Proton-coupled erythromycin antiport at rat blood-placenta barrier

Yoshimichi Sai, Tomohiro Nishimura, Kaori Ochi, Noriaki Tanaka, Akinori Takagi, Masatoshi Tomi, Noriko Kose, Yasuna Kobayashi, Naoki Miyakoshi, Shinji Kitagaki, Chisato Mukai, Emi Nakashima

Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan (Y.S., T.N., K.O., N.T., A.T., M.T., N.K., E.N.)

Department of Pharmacy, Kanazawa University Hospital, 13-1 Takara-machi, Kanazawa 920-8641, Japan (Y.S.)

Department of Clinical Pharmacy, School of Pharmacy, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan (Y.K.)

Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan (N.M., S.K., C.M.)
Running Title

Erythromycin/H⁺ antiport at placenta

To whom correspondence should be addressed:

Professor Emi Nakashima, Ph.D.

Faculty of Pharmacy

Keio University

1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan.

PHONE: +81-3-5400-2660 / FAX: +81-3-5400-2553

E-mail: nakashima-em@pha.keio.ac.jp

Number of text pages: 31
Number of figures: 6
Number of tables: 0
Number of cited references: 34
Number of words in abstract: 213
Number of words in introduction: 329
Number of words in discussion: 1197
Abbreviations

ALP, alkaline phosphatase; BCRP, breast cancer resistance protein; BBMV, brush-border membrane vesicles; EIPA, ethylisopropylamiloride; CsA, cyclosporin A; FTC, fumitremorgin C; MATE, multidrug and toxin extrusion; MRP, multidrug resistance protein; NHE, Na+/H+ exchanger; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCTN1, organic cation/ergothioneine transporter; OSCP, organic solute carrier protein; PEPT1, oligopeptide transporter; P-gp, P-glycoprotein; SLC, solute carrier
Abstract

The aim of the present study was to characterize the mechanism of erythromycin transport at the blood-placenta barrier, using TR-TBT 18d-1 cells as a model of rat syncytiotrophoblasts. [14C]Erythromycin was taken up by TR-TBT 18d-1 cells with a Michaelis constant of 466 µM. Although the uptake was not dependent on extracellular Na⁺ or Cl⁻, it was increased at weakly alkaline pH. Significant overshoot of [14C]erythromycin uptake by placental brush-border membrane vesicles was observed in the presence of an outwardly directed proton gradient. These results indicate that erythromycin is transferred by H⁺ coupled transport system in syncytiotrophoblasts. To address the physiological transport of erythromycin in rat placenta, fetal-to-maternal transport clearance was estimated by means of the single placental perfusion technique. Clearance of [14C]erythromycin was higher than that of [14C]inulin, a paracellular pathway marker, and was decreased by the addition of 5 mM erythromycin, indicating that saturable efflux system from fetus to mother is involved. The effect of various transporter inhibitors on [14C]erythromycin efflux from TR-TBT 18d-1 cells was evaluated. CsA, FTC and probenecid had no effect, while EIPA, a specific inhibitor of Na⁺/H⁺ exchangers (NHEs), was significantly inhibitory. These results suggest that erythromycin efflux transport at the rat blood-placenta barrier is mediated by an erythromycin/H⁺ antiport system, driven by H⁺ supplied by NHEs.
Introduction

Erythromycin transport across the placenta is restricted by the blood-placenta barrier. The fetal plasma concentration of erythromycin is 3-10% of the maternal concentration in humans (Kiefer et al., 1955; Heikkinen et al., 2000), and the placental and fetal distribution of erythromycin is also quite low in rats (Kibwage et al., 1989). Therefore it is likely that an efflux transporter(s) is involved in erythromycin transport at the placenta. Erythromycin is used to treat pregnant women because it is considered to present little risk to fetal development. It has, however, been reported that some infants exposed to erythromycin as fetuses showed pyloristenosis (Kallen et al., 2005). Since the fetal toxicity of erythromycin is unclear, it is important to clarify the molecular mechanism of erythromycin transport at the blood-placenta barrier.

