Effects of Antiviral Drugs on Organic Anion Transport in Human Placental BeWo Cells

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Placental drug transfer is important for achieving better pharmacotherapy in pregnant women and in fetuses. In the present study, we examined the effects of anti-hepatitis C virus (HCV) and anti-HIV drugs on organic anion transport in human placental BeWo cells. The cellular uptake of two fluorescence organic anions, 8-(2-[fluoresceinyl]aminoethylthio)adenosine-3',5'-cyclic monophosphate (8-FcAMP) and fluorescein, was temperature and concentration dependent. The Michaelis constant (Km) and the maximum uptake rate (Vmax) for 8-FcAMP transport in BeWo cells were estimated to be 6.45 ± 0.75 μM and 25.55 ± 5.93 pmol/mg protein/10 min, respectively. The Km and Vmax values for fluorescein uptake were estimated to be 31.2 ± 11.8 μM and 510.9 ± 90.6 pmol/mg protein/10 min, respectively. Several known substrates of organic anion transporters in human placenta, including atorvastatin, glibenclamide, estrone-3-sulfate, and rifampin, inhibited cellular uptake of 8-FcAMP and fluorescein in BeWo cells. Transport of 8-FcAMP and fluorescein was inhibited by the antiviral drugs boceprevir, telaprevir, elvitegravir, and maraviroc. These findings suggest that some antiviral drugs are sufficiently potent to influence placental drug transfer and cause drug-drug interactions.

The placenta plays a critical role in the survival and growth of a fetus, which involves the delivery of nutrients and oxygen and removal of waste compounds from the fetal compartment. The placenta forms an interface between the maternal and fetal circulations and acts as a functional barrier termed the blood-placental barrier, which protects the developing fetus from harmful endogenous and exogenous compounds (1–4). Drug transporters are present in the maternal-facing (apical) membrane and fetal-facing (basal) membrane of human syncytiotrophoblast cells and play important roles in drug transport across the placenta. Organic anion transporter 4 (OAT4; SLC22A14), organic anion transporting polypeptide 2B1 (OATP2B1; SLC22A11), and multidrug resistance protein 2 and/or 4 (MRP2/4; ABCC2/4) are expressed in the human placenta (1–7). Clinically important drugs, such as rosuvastatin, pravastatin, pitavastatin, valsartan, olmesartan, telmisartan, trichlormethiazide, probenecid, and benzylpenicillin, are transported by the organic anion transport system (5). P-glycoprotein (P-gp; MDR1 and ABCB1) and breast cancer resistance protein (BCRP; ABCG2) are placental drug transporters that have been well-studied with a variety of techniques, including the use of plasma membrane vesicles from human placenta, the placenta perfusion model, and the in vivo animal model (1–4). A CF-1 mouse strain contains the Mdr1a gene mutation and leads to a lack of P-gp expression in the placenta (8). Fetuses of P-gp-deficient (−/−) mice are 100% susceptible to teratogenic photoisomers of avermectin, and this deficiency produces cleft palates (8). In humans, mothers carrying the MDR1 3435C → T polymorphism and using medication showed a 6.2-fold increased risk of having a child with a cleft lip and/or a cleft palate (9). These studies clearly demonstrate that P-gp plays an important role in limiting fetal exposure to harmful drugs and xenobiotics. Less information is available regarding organic anion transporters in the placenta. BeWo cells are human origin placental choriocarcinoma epithelial cells and serve as a model of syncytiotrophoblasts (10). BeWo cells are commonly used in studies of the blood-placental barrier and drug transporters (11, 12). Newly developed direct-acting antiviral agents against hepatitis C virus (HCV) proteins have dramatically improved clinical outcomes in chronic hepatitis C therapy. Boceprevir and telaprevir are new orally administered HCV nonstructural protein 3/4A protease inhibitors approved for the treatment of HCV infections (13, 14). Combination antiretroviral therapy has dramatically decreased the mortality of patients with human immunodeficiency virus (HIV) infections (15, 16). Because antiretroviral therapy has improved and permitted patients to live near-normal life spans, HIV infection has shifted from being an acute disease to being a chronic and manageable disease. Combination antiretroviral therapy requires several classes of antiviral agents (6, 13, 17). Elvitegravir and raltegravir are potent HIV-1 integrase inhibitors and represent a new class of antiretroviral treatment. Maraviroc and vicriviroc are novel chemokine receptor CCR5 antagonists. Rilpivirine and etravirine are new nonnucleoside reverse transcriptase inhibitors approved for the treatment of HIV infections (6, 13, 17). Most newly developed anti-HCV and anti-HIV drugs are organic anions at physiological pH. The organic anion transport system in the human placenta may be the target of drug-drug interactions by these antiviral drugs. Drug transfer from the mother to the fetus must be monitored to minimize drug exposure to the fetus. More detailed knowledge of placental drug transport is necessary to improve drug therapy in pregnant women. In this study, we examined the effects of antiviral drugs on organic anion transport in the human placenta using fluorescent organic anions and human syncytiotrophoblast BeWo cells.
and were counted using a TC20 automated cell counter (Bio-Rad Laboratories). BeWo cells were subcultured using TrypLE express (Life Technologies) and glibenclamide (Wako Pure Chemical Industries, Ltd., Osaka, Japan); fetal bovine serum (FBS), penicillin, and streptomycin (Life Technologies, Carlsbad, CA); 8-[(2-[fluoresceinyl]aminoethylthio)adenosine-3',5'-cyclic monophosphate (8-FcAMP) (Biolog Life Science Institute, Bremen, Germany); sodium fluorescein, rifampin, estrone-3-sulfate, atorvastatin, and p-aminomhippurate (PAH) (Sigma-Aldrich, St. Louis, MO); MK-571 and maraviroc (Cayman Chemical, Ann Arbor, MI); boceprevir, telaprevir, elvitegravir, and etravirine (ChemScence, LLC, Monmouth Junction, NJ); raltegravir (ChemieTek, Indianapolis, IN); rilpivirine (Carbosynth Limited, Berkshire, United Kingdom); vicriviroc (AdooQ BioScience, Irvine, CA); taurocholate (Phoenix Pharmaceuticals, Inc., Burlingame, CA); and furosemide (Nacalai Tesque Inc., Kyoto, Japan). All other chemicals used were of the highest purity available.

Cell culture. BeWo cells (CCl-98, lot number 59368210) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). BeWo cells were cultured in F-12K supplemented with 10% FBS, 100 U/ml of penicillin, and 100 ng/ml of streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO2–95% air.

measurement of cellular uptake of fluorescent organic anions. BeWo cells were subcultured using TrypLE express (Life Technologies) and were counted using a TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA). The cells (2 × 10^6 cells/tube) were suspended in Hanks’ balanced salt solution (HBBS; pH 7.4). The composition of HBBS was 1.26 mM CaCl2, 0.81 mM MgCl2, 5.33 mM KCl, 0.44 mM KH2PO4, 4.17 mM NaHCO3, 138 mM NaCl, 0.33 mM Na2HPO4, 5.56 mM D-glucose, and 10 mM HEPES (pH 7.4). The cell suspensions were preincubated at 37°C for 10 min. Then 8-FcAMP (final concentration, 1 μM) or fluorescein (final concentration, 1 μM) was added, and the suspensions were further incubated for 10 to 30 min at 37°C. For the concentration-dependent studies, various concentrations of 8-FcAMP or fluorescein were incubated with the cell suspensions for 10 min at 37°C, 0°C, or 4°C. To evaluate the effects of antiviral drugs on cellular uptake of the fluorescent organic anions, 100 μM concentrations of various compounds and cell suspensions (2 × 10^6 cells/tube) in HBBS were incubated for 10 min at 37°C and then were further incubated with 1 μM 8-FcAMP or fluorescein for 10 min. After the incubation, the tubes were centrifuged (1,000 × g) for 2 min at 4°C. Supernatants were removed by aspiration. The cells were resuspended in ice-cold HBBS and centrifuged again. After removing the supernatant, the cells were lysed with 0.5% Triton X-100 in HBBS. Fluorescence intensity was measured using a microplate fluorometer (DTX-880; Beckman Coulter, Inc., Indianapolis, IN). The excitation and emission wavelengths were 485 and 535 nm, respectively. Protein concentrations were measured using the detergent-compatible modified Lowry method and a Bio-Rad DC protein assay kit (Bio-Rad Laboratories), with bovine serum albumin as the standard. Cellular uptake kinetics were determined by fitting the Michaelis-Menten equation to the net uptake values obtained by subtracting the uptake measured at 4°C from the uptake measured at 37°C.

FIG 1 Cellular uptake of 8-FcAMP by BeWo cells. The cells were incubated with 1 μM 8-FcAMP for 10 to 30 min at 37°C. Data are the mean ± SD of six determinations from three independent experiments.

FIG 2 Cellular uptake of fluorescein by BeWo cells. The cells were incubated with 1 μM fluorescein for 10 to 30 min at 37°C. Data are the mean ± SD of six determinations from three independent experiments.

RESULTS

Characterization of cellular uptake of fluorescent organic anions by BeWo cells. Figure 1 shows the uptake of 8-FcAMP in human placental BeWo cells. The cellular uptake of 8-FcAMP increased linearly with increasing incubation time. Fluorescein, another fluorescent organic anion, was time dependently taken up by BeWo cells (Fig. 2). Transport of 8-FcAMP and fluorescein in BeWo cells was not saturated for 30 min; therefore, we selected a 10 min incubation time as the initial uptake period for further studies.

Cellular uptake of 8-FcAMP by BeWo cells was low when the cells were incubated at 4°C (Fig. 3). Uptake of 8-FcAMP at 4°C increased linearly with increasing substrate concentrations. In contrast, uptake of 8-FcAMP at 37°C was high and showed Michaelis-Menten-type kinetics. Cellular uptake of fluorescein was high at 37°C and was saturated at higher concentrations (Fig. 4). The apparent Michaelis constant (Km) and the maximum uptake rate (Vmax) for 8-FcAMP were found to be 6.45 ± 0.75 μM and 25.55 ± 5.93 pmol/mg protein/10 min, respectively. The Km and Vmax values for fluorescein uptake were estimated to be 31.2 ± 11.8 μM and 510.9 ± 90.6 pmol/mg protein/10 min, respectively. Transport of 8-FcAMP and fluorescein in the BeWo cells showed time-dependent, temperature-dependent, and concentration-dependent characteristics. These results indicate that the cellular up-
take of 8-FcAMP and fluorescein by BeWo cells was mediated by a specific transport system.

Effects of antiviral drugs on cellular uptake of fluorescence organic anions in BeWo cells. Figure 5 shows the effects of various compounds on the cellular uptake of 8-FcAMP by BeWo cells. Known OAT substrates, including rifampin, taurocholate, estrone-3-sulfate, glibenclamide, and atorvastatin, significantly inhibited the uptake of 8-FcAMP by BeWo cells. MK-571, an inhibitor of the efflux transporter MRP, did not increase cellular accumulation of 8-FcAMP. The OAT inhibitor, furosemide, had no effect. Furthermore, the OAT substrates, rifampin, estrone-3-sulfate, glibenclamide, and atorvastatin, significantly inhibited the uptake of fluorescein by the BeWo cells, but the OAT substrate, PAH, increased uptake (Fig. 6). These results indicate that cellular uptake of 8-FcAMP and fluorescein by BeWo cells was probably mediated by OATP rather than by MRP and OAT.

We examined the effects of newly developed anti-HCV and anti-HIV drugs on fluorescent organic anion transport in human placental BeWo cells. Boceprevir, telaprevir, elvitegravir, raltegravir, maraviroc, and vicriviroc significantly inhibited the uptake of 8-FcAMP by BeWo cells (Fig. 5). Furthermore, boceprevir, telaprevir, elvitegravir, and maraviroc significantly inhibited the cellular transport of fluorescein (Fig. 6). These results suggest that several antiviral drugs, including boceprevir, telaprevir, elvitegravir, and maraviroc, inhibit the organic anion transport system in the human placenta.

**DISCUSSION**

In the present study, the cellular uptake of fluorescein organic anions was characterized in BeWo cells. The uptake of 8-FcAMP and fluorescein by suspended BeWo cells was temperature and concentration dependent (Fig. 1 to 4). The rank order of the inhibitory effects of anionic drugs on the uptake of 8-FcAMP was atorvastatin > glibenclamide > estrone-3-sulfate > rifampin; this is consistent with that for fluorescein uptake (Fig. 3, 4). It is thought that OATP2B1 and OAT4 take up anionic drugs from fetal blood and that MRP2/4 exports them to maternal blood (3–6). We previously reported that adefovir, cidoflovir, and tenofovir are substrates of human OAT1 (SLC22A6) and OAT3 (SLC22A8) (18). OAT1 and OAT3 are mainly expressed in the kidneys and are not present in placenta (5). OAT4 has been reported to be expressed in the fetal-facing membrane of human syncytiotrophoblasts (3, 5). However, the human OAT4 protein is not expressed in wild-type BeWo cells; therefore, Zhou et al. (19) generated hOAT4-expressing BeWo cells and used them to study the regulation of hOAT4 activity. The present findings suggest that 8-FcAMP and fluorescein transport in the fetal-facing membrane of BeWo cells is mediated by a specific organic anion transport system, probably by OATP2B1. BeWo cells are useful for the study of placental drug transporters. However, as is seen from the example of OAT4, the expression of drug transporters in the human placenta and in BeWo cells is not exactly matched. It is reported that genetic polymorphisms cause variation in the activity and expression of P-gp and BCRP in the human placenta (3, 4). BeWo cells seem to have limitations in the study of the interindividual differences of drug transporters.

Sodium fluorescein has been reported to be a substrate of the hepatic organic anion transporters OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3) (20, 21). The kinetic values for fluorescein transport in OATP1B1-transfected CHO cells have been reported to be 4.2 ± 0.8 µM and 30.9 ± 10.5 pmol/mg protein/min, respectively. The kinetic values for fluorescein transport in OATP1B3-transfected CHO cells were 30.7 ± 10.1 µM and 135 ± 20 pmol/mg protein/min, respectively (20). De Bruyn et al. (21) reported that the kinetic values for fluorescein transport in OATP1B1-transfected or OATP1B3-transfected CHO cells were 12.2 ± 4.9 and 33.2 ± 3.4 µM, respectively. The kinetic values for OATP1B1-transfected or OATP1B3-transfected CHO cells were 30.7 ± 3.8 and 195.6 ± 16.6 pmol/mg protein/min, respectively (21). These values were
comparable to our estimated \( K_m \) and \( V_{\text{max}} \) values in BeWo cells. In addition, it has been reported that 8-FcAMP was a substrate of OATP1B1 and OATP1B3 (22). The \( K_m \) values of 8-FcAMP uptake in OATP1B1-transfected or OATP1B3-transfected CHO cells were 2.9 ± 0.40 and 1.8 ± 0.47 \( \mu M \), respectively (22). These values are similar to our \( K_m \) for 8-FcAMP, which was estimated to be 6.45 ± 0.75 \( \mu M \). Uptake of 8-FcAMP or fluorescein in OATP1B1-transfected or OATP1B3-transfected CHO cells has been reported to be inhibited by rifampin, estrone-3-sulfate, glibenclamide, and atorvastatin (20–22). These results support our hypothesis that 8-FcAMP and fluorescein are transported by the OATP transporter in human BeWo cells. We are now in the process of establishing OATP2B1-transfected HEK293 cells and examining 8-FcAMP and fluorescein transport in HEK/OATP2B1 cells.

Some antiviral drugs have been reported to be substrates and/or inhibitors of OATP1B1 or OATP1B3 (5). The HIV protease inhibitors, lopinavir, ritonavir, nelfinavir, darunavir, and saquinavir, have been shown to inhibit OATP1B1-mediated and OATP1B3-mediated fluorescein transport (20, 21). Recently, Furihata et al. (23) showed that simvastatin, asunaprevir, daclatasvir, and sofosbuvir inhibited OATP1B1 or OATP1B3 functions. In addition, they showed that simvastatin and asunaprevir have long-lasting preincubation inhibitory effects on OATP1B1 activity (23). Zembruski et al. (24) reported that elvitegravir and vicriviroc inhibited P-gp and BCRP but that raltegravir and maraviroc did not. To the best of our knowledge, the present study is the first to investigate the effects of newly developed anti-HCV and anti-HIV drugs on placental drug transport.

We previously reported changes in drug transporters in the placenta taken during the first and second trimesters of pregnancy and at full term (7). Expression of OATP2B1 mRNA in the human placenta changed with gestational age. OATP2B1 in the term placenta showed 2.5-fold higher expression than in the first trimester placenta (7). Antiretroviral therapy is necessary for HIV-infected pregnant women. In addition to their importance in the mothers’ health, such regimens are important for preventing perinatal transmission of HIV to the fetus (4, 6). Therefore, further studies, such as in vivo pharmacokinetic studies of drug transfer in the developing placenta, are needed to achieve better pharmacotherapy for pregnant women and their fetuses.

In conclusion, cellular uptake of the fluorescence organic anions, 8-FcAMP and fluorescein, from the fetal-facing membrane of human placental BeWo cells was temperature and concentration dependent. Transport of 8-FcAMP and fluorescein in BeWo cells was inhibited by known OATP substrates, including atorvastatin, glibenclamide, estrone-3-sulfate, and rifampin. Antiviral drugs, including boceprevir, telaprevir, elvitegravir, and maraviro, inhibited 8-FcAMP and fluorescein transport in BeWo cells. These findings suggest that some antiviral drugs may cause drug-drug interactions by inhibiting the organic anion transport system in the human placenta.

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REFERENCES

