Placental Transfer of Maraviroc in an Ex Vivo Human Cotyledon Perfusion Model and Influence of ABC Transporter Expression


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Nowadays, antiretroviral therapy is recommended during pregnancy to prevent mother-to-child transmission of HIV. However, for many antiretroviral drugs, including maraviroc, a CCR5 antagonist, very little data exist regarding placental transfer. Besides, various factors may modulate this transfer, including efflux transporters belonging to the ATP-binding cassette (ABC) transporter superfamily. We investigated maraviroc placental transfer and the influence of ABC transporter expression on this transfer using the human cotyledon perfusion model. Term placentas were perfused ex vivo for 90 min with maraviroc (600 ng/ml) either in the maternal-to-fetal (n = 10 placentas) or fetal-to-maternal (n = 6 placentas) direction. Plasma concentrations were determined by ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). Fetal transfer rates (FTR) and clearance indexes (CLI) were calculated as ratios of fetal to maternal concentrations at steady state (mean values between 30 and 90 min) and ratios of FTR of maraviroc to that of antipyrine, respectively. ABC transporter gene expression levels were determined by quantitative reverse transcription (RT)-PCR and ABCB1 protein expression by Western blotting. For the maternal-to-fetal direction, the mean FTR and CLI were 8.0% ± 3.0 and 0.26 ± 0.07, respectively, whereas the mean CLI was 0.52 ± 0.23 for the fetal-to-maternal direction. We showed a significant inverse correlation between maraviroc CLI and ABCC2, ABCC10, and ABCC11 placental gene expression levels (P < 0.05). To conclude, we report a low maraviroc placental transfer probably involving ABC efflux transporters and thus in all likelihood associated with a limited fetal exposition. Nevertheless, these results would need to be supported by in vivo data obtained from paired maternal and cord blood samples.

N owadays, mother-to-child transmission (MTCT) is the main cause of pediatric HIV infection. This transmission may occur in utero during pregnancy, intra partum at the time of delivery, or during breastfeeding (1). Maternal plasma HIV viral load has been shown to be the best predictor of vertical HIV transmission. Therefore, the role of appropriate antiretroviral treatment allowing the obtaining of undetectable viral loads at delivery is crucial to limit MTCT. The use of antiretroviral (ARV) treatment, which besides allowing for the protection of mother’s health, associated with caesarean section and avoidance of breastfeeding, actually allowed for the reduction of the risk of HIV-1 transmission from 15 to 40% to 1.5 to 2% (2–4).

However, the growing emergence of HIV resistance to various antiretroviral drugs is a major cause of treatment failures. New treatment options for salvage therapy are therefore of considerable importance. Maraviroc belongs to a recent class of antiretroviral agents acting as entry inhibitors. Indeed, it is a selective non-competitive inhibitor of the CCR5 chemokine receptor, which plays an important role in the process of HIV-1 entry into cell hosts. Its binding to this cell surface protein results in blocking HIV-1 attachment to the coreceptor and prevents the virus from entering CD4+ cells (5).

Transplacental exchanges involve passive transfer, facilitated diffusion, and active transport (6). Membrane transporters participate in the protection of the fetus against endogenous and exogenous toxic substances, including drugs, by rejecting them from the fetal to the maternal compartment. In particular, efflux transporters belonging to the ATP-binding cassette (ABC) transporter superfamily, such as ABCB1/P-glycoprotein (P-gp), the ABCCs/ multidrug-associated resistance proteins (MRPs), and ABCG2/breast cancer resistance protein (BCRP), play a major role. They have all been detected in the human placenta, with various expression levels. ABCG2, ABCC5, and ABCC10 genes have been reported to have the highest expressions. The ABCC3 gene appears to be almost undetectable, and the ABCB1 gene and the other ABC genes appear to have intermediary expressions, with rather great variabilities between studies (7–16). Most antiretroviral drugs have been identified as substrates of at least one ABC transporter (17, 18). Notably, maraviroc has been reported to be an ABCB1 substrate (5).

At this time, the estimation of the materno-fetal risk associated with antiretroviral drug administration during pregnancy is based only on animal studies because of the complexity to conduct clinical studies in pregnant women. However, animal data cannot be extrapolated to humans because of significant interspecies differences of placental structures (19). In this context, maraviroc has been classified by the U.S. Food and Drug Administration (FDA)
in “Pregnancy category B,” which means that no fetal risk was detected by reproduction studies in animals but that adequate in vivo studies involving pregnant women would be required to assert a lack of fetal risk in humans. Available data regarding in vivo placental transfer of maraviroc are still lacking, while HIV-infected women may already receive this treatment during pregnancy. It seems therefore essential to estimate this transfer with other reliable approaches. To that end, the ex vivo perfused human placental cotyledon model, which allows reproducing the conditions of the third trimester of pregnancy, is the gold standard method.

The purpose of this study was thus to investigate the placental transfer of maraviroc by using the ex vivo gold standard method of the human perfused cotyledon to estimate the fetal risk exposure in case of maternal treatment and determine the role of ABC transporters in this transfer.

MATERIALS AND METHODS

Materials. Maraviroc base was kindly provided by Pfizer/ViiV Healthcare Laboratories France (Paris, France). The antipyrine–phosphate-buffered saline (PBS) solution and Bradford reagent were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Other chemicals and reagents were purchased from Invitrogen (Cergy Pontoise, France).

Placenta tissue collections. Sixteen term placentas from normal pregnancies (from 38 to 42 weeks of gestational age) were obtained from Louis Mourier Hospital (Colombes, France) after uncomplicated vaginal delivery (n = 12) or caesarean section (n = 4). All mothers were seronegative for HIV infection, were noninfected by hepatitis B or C, and had taken no medication other than oxytocin or epidural anesthesia during labor. Their geographic origins were distributed as follows: n = 7 Caucasians, n = 8 from North Africa, and n = 1 from Sub-Saharan Africa. Written informed consents were obtained for all participants in the study.

Placental perfusion. Placentas were perfused in an open double circuit according to a method modified from that of Schneider et al. and Forester et al. (20, 21). Perfusion experiments were started within 20 min after delivery. After a visual examination for lack of evident lesions on the chorionic plate and selection of a visually unimpaired isolated cotyledon, a truncal branch of the chorionic artery and the associated vein were cannulated. The establishment of a fetal venous flow in return to that of the fetal artery flow confirmed the viability of the cotyledon first assessed by visual examination. Indeed, given the fact that the fetal artery flow was known (induced by the peristaltic pump), if a difference in pressure was noticed between the fetal artery and vein, the balance of flows was subsequently found to be incorrect and the perfusion procedure was discontinued. On the maternal side, the perfused area progressively whitened, which allowed visualization of the chosen cotyledon. The cotyledon was placed into the perfusion chamber and maintained at 37°C, with the maternal side upward. The intervillous space on the maternal side was perfused by two needles piercing the basal plate. Both circuits were pumped separately by peristaltic pumps (Minipuls 3; Gilson Medical Electronics, Villiers le Bel, France). The fetal and maternal flows were 6 and 12 ml/min, respectively, and the perfusion length was 90 min. Indeed, the system reached steady state after 30 min and then remained stable, allowing the calculation of the parameters of transfer as averages of the measurements from 30 to 90 min. The pH of maternal and fetal solutions, prepared with Earle medium, had been adjusted to 7.4 ± 0.1 and 7.2 ± 0.1, respectively. Maraviroc’s target concentration of 600 ng/ml was used. Antipyrine, a freely diffusion marker requested to allow the validation of the cotyledon’s viability through the experiment, was used at 20 μg/liter. Maraviroc and antipyrine were perfused into the maternal reservoir. Samples were collected every 5 and 15 min from the fetal and maternal compartments, respectively, to determine their concentrations. They were then stored at −20°C until analysis. Reverse perfusions were done similarly but in the opposite way, which means that drugs were infused into the fetal artery and placental transfer was carried out in the fetal-to-maternal direction.

Pharmacokinetic analysis. Maraviroc concentrations in maternal and fetal samples were determined using ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS; Acquity UPLC/TQD) as previously described (7). Antipyrine concentrations were determined by high-performance liquid chromatography with UV detection at 290 nm. The mobile phase was made of 0.05 mol/liter phosphate buffer (pH 3)-methanol-tetrahydrofuran (75:25:0.9 [vol/vol/ vol]). Standard curves were prepared for antipyrine concentrations that ranged from 0.5 to 20 μg/liter.

Standard parameters were calculated according to the formulas of Challier et al. (8). For maternal-to-fetal transfer, a fetal transfer rate (FTR) and clearance index (CLI) were calculated under steady-state conditions as the ratios of fetal-to-maternal concentrations and the FTR of maraviroc to that of antipyrine, respectively. An FTR of antipyrine of ≥20% was required to validate each experiment. In the fetal-to-maternal direction, inverse FTRs were calculated as the ratios of maternal-to-fetal concentrations of maraviroc and antipyrine, and the placental transfer was estimated as a CLI calculated as ratios of the inverse FTRs of maraviroc to that of antipyrine.

Determination of ABC transporter gene expression levels by quantitative RT-PCR. Biopsy samples (small cubes of 75 mm3) were collected from each placenta, with the maternal side upward, halfway between the placental edge and the cord blood’s insertion, at a depth of 1.5 cm to avoid any contamination by the decidua. They were cut and washed in PBS solution and then stored at −80°C until RNA and Western blot analysis.

Part of the samples was homogenized using the Tissuemizer system, and then total RNA was purified and extracted using an RNasy minikit according to the manufacturer’s instructions (Qiagen SA, Courtaboeuf, France). Genomic DNA was eliminated by DNase I treatment (RNase-free DNase set; Qiagen SA). The concentrations and purity of RNA samples were assessed spectrophotometrically by optical density at 260 nm (OD260/OD280 ratios using the Nanodrop ND-1000 instrument [Nanodrop Technologies, Wilmington, DE]). For each sample, reverse transcription (RT) was performed using 500 ng of total RNA in a final reaction mixture of 20 μl containing 500 μM each deoxynucleoside triphosphate (dNTP), 10 mM dithiothreitol, 1.5 μM random hexanucleotide primers (Amersham Biosciences, Orsay, France), 20 U RNasin RNase inhibitor (Promega, Charbonnières-les-Bains, France), and 100 U superscript II reverse transcriptase (Invitrogen). All samples were incubated at 25°C for 10 min and then at 42°C for 30 min and 99°C for 5 min on a thermal cycler (PTC-100 programmable thermal controller; MJ Research Inc., Waltham, MA). cDNAs were stored at −80°C until analyses. The expression of the target genes (ABCR/UP-gp, ABCG2/BCRP, ABCCI to ABCCI6/MRP1 to MRP6, ABCCl0 to ABCCl2/MRP7 to MRP9 genes) and that of the housekeeping gene encoding the TATA box-binding protein (TBP) were analyzed by quantitative PCR performed using an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). PCR was performed using the DNA Master SYBR green mix (Applied Biosystems). The thermal cycling conditions comprised an initial Thermus aquaticus polymerase activation step at 95°C for 15 min, 50 cycles at 95°C for 15 s, 60°C for 45 s, and a dissociation stage. Specific primers were designed for each gene using OLiGO 6.42 software (Med Probe, Oslo, Norway). For each primer pair, we performed control assays in the absence of the template, for which signals were negligible. Melting curve analysis also assessed the specificity of each reaction. Primer sequences are shown in Table 1.

Gene expression was evaluated for each sample using the cycle threshold value (Ct), defined as the fraction cycle number at which the fluorescence generated by SYBR green dye-amplicon complex formation passes a fixed threshold above the baseline. The expression of the target genes in each sample was normalized on the basis of the expression of the TBP gene, used as endogenous gene control. As the efficacy of each PCR for all genes was higher than 95%, the comparative variable ΔΔCt was calculated.
from the following formula: $\Delta C_T = (C_T\text{ target gene} - C_T\text{ TBP gene})$ sample $- (C_T\text{ target gene} - C_T\text{ TBP gene})$ calibrator. The $2^{-\Delta\Delta C_T}$ values represented the fold change in mRNA in samples related to the calibrator composed half and half of placental and hepatic cell RNA, arbitrarily set at 1. We controlled our results with a second reference gene, the ribosomal protein large P0 (RPLP0) gene, which led to similar conclusions.

**Immunodetection of ABCB1 transporter by Western blotting.** The remaining placental samples were sonicated at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% Triton X-100, and complete protease inhibitor complex (Roche Diagnostics, Meylan, France). Homogenates were centrifuged at 4°C for 10 min at 10,000 × g, and supernatants were stored. The protein content of samples was determined with the Bradford reagent and a bovine serum albumin calibration curve. Samples were normalized for equal amounts of protein (50 μg for placental cells, -20 μg for HL60-DNR cells) and were separated using 8% SDS-PAGE gels and electrotransferred onto nitrocellulose membranes HYBOND (Bio-Rad, Marne la Coquette, France). Nonspecific binding sites were blocked overnight at 4°C with Tris base buffer containing 0.1% Tween 20 (TBST) and 5% nonfat milk. The membranes were immunoblotted with the C-219 (diluted 1:200) antibody (Enzo Life Sciences, Lyon, France) for 2 h at room temperature, washed several times in TBST, and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:10,000) for 45 min at room temperature (Enzo Life Sciences). Membranes were exposed to an Amersham ECL system. Signals were revealed with the Bio-Rad ChemiDoc XRS imaging device. The blot was then stripped by immersion in acetic acid, 0.1 M, for 2 h and reprobed with monoclonal mouse anti-β-actin antibody (1:50,000) (Enzo Life Sciences). HL60-DNR cells were used as a positive control for ABCB1 expression.

**Statistical analysis.** Data are presented as means ± standard deviation (SD) and were analyzed using NCSS version 07.1.4 software (NCSS, Kaysville, UT). A one-way analysis of variance (ANOVA) followed by a Bonferroni test was applied for the comparison of placental ABC transporter expression levels. For the correlation study, correlation coefficients of Pearson were calculated, and a t test was used to establish potential significance. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Investigation of maraviroc placental transfer.** Ten placentas were collected for the maternal-to-fetal transfer study. The placentas were all validated, with FTR of antipyrine ranging from 22% ± 3% to 49% ± 6%. The mean FTR and CLI of maraviroc were 8.0% ± 3.0% and 0.26 ± 0.07, respectively. In the fetal-to-maternal direction, the mean maraviroc CLI was 0.52 ± 0.23 (n = 6 placentas).

**Correlation between maraviroc placental transfer and ABC transporter placental gene expression levels.** ABC transporter gene expression levels could be measured in 9 of the 10 placentas perfused in the maternal-to-fetal direction. We demonstrated that ABCB1, ABC5, ABC10, and ABCG2 might present significantly higher expressions than ABC2, ABC3, ABC4, ABC11, and ABC12. ABC6 was found to be almost undetectable (P < 0.05; Fig. 1).

We showed a significant inverse correlation between the maraviroc CLI measured in the maternal-to-fetal direction and ABC2, ABC10, and ABC11 placental gene expression levels (P < 0.05). We also observed a trend of inverse correlation between this maternal-to-fetal CLI and ABC4 and ABC5 gene expression, as well as a trend of positive correlation between this CLI and ABC3 gene expression.

**Correlation between maraviroc placental transfer and ABCB1 protein placental expression.** ABCB1 was detected at the protein level in all placental samples with an important interindividual variability. No correlation was found between maraviroc CLI and ABCB1 protein expression, as already observed at the mRNA level (r = -0.15, P = 0.70) (Fig. 2).

**DISCUSSION**

The present study is the first one that investigates the human placental transfer of maraviroc using the *ex vivo* human perfusion model, which reproduces the conditions of the third trimester of pregnancy. The maraviroc target concentration at steady state in the maternal compartment corresponds to the mean maximal plasma concentration (C<sub>max</sub>) reported in patients who receive a standard dose of 300 mg twice daily (22). The C<sub>max</sub> concentration was chosen to reproduce the conditions associated with the highest risk of maternal-to-fetal transfer. The medium flow in the intervillous space was maintained at a constant rate throughout the experiment. The FTR of antipyrine was monitored on the one hand to control the integrity of the placental barrier and on the other hand to normalize the data of drug transfer by accounting for interindividual variations (23). Using this method for maraviroc, we measured low FTR and CLI. These results suggest a weak maternal-to-fetal transfer of the drug across the human placenta, which is in accordance with data obtained in a study conducted in rhesus macaques. Actually, maraviroc plasma concentrations in the newborn macaques were less than 1% of the mothers’ plasma concentrations (24). Besides, maraviroc presents a relatively high molecular weight, superior to 500 Da (514 Da), in accordance with a low placental transfer. Nucleoside reverse transcriptase-in
Hibitors, which are smaller molecules, are conversely known for their higher placental passage (molecular weight [MW] 286 Da and CLI 0.50 for abacavir; MW 671 Da and CLI 0.40 for zidovudine) (25, 26). Interestingly, protease inhibitors, which have higher molecular weights and are more lipophilic than maraviroc, present even lower placental transfer (MW 671 Da and CLI 0.05 for saquinavir; MW 629 Da and CLI 0.10 for lopinavir) (21, 27).

We then investigated whether ABC transporters could be involved in the weak maraviroc placental transfer we observed. First, the observation of a higher drug transfer in the fetal-to-maternal direction than in the maternal-to-fetal direction strongly suggests the involvement of efflux transporters localized at the apical side of the placental barrier. Second, we showed a significant inverse correlation between ABC2, ABC10, and ABC11 gene expressions and the clearance index of maraviroc. ABC10 and ABC11 placental localizations have never been clearly identified. However, it is interesting to observe that ABC2, through its localization on the apical side of the polarized syncytiotrophoblasts, may participate to explain the weak maraviroc placental transfer by expulsing the drug in the fetal-to-maternal direction. To our knowledge, ABC2 has never been shown to be a maraviroc transporter. Nevertheless, to our knowledge, only one recent study has clearly been interested in determining the complete profile of maraviroc as the substrate but also inducer and inhibitor of ABC2 in the literature. Indeed, Zembruski et al. demonstrated that maraviroc could stimulate ABC2 transport function (28). However, they explained they could not determine whether it was an ABC2 substrate because of having used proliferation assays for investigation of substrate characteristics, which usually are a suitable tool to detect substrates. Yet the low toxicity of maraviroc rendered it impossible to evaluate whether it is transported by ABC2. Therefore, given the lack of the data, it appears not impossible that maraviroc could be an ABC2 substrate (28). Conversely, maraviroc is known to be a high-affinity ABCB1 substrate. Moreover, ABCB1, like ABC2, is known to be expressed at the apical surface of placental trophoblasts and thus to similarly prevent the entry of xenobiotics into the fetal circulation (29, 30).

However, we found no correlation between maraviroc placental transfer and ABCB1 gene expression level and confirmed this lack of correlation at the protein level (31, 32). We could not conduct any other protein study because of the lack of residual placental material after investigation of all transporters’ mRNAs and ABCB1 protein. We are aware it could represent a limit for the interpretation of the correlation found for ABC2 and that the choice of investigating ABCB1 rather than ABC2 protein is open to criticism. However, our choice is supported by the correlation between ABC2 mRNA and protein expression levels reported by

**FIG 1** Relative gene expression of ABC transporters in placentas. ABCB1, ABCG2, ABCC1 to -5, and ABCC10 to -12 relative gene expressions were determined using quantitative RT-PCR, with normalization based on the expression of the housekeeping gene TBP. ABCC6 is not represented because the mRNA expression levels were too low in comparison with those of the other transporters. Results are means (±SD) of individual samples of placental homogenates isolated from eight placentas, with all measurements done in duplicates. *, P < 0.05.

**FIG 2** Determination of ABCB1 protein expression in placentas. ABCB1 protein expression was examined by Western blotting (n = 9 samples). HL60-DNR cells provided a positive control. Placental samples were loaded with 50 μg of proteins. The autoradiographs of ABCB1 protein were analyzed by densitometry using Quantity One software from Bio-Rad. ABCB1 expression levels are formulated in arbitrary units of densitometry (AUD). MW, molecular weight; CLI, clearance index.
Meyer zu Schwabedissen et al. in the literature (9). Finally, these results suggest that maraviroc might be a substrate of ABCB2. Yet, it has to be experimentally demonstrated. Besides, it could also be suggested that maraviroc inductive properties toward ABCB2 could counterbalance ABCB2 rather low placental expression (28). Regarding ABCB1, one hypothesis that could partly explain the lack of correlation we observed could be the variability of frequency between ethnic groups of the 3435C→T substitution, which has been associated with a decrease of ABCB1 mRNA and protein expression (33, 34). Indeed, in European Caucasians, 3435T frequencies of 0.52 to 0.57 were reported, whereas in Africa, the prevalence is much lower, 0.17 to 0.27 (35, 36). Since in the present work half the participants are North African and half are European Caucasians, this polymorphism might have played a role in the lack of correlation found for ABCB1, which we cannot check given the absence of remaining placental material.

In pregnant women, very scarce information is available since no clinical studies are conducted. Animal investigations are required to provide minimum safety data, but those cannot be extrapolated to humans because of too many interspecies differences. In the present study, we used the gold standard ex vivo human placental cotyledon perfusion model to evaluate the in vivo placental transfer of maraviroc by reproducing the conditions of the last trimester of pregnancy (37). The concordance between the results obtained with the ex vivo placental perfusion model and the cord-to-maternal plasma (C:M) ratios has already been shown for various antiretroviral agents of the nucleoside reverse transcriptase inhibitor and protease inhibitor classes. Indeed, the results obtained with the perfused cotyledon model and suggesting a relatively high and low placental transfer for nucleotide reverse transcriptase inhibitors and protease inhibitors, respectively, are in accordance with those obtained using the perfused cotyledon model and with calculation of ratios in paired maternal and cord blood samples.

In conclusion, the present study reports original data on the placental transfer of maraviroc using the ex vivo human cotyledon perfusion model. Maraviroc placental transfer appears to be weak and may probably involve efflux transporters localized on the trophoblasts’ apical side of the placental barrier, in particular ABCB2. Nevertheless, in vivo data with paired maternal and cord blood samples would be required to confirm these results.

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REFERENCES