Several drug transporters can transport erythromycin. For example, erythromycin is a substrate and an inhibitor of P-gp (Hofsli and Nissen-Meyer, 1989; Dey et al., 2004). Although P-gp function at the blood-placenta barrier is an important determinant of fetal drug exposure (Smit et al., 1999), functional involvement of P-gp in erythromycin transfer in the placenta has not been reported. MRP2 (ABCC2) also recognizes erythromycin (Karla et al., 2007). Further, some solute carriers (SLC) function as efflux transporters. Therefore, possible involvement of SLC and ABC transporters in erythromycin transport in the placenta should be clarified.

The aim of the present study was to characterize erythromycin transport at the blood-placenta barrier by using TR-TBT 18d-1 cells as a model of rat syncytiotrophoblasts, which compose the blood-placenta barrier. It has been established that TR-TBT 18d-1 show transport function for nucleosides, anti-retroviral drugs, and β-amino acids (Kitano et al., 2002;
Chishu et al., 2008; Nishimura et al., 2008; Sai et al., 2008; Sato et al., 2009). Therefore, 
TR-TBT 18d-1 cells should be a suitable model to characterize the molecular mechanism of 
drug transport at the blood-placenta barrier. Fetal-to-maternal clearance of erythromycin in vivo 
in rats and erythromycin uptake by placental brush-border membrane vesicles were also 
examined.
Materials and Methods

Chemicals and Animals.

[^14C]Erythromycin (2.0 GBq/mmol) and[^14C]inulin (74 MBq/g) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Ko143 was synthesized as described previously (Asashima et al., 2006). All other chemicals were commercial products of analytical grade. Wistar female rats were purchased from Japan SLC (Hamamatsu, Japan). Animal studies were performed in accordance with the Guideline for the Care and Use of Laboratory Animals of Keio University School of Medicine.

Cell Culture.

TR-TBT 18d-1 cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS), 100 U/ml benzylpenicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Invitrogen, San Diego, CA) on 100-mm culture dishes (Corning Life Sciences, Lowell, MA) in a humidified incubator at 33 °C under an atmosphere of 5% CO₂ in air. For the uptake and efflux studies, TR-TBT 18d-1 cells were seeded on 4-well plates (Nalge Nunc International, Naperville, IL) coated with porcine skin collagen type I (Nitta Gelatin) at a density of 1×10⁵ cells/well. After incubation for 3 days at 33 °C, the cells were cultured at 37 °C for a further 4 days.

Uptake and efflux of[^14C]erythromycin by TR-TBT 18d-1 cells, and effect of other macrolide drugs on uptake.
Transport medium contained 122 mM NaCl, 25 mM NaHCO₃, 3 mM KCl, 1.4 mM CaCl₂, 1 mM MgSO₄, 0.4 mM KH₂PO₄, 10 mM d-glucose, and 10 mM Hepes at pH 7.4. Na⁺-free transport medium was prepared by replacing Na⁺ with choline⁺, Li⁺, K⁺, or N-methyl-D-glucamine⁺ (NMG⁺). Cl⁻-free transport medium was prepared by replacing Cl⁻ with Br⁻, NO₃⁻, and thiocyanate (SCN⁻). Medium pH was adjusted at 6.4 - 8.4 with 1 N HCl or 1 N NaOH.

For the uptake study, TR-TBT 18d-1 cells were washed twice with the transport medium. After a 10 min preincubation in the above medium, the transport medium was replaced with drug solution containing [¹⁴C]erythromycin (10 µM) in the absence or presence of an inhibitor to initiate the uptake reaction. For the efflux study, TR-TBT 18d-1 cells were incubated with the transport medium containing [¹⁴C]erythromycin (10 µM) for 15 min. These cells were washed twice with the transport medium and further incubated in fresh medium (pH 7.4). At the designated time, both the uptake and efflux reaction was terminated by addition of ice-cold transport medium and the cells were washed twice in the same medium.

The cells were solubilized in 500 µL of 0.1 M NaOH/1% Triton-X 100 solution. Then 400 µL of the cell lysate was mixed with 3 mL of scintillation cocktail (Clearsol-I, Nacalai Tesque, Kyoto, Japan), and radioactivity was measured with a liquid scintillation counter (TRI-CARB 317TR/SL, Packard Instrument Company, Meriden, CT). Cellular protein content was quantified by means of the BCA method using a protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

For the study of the inhibitory effect of other macrolide drugs, spiramycin, azithromycin, clarithromycin and josamycin, on initial uptake of [¹⁴C]erythromycin (10 µM) by
TR-TBT 18d-1 cells, measurements were made in the uptake buffer containing an inhibitor and 1% DMSO. Inhibitor concentrations were 1 mM except for clarithromycin (0.4 mM).

Rat placental single perfusion.

Pregnant rats at gestation day 19-20 were anesthetized with an intraperitoneal injection of pentobarbital (40-50 mg/kg). The abdominal cavity and the uterus was opened to expose the fetus in a warm bath. The umbilical cord artery and vein were cannulated with an indwelling catheter and needle (24-gauge; Terumo Co., Ltd., Tokyo, Japan). The placenta was perfused with Krebs-Ringer solution containing 1% bovine serum albumin at the rate of 1.0 mL/min. Drug concentration in the perfusate was measured with a liquid scintillation counter. Fetal-to-maternal clearance ($CL_{fm}$) was calculated based on the extraction ratio and perfusion flow rate by use of the following equation:

$$CL_{fm} = (1 - \frac{[v]}{[a]}) \cdot \frac{Q_f}{W_p}$$

where $[v]$, $[a]$, $Q_f$ and $W_p$ represent drug concentration in the umbilical vein and in the artery, perfusion flow rate and placenta weight, respectively. The purity of BBMVs was checked by measuring alkaline phosphatase (ALP) activity as an apical membrane marker, and Na⁺-K⁺ ATPase activity as a basolateral membrane marker.

Preparation of rat placental BBMVs.

Rat placental BBMVs were prepared according to the reported method with some modifications (Alonso de la Torre et al., 1991). The placentas were isolated from pregnant rats.
at gestation day 18 under anesthesia with ether, and were homogenized (H1) in 10 volumes of buffer containing 300 mM sorbitol, 1 mM EGTA, 0.1 mM PMSF, 20 mM Tris/Mes, pH 7.4) in a Teflon-glass homogenizer (Iwaki, Tokyo, Japan) with 10 strokes at 2,500 rpm. MgCl₂ was added at the final concentration of 10 mM, and the homogenate was incubated for 30 min. Supernatant (S1) and pellet (P1) were obtained by centrifugation at 800×g for 10 min. P1 was homogenized again in the 10 mM MgCl₂ buffer. Supernatant (S1') obtained by centrifugation at 800×g for 10 min was mixed with S1, and the mixture was centrifuged at 10,400×g for 20 min. The resulting supernatant was further centrifuged at 110,000×g for 45 min. The final pellet was suspended in the buffer containing 300 mM sorbitol, 20 mM Tris/Mes (adjusted to pH 7.4) via a 25-gauge needle, and 100 µL aliquots were frozen in liquid nitrogen and stored at -80 °C until use. The tissue was handled on ice during all procedures.

Protein content was measured by the SDS-Lowry method with bovine serum albumin as a standard (Lowry et al., 1951). Lowry reagent (1 mL) containing 2% Na₂CO₃, 0.4% NaOH, 1% CuSO₄, and 2% Na,K-tartrate was added to a 200 µL aliquot of diluted of BBMVs, and the mixture was incubated for 10 min at room temperature. Then 100 µL of 2-fold diluted Folin and Ciocalteu's phenol reagent (Sigma, St. Louis, MO) was added. The absorbance at 750 nm was measured after 30 min incubation at room temperature.

For ALP assay, the reported method was employed (Hirohashi et al., 2000). Briefly, 45 µL of the diluted BBMVs solution and the first homogenate (H1) was mixed with 5 µL of Triton-X 100, and the mixture was incubated for 30 min at room temperature. Then 900 µL of the buffer ALP containing 50 mM 2-amino-2-methyl-1-propanol, 5 mM MgCl₂, 300 mM sorbitol and 50 µL of substrate solution containing 100 mM p-nitrophenyl phosphate in the
buffer ALP were added to the sample and the whole was incubated for 30 min at 37 °C. To terminate the reaction, the sample was mixed with 1 mL of 10% trichloroacetic acid. The mixture was centrifuged at 12,000×g for 5 min and the absorbance of the supernatant was measured.

Ouabain-sensitive Na⁺-K⁺ ATPase assay was conducted according to the reported method (Scharschmidt et al., 1979). A 10 µL aliquot of the diluted BBMVs solution and H1 was mixed with 790 µL of the buffer containing 200 mM MgCl₂, 126 mM NaCl, 13 mM KCl, 5.2 mM NaN₃, 5 mM ATP, 1.1 mM EGTA, 131 mM Tris, pH 7.4 in the absence or presence of 1.1 mM ouabain. The mixture was incubated for 10 min at 37 °C. To terminate the reaction, 200 µL of 35% trichloroacetic acid was added. The supernatant was obtained by centrifugation at 12,000×g for 5 min, and a 200 µL aliquot was mixed with 40 µL of 10 N H₂SO₄, 40 µL of ammonium molybdate, and 40 µL of filtered reagent containing 0.25% 1-amino-2-naphthol-4-sulfonic acid, 15% sodium bisulfite anhydrous, 0.5% sodium sulfite anhydrous, and 680 µL of distilled water. The mixture was incubated for 7 min at 37 °C, and the absorbance at 660 nm was measured.

BBMVs solution that showed a 10-times greater ALP activity and a 2-times lower Na⁺-K⁺ ATPase activity than H1 was used for the drug uptake study.

Rapid filtration technique for evaluating drug uptake by BBMVs

A 20 µL aliquot of the BBMVs solution was preincubated for 10 min at 37 °C. The reaction was initiated by adding 80 µL of the transport medium, 300 mM sorbitol and 10 mM Hepes/Tris, pH 7.4 or 8.4, containing a designated concentration of radiolabeled compound to
the BBMV solution. To terminate the uptake, 900 µL of ice-cold transport medium containing 1 mM erythromycin was added; the unlabeled erythromycin served to reduce nonspecific binding of the radiolabeled compound to the filter. The mixture was immediately filtered through a HAWP 0.45 µm filter (Millipore Corporation, Billerica, MA). The filter was washed with 8 mL of the ice-cold transport medium and solubilized in 4 mL of Clearsol-I. The radioactivity was measured with a liquid scintillation counter.

mRNA expression of Na+/H+ exchangers, NHE1 and NHE3

Total RNA was isolated from the placenta of a rat at gestational day 18 and from TR-TBT 18d-1 cells cultured as described for the uptake study, using an RNeasy mini kit (QIAGEN, Valencia, CA). cDNA was synthesized by reverse transcriptase (ReverTra Ace; Toyobo Co., Ltd., Osaka, Japan). PCR was performed using Platinum PCR SuperMix (Invitrogen, San Diego, CA). Specific primers were designed for rat NHE1 and NHE3 as follows; 5'-GAACATCCACCCCAAGTCTG-3' for NHE1 forward, 5'-CAGTGGGTCTGAGCCTATGC-3' for NHE1 reverse, 5'-ACTGCTTAA TGACGCGGTGACTGT-3' for NHE3 forward and 5'-AAAGACGAAGCCAGGCTCGATG-3' for NHE3 reverse.

Data analysis.

Kinetic analysis was done using the following Michaelis-Menten equation:

\[ v = \frac{V_{\text{max}} \times S}{K_m + S} + K_n \times S \]
where \( v, S, V_{\text{max}}, K_m, \) and \( K_{nt} \) represent initial uptake velocity, substrate concentration, maximum uptake velocity, Michaelis constant, and non-saturable uptake constant, respectively. The fitting was performed by nonlinear least-square regression analysis using Delta Graph (version 5.5.5, RedRock Software Inc., Salt Lake City, UT).

Statistical analysis was performed by the use of Student's \( t \)-test or analysis of variance (ANOVA) with Dunnett's post-hoc test. \( P < 0.05 \) was considered significant.
Results

Cellular uptake of $^{14}$C]erythromycin by TR-TBT 18d-1 cells

To address the transporter-mediated cellular uptake of $^{14}$C]erythromycin across the blood-placenta barrier, $^{14}$C]erythromycin uptake by TR-TBT 18d-1 cells was measured. $^{14}$C]Erythromycin was strongly taken up by TR-TBT 18d-1 cells, and the uptake was reduced by the addition of an excess of unlabeled erythromycin, indicating that the $^{14}$C]erythromycin uptake was predominantly mediated by a transport system(s) (Fig. 1A). To characterize the transport system, its Na$^+$ and Cl$^-$-dependency was examined. No significant difference was observed upon replacing these ions with other monocations and monoanions, respectively (Fig. 1B). To further characterize $^{14}$C]erythromycin uptake, the uptake kinetics was analyzed based on the Michaelis-Menten equation. Erythromycin uptake showed a clear saturable curve and the Eadie-Hofstee plot was linear. The estimated kinetic parameters ($K_m$, $V_{max}$ and $K_{ns}$) were 466 µM, 48.8 nmol/mg protein/10 min, and 0.514 µL/mg protein/10 min, respectively. These results indicate that a single transporter is involved in $^{14}$C]erythromycin uptake.

Response to pH gradient of erythromycin transport in syncytiotrophoblasts

To investigate the response to extracellular pH, $^{14}$C]erythromycin uptake was measured in medium at various pH values in the range of 6.4 - 8.4. Compared to the uptake at pH 6.4, saturable uptake was gradually increased at higher pH, suggesting that the saturable transport of $^{14}$C]erythromycin is enhanced by an outwardly directed proton gradient (Fig. 3A). To clarify the effect of such a gradient on erythromycin transport in syncytiotrophoblasts, $^{14}$C]erythromycin transport by BBMVs was measured in the presence and absence of an
outwardly directed proton gradient. Significant overshoot was observed only in the presence of the proton gradient, indicating that [14C]erythromycin is counter-transported with protons (Fig. 3B).

Specificity of the erythromycin transport system in syncytiotrophoblasts

To examine whether or not the erythromycin transport system is specific for erythromycin, the inhibitory effect of other macrolide drugs on erythromycin uptake by TR-TBT 18d-1 cells was investigated. Josamycin was ineffective, while spiramycin, azithromycin, clarithromycin and erythromycin significantly inhibited [14C]erythromycin uptake to about 25-40% of the control (Fig. 4). These results suggest that the transport system has broad specificity for erythromycin-like structure.

Fetal-to-maternal transport of erythromycin across rat placenta

To address the physiological transport of erythromycin in rat placenta, fetal-to-maternal transport clearance was estimated by means of the single placental perfusion technique. [14C]Inulin showed minimal transport clearance, corresponding to clearance via the paracellular pathway transport (Fig. 5). Compared to [14C]inulin clearance, clearance of [14C]erythromycin was quite high, and was saturated by the addition of 5 mM erythromycin (Fig. 5). These results suggest that a fetus-to-mother-oriented efflux transport system is involved in physiological trans-placental transport of erythromycin.

Erythromycin efflux from TR-TBT 18d-1 cells
To address the efflux transport of erythromycin in TR-TBT 18d-1 cells, the effect of transporter inhibitors were investigated. TR-TBT 18d-1 cells were preincubated with $[^{14}\text{C}]$erythromycin, and the time course of efflux was followed for 30 min. $[^{14}\text{C}]$Erythromycin was 90% eliminated from the cells within 30 min and 60% was eliminated within 1 min (data not shown). Therefore, efflux of $[^{14}\text{C}]$erythromycin was characterized at 1 min. $[^{14}\text{C}]$Erythromycin efflux was not inhibited by representative ABC efflux transporter inhibitors (CsA, FTC and probenecid) or a V-type ATPase inhibitor, bafilomycin A1 (Fig. 6A). Erythromycin itself did not inhibit $[^{14}\text{C}]$erythromycin efflux, but rather tended to increase the efflux, though this was not statistically significant. On the other hand, EIPA, a specific inhibitor of Na$^+$/H$^+$ exchangers (NHEs), was significantly inhibitory (Fig. 6A). mRNA expression of NHE1 and NHE3, representative NHEs, was examined. Rat placenta and TR-TBT 18d-1 cells both expressed NHE1 and NHE3 (Fig. 6B).
Discussion

P-gp and BCRP are expressed at the blood-placenta barrier, and consequently the transfer of their substrates, such as digoxin, saquinavir, paclitaxel, topotecan and mitoxantrone, from mother to fetus is limited (Smit et al., 1999, Jonker et al., 2000, Kolwankar et al., 2005), although administration of these drugs in early pregnancy, i.e., before development the blood-placenta barrier, presents a high risk to the fetus. However, other drugs can be transported through the blood-placenta barrier and are transferred to the fetus. Thus, drug therapy for pregnant women is usually to be avoided. Our findings here indicate that the limited fetal transfer of erythromycin involves a proton antiport system cooperating with a sodium/proton exchanger(s). This proton antiport efflux system, which is distinct from ABC transporters as mentioned above, represents a novel mechanism restricting drug transfer at the blood-placenta barrier, especially in the brush-border membrane of syncytiotrophoblast. These findings imply that low-risk drug therapy for pregnant women may be possible using drugs that are transported by this system. Therefore, it is important to identify other substrates of this efflux system and to fully characterize the transport protein.

TR-TBT 18d-1 cells showed clear erythromycin uptake activity (Fig. 1). The concentrative cell-to-medium ratio indicated the involvement of uphill transport. Although membrane transporters driven by Na⁺ and Cl⁻ gradients across the plasma membrane generally mediate such transport, the erythromycin transport system did not require a Na⁺ or Cl⁻ gradient. Kinetic study revealed relatively low-affinity transport, compared to clinically and experimentally achievable plasma concentrations of erythromycin (Fig. 2), indicating that the transport system is likely to be involved in physiological placental disposition of erythromycin,
at least in rats. The transport process appeared to involve a single transporter (Fig. 2B).

Known SLCs seem unlikely to be involved in placental erythromycin transport. OAT2 does transport erythromycin (Kobayashi et al., 2005a), but the reported affinity ($K_m$ : 18.5 µM) is different from that found in the present study. In addition, OAT2 mRNA is only weakly expressed in the placenta (Leazer and Klaassen, 2003; Nishimura and Naito, 2005). OATP1A2 and OATP2B1 recognize erythromycin at least as an inhibitor, but the affinity was lower than that found in the present study (EC$_{50}$ > 1,600, 1100 µM, respectively) (Lan et al., 2009). It has also been reported that OSCP1 accepts erythromycin as a substrate (Izuno et al., 2007; Kobayashi et al., 2007). We investigated the expression of OSCP1 in rat placenta. Placenta isolated at gestational day 18 and TR-TBT 18d-1 cells, as well as the testis (positive control), showed protein expression of OSCP1, but placental BBMVs did not contain OSCP1 (data not shown). These results indicate that OSCP1 is expressed in the placenta, but not in the brush-border membrane of rat syncytiotrophoblasts, in accordance with the subcellular localization in basolateral membrane of human syncytiotrophoblasts (Kobayashi et al., 2005b). Consequently, the contribution of these SLC transporters to erythromycin transport in brush-border membrane in syncytiotrophoblasts is considered to be negligible.

Erythromycin transport was significantly elevated under weakly alkaline conditions (Fig. 3A). This result can be interpreted in two ways: 1) the optimal pH is in the alkaline range, or 2) the transport system is driven by an outwardly directed H$^+$ gradient. To examine these possibilities, we prepared rat placental BBMVs, since BBMVs are useful for analysis of the driving force of transporters. An outwardly directed H$^+$ gradient showed overshoot transport of erythromycin, indicating that a H$^+$ antiporter contributes to erythromycin uptake in rat
syncytiotrophoblasts (Fig. 3B). An erythromycin/H⁺ antiport system has previously been found only in bacteria, such as *S. aureus* and *E. coli* (Kodama et al., 1998; Zgurskaya and Nikaido, 1999).

It has been reported that OCTN1 shows organic cation/H⁺ antiport function (Tamai et al., 2004). mRNA expression of OCTN1 in placenta is similar in rats and 4 times lower in humans when compared to that in kidney, which shows the maximum expression (Leazer and Klaassen, 2003; Nishimura and Naito, 2005). OCTN1 generally transports lower-molecular-weight compounds than erythromycin (MW: 734), such as tetraethylammonium (MW: 130) and ergothioneine (MW: 229) (Tamai et al., 2004; Grundemann et al., 2005). However, we can not exclude the involvement of OCTN1 in erythromycin transport in the placenta. MATE1 is also known to be an organic cation/H⁺ antiporter (Otsuka et al., 2005). The mRNA expression of MATE1 is, however, quite low in the placenta, at least in mice (Aleksunes et al., 2008). Thus, it seems unlikely that functional expression of MATE1 is significant in the placenta.

To investigate whether or not the erythromycin transport system in TR-TBT 18d-1 cells is specific for erythromycin, the inhibitory effects of several other macrolide drugs were examined. Spiramycin, azithromycin and clarithromycin significantly reduced erythromycin uptake, but josamycin did not (Fig. 4). Spiramycin, azithromycin and clarithromycin, like erythromycin, have two separate sugar moieties, whereas josamycin has one linked sugar moiety. However, further study will be needed to establish whether or not the difference of inhibitory effect is related to this structural feature.

We studied *in vivo* efflux transport of erythromycin in rat placenta. Fetus-to-mother
transport clearance of erythromycin was higher than that of inulin, a paracellular transport marker, and was significantly reduced by excess erythromycin (Fig. 5), implying that efflux transport of erythromycin transport occurs at the placenta. This finding is consistent with the restricted transfer of erythromycin from mother to fetus (Kibwage et al., 1989).

To examine the possible involvement of ABC efflux transporters, such as P-gp, BCRP and MRPs, the effects of inhibitors of these transporters was examined, though there is no report indicating that erythromycin is a substrate of BCRP. CsA, FTC and probenecid showed no significant inhibition, indicating that the contribution of P-gp, BCRP and MRPs to erythromycin efflux is minimal, at least in TR-TBT 18d-l cells (Fig. 6A). An excess concentration of erythromycin did not reduce the efflux, but tended to increase it (Fig. 6A). This might be explained by a trans-stimulatory effect of erythromycin on the efflux of preloaded \[^{14}C\]erythromycin. This would be consistent with the idea that an erythromycin/H\(^+\) exchange mechanism is functional at the blood-placenta barrier. Inhibition by EIPA indicates that NHEs are involved in erythromycin transport (Fig. 6A). Considering the substrate specificity of NHE, it is unlikely that NHEs are directly involved in transporting erythromycin as a substrate. Since the erythromycin transport was driven by a H\(^+\) gradient, it seems likely that NHEs supply the driving force for the erythromycin transport system, as NHE3 functionally couples with PEPT1 (Watanabe et al., 2005). NHE1 and NHE3 expression was detected at the transcriptional level in both rat placenta and TR-TBT 18d-l cells (Fig. 6B). Further studies are required to determine whether or not a similar mechanism is involved in erythromycin transport at the placenta. The coupling with NHEs may provide a clue to identify the erythromycin transporter itself.

To conclude, we have found a H\(^+\)-gradient-driven erythromycin transport system in
rat placenta. Although TR-TBT 18d-1 cells showed \[^{14}\text{C}]\text{erythromycin}\) uptake under weakly alkaline conditions, it has been supposed that \[^{14}\text{C}]\text{erythromycin}\) transfer from mother to fetus is restricted. Therefore, it is possible that the present transport system acts as a part of the blood-placenta barrier, mediating erythromycin efflux by using \(\text{H}^{+}\) supplied by NHE1 or NHE3 as the driving force.
Acknowledgements

We thank Ms. Yui Inamori and Ms. Kanako Hosoda for technical assistance.
References


Gynaecol 107:770-775.


Eur J Drug Metab Pharmacokinet 14:7-14.


Kolwankar D, Glover DD, Ware JA and Tracy TS (2005) Expression and function of ABCB1 and ABCG2 in human placental tissue. Drug Metab Dispos 33:524-529.

Lan T, Rao A, Haywood J, Davis CB, Han C, Garver E and Dawson PA (2009) Interaction of
macrolide antibiotics with intestinally expressed human and rat organic
anion-transporting polypeptides. Drug Metab Dispos 37:2375-2382.


Drug Metab Dispos 31:153-167.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin

ATP-binding cassette and solute carrier transporter superfamilies. Drug Metab
Pharmacokinet 20:452-477.

Nishimura T, Seki Y, Sato K, Chishu T, Kose N, Terasaki T, Kang YS, Sai Y and Nakashima E
syncytiotrophoblast cell line TR-TBT 18d-1. Drug Metab Dispos 36:2080-2085.

transporter protein that mediates the final excretion step for toxic organic cations. Proc

Sai Y, Nishimura T, Shimpo S, Chishu T, Sato K, Kose N, Terasaki T, Mukai C, Kitagaki S,
zidovudine uptake by rat conditionally immortalized syncytiotrophoblast cell line

mechanism of 2',3'-dideoxyinosine and uridine at the blood-placenta barrier. Placenta
30:263-269.


Footnotes

The present study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan; a grant from the "High-Tech Research Center" and "Open Research Center" Project for Private Universities matching fund subsidy; and a grant for a Joint Research Project under the Japan-Korea Basic Scientific Cooperation Program of the Japan Society for the Promotion of Science (JSPS) and Korea Science & Engineering Foundation (KOSEF).
Legends

Figure 1

$[^{14}\text{C}]$Erythromycin uptake by TR-TBT 18d-1 cells

$[^{14}\text{C}]$Erythromycin (10 µM) uptake by TR-TBT 18d-1 cells was measured for 30 min in the absence (○) and presence (●) of an excess concentration (5 mM) of unlabeled erythromycin (A). Na$^+$ and Cl$^-$ dependency of initial $[^{14}\text{C}]$erythromycin uptake was measured by replacing Na$^+$ and Cl$^-$ with other monocations and monoanions as shown (B). Data represent the mean ± S.E.M. of 4 determinations. A significant difference from the corresponding control ($p < 0.05$) is indicated by an asterisk (*).

Figