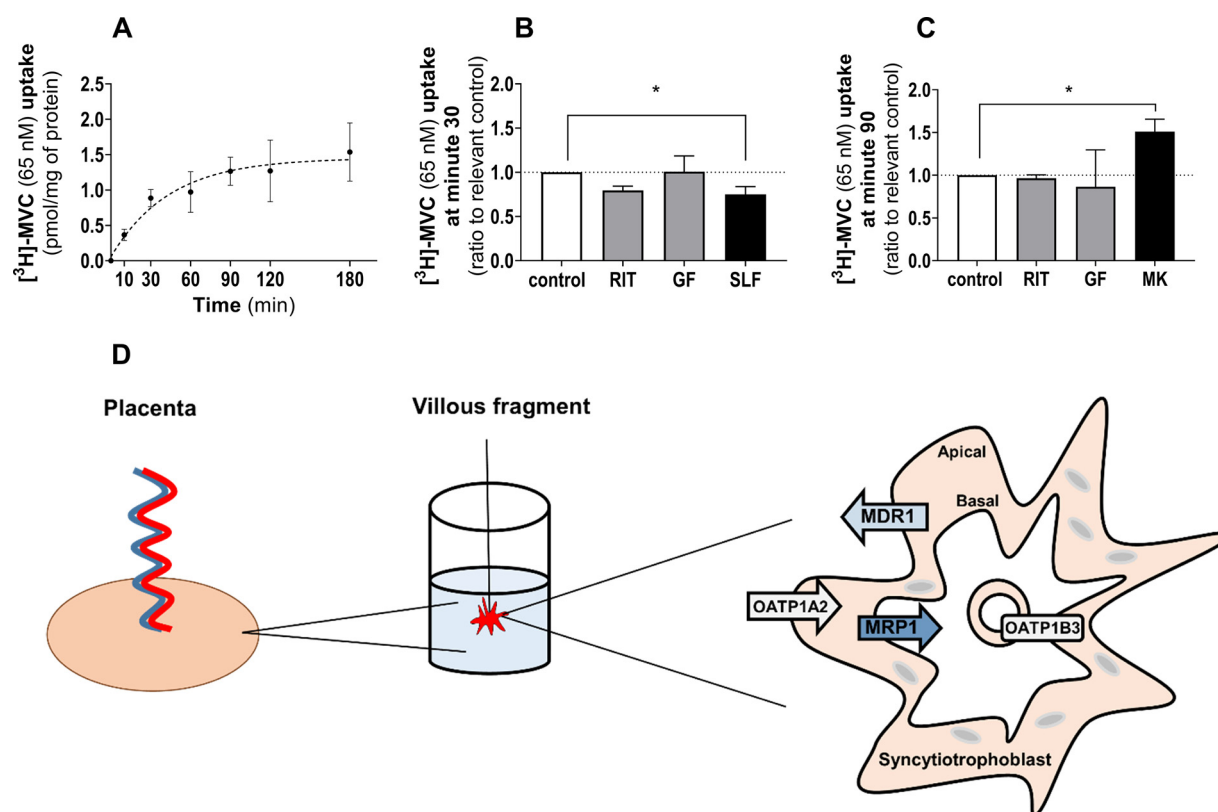


Fig. 6. Uptake of [³H]-MVC (65 nM) into fresh villous fragments of human term placenta.

A: Time-dependent accumulation of maraviroc in fresh villous placental fragments. **B, C:** Accumulation of maraviroc in presence of elacridar (GF, 2 μM), ritonavir (RIT, 10 μM), and sulfasalazine (SLF, 100 μM) is shown at minute 30 (**B**) and at minute 90 (**C**) in presence of MK-571 (MK, 50 μM). **D:** Illustration of the experimental setup showing transporters involved in transfer of maraviroc in syncytiotrophoblast. Data are presented as means ± SD ($n \geq 4$). Statistical significance was evaluated by Mann–Whitney test (* $p \leq 0.05$).

OATP1B1 [73] and maraviroc-induced inhibition of uptake of the nonspecific OATPs fluorescent substrate 8-(2-[fluoresceinyl]aminoethylthio)adenosine-3',5'-cyclic monophosphate (8-FcAMP) into BeWo cells [74] suggest the possible involvement of a member of the OATP transporter family.

Based upon the mRNA expression profile of OATP transporters in all perfused placentas (Table 1), *in vitro* studies were carried out in several A431 cell lines overexpressing OATP transporters to evaluate the interaction of maraviroc and the inhibitors used with these uptake transporters.

The accumulation assays revealed uptake of maraviroc by OATP1A2 and OATP1B3 when applied at low (65 nM) concentration. This effect obviously remained hidden in the experimental setup of a recent study that evaluated maraviroc in micromolar concentrations only using OATP1B3-transfected HEK 293 cells [75]. The number of OATP1A2 encoding mRNA transcripts (*SLCO1A2*) found in trophoblast cells was comparable to the expression of MDR1 (*ABCB1*) and MRP1 (*ABCC1*), while the expression of OATP1B3 encoding gene *SLCO1B3* was one order of magnitude lower. Expression of *SLCO1B3* more than one order of magnitude lower compared to that of *SLCO1A2* was found also in the perfused placental tissue and in isolated fetal trophoblast cells, indicating that OATP1B3 can play a less important role in placental transfer of maraviroc than does OATP1A2. Significant expression of *SLCO1A2* was revealed also in BeWo cells, thus indicating OATP1A2 to be the OATP member most probably responsible for the observed uptake of maraviroc to the BeWo cells (Fig. 2D). Decreased uptake was then observed in the presence of elacridar, ritonavir, and higher concentration of maraviroc, the inhibitors of OATP1A2 as demonstrated in inhibitory assays using OATPs overexpressing A431 cells (Fig. 5B). Since verapamil is also a potent inhibitor of OATP1A2 [76], significant

decrease in maraviroc accumulation in BeWo cells in the presence of verapamil can be also attributed to inhibited OATP1A2-mediated uptake. These findings help to explain not only maraviroc's behavior during accumulation in BeWo cells, but also the drop of maraviroc levels in maternal perfusates and their consequent increase after the washout and pre-incubation period during placental perfusions, in which inhibition of the OATP-mediated uptake obviously enables releases of accumulated maraviroc from the tissue.

Identification of maraviroc as a substrate of other placental transporters helps to explain its accelerated transport in the apical-to-basolateral direction observed in BeWo cell monolayers. These indicate vectorial transport of maraviroc provided by OATP1A2-mediated influx in the apical membrane followed by MRP1-mediated efflux across the basolateral membrane (Fig. 2A). Elacridar (GF) blocked only the apically expressed OATP1A2 transporter, thereby decreasing the apical-to-basolateral transport of maraviroc while having no effect on basolateral-to-apical transport (Fig. 2B). In contrast, ritonavir, inhibiting both OATP1A2 and MRP1, as shown in Fig. 5A,B, enabled higher permeability of maraviroc in the basolateral-to-apical direction (Fig. 2C).

Time-dependent accumulation of maraviroc was then shown in fresh villous placental fragments representing primarily the situation in syncytiotrophoblast as depicted in Fig. 6D. The absence of significant effect of ritonavir or elacridar may be caused by inhibition not only of OATP1A2 and MRP1, but also of MDR1 expressed in the apical membrane (Fig. 6D). The contribution of OATP1B3 to this transport is probably only marginal, because, in spite of the protein staining described in the vasculosyncytial membrane [31], only very low expression was found in the placental tissue, as well as in isolated trophoblasts and endothelial cells.

In summary, here we identified for the first time maraviroc as a

substrate of three different transporters – MRP1, OATP1A2, and OATP1B3 – while excluding its interaction with OATP2A1, OATP2B1, OATP3A1, OATP4A1, and indirectly also with OCTN transporters. Viewed in the context of MRP1 and OATP expression in human placental tissue, isolated trophoblasts, and fetal endothelial cells, our data can contribute to a better understanding of maraviroc transplacental pharmacokinetics and the drug's safety during pregnancy. In addition to the novel findings presented here, we question the generally accepted concept that considers MDR1 substrates always as compounds with diminished maternal-to-fetal transfer. By the example of maraviroc, we describe a situation wherein MDR1 activity was likely surpassed by the interplay of other placental transporters, the greatest contribution to which we attribute to MRP1 and OATP1A2. In contrast to the localization of MDR1 in the apical membrane, MRP1 was found in basal membrane of polarized cells with the oppositely oriented transport direction [77]. Basolateral localization of MRP1 has been confirmed in trophoblast cells [17,18,78] and reported also in fetal endothelial cells [18]. Therefore, we assume bidirectional flow of maraviroc toward placental interstitium provided by the MRP1-mediated efflux and further encouraged by OATP1A2- and, to a lower extent, also by OATP1B3-mediated uptake. This theory actually corresponds with our perfusion data and, consequently, it is not in conflict with the low umbilical-cord-to-maternal-blood ratio of maraviroc (0.33) reported by Colbers et al. [42]. Obviously, the impact of transporters interplay on transplacental drug transfer depends on the preferential affinity of the drug to the particular transport proteins and on the transporter expression profile across the pregnancy. Interestingly and in line with our hypothesis, the well-known MRP1 substrate saquinavir showed low penetration to fetus and retention within the placental tissue up to only 1.6 % in a study using an open-circuit placenta perfusion experiment in the maternal-to-fetal direction [79]. Subsequently, it was found to be a high affinity substrate of MDR1 and MRP2 [61], the transporters providing efflux on the apical microvillous membrane back to maternal circulation and thereby overwhelming potential MRP1-mediated transport of saquinavir in the opposite direction. Interestingly, reduction of saquinavir transport in the fetal-to-maternal direction by as much as 43 % observed in other study, when MK-571 was added to the perfusion buffer [80], could be explained as inhibition of MRP1 efflux activity in fetal endothelial capillaries. Since MRP1 expression is significantly increased by the end of gestation compared to at mid-gestation [81,82] while MDR1 is known to follow the opposite pattern [83], the contribution of MRP1 might be increased by the end of gestation. Indeed, Coles et al. have suggested that gestational-induced changes in the transporters lead to greater uptake of saquinavir into mice placenta at term [81]. Taking all these findings together, it seems that the MRP1-mediated flux of maraviroc to the placenta is ensured in particular by the end of pregnancy and that similar behavior might obviously be expected in many other MRP1 substrates. Even though MRP1 is known to transport several endogenous compounds, it is not yet clear what role it fulfils when leading to the retention of its substrates within the placental tissue. We could speculate that by the end of pregnancy the inner placental tissue might serve as space for disposal storage and additional protection of the fetus and its mother shortly before delivery, during which the organ is released. This hypothesis might be further supported by findings of high levels of methylmercury in placentas of women exposed to this toxin during pregnancy, as it, too, recently was identified as an MRP1 substrate [84,85]. Detailed studies would be needed to consider the possibility that additional drugs described as MRP1 substrates could be retained in human placental tissue when possessing lower affinity to efflux transporters localized in the trophoblast microvillous membrane. Importantly, when evaluating the maternal-to-fetal transfer of drugs and other xenobiotics, the involvement of additional placental transporters and their interplay affecting the permeability of an investigated compound must be borne in mind and considered critically.

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CRediT authorship contribution statement

Lenka Tupova: Investigation, Formal analysis, Writing - original draft. **Birgit Hirschmugl:** Investigation, Writing - review & editing. **Simona Sucha:** Investigation, Formal analysis. **Veronika Pilarova:** Investigation. **Virág Székely:** Investigation. **Éva Bakos:** Methodology. **Lucie Novakova:** Resources, Formal analysis, Supervision. **Csilla Özvegy-Laczka:** Resources, Formal analysis, Supervision, Writing - review & editing. **Christian Wadsack:** Resources, Methodology, Supervision, Writing - review & editing. **Martina Ceckova:** Conceptualization, Methodology, Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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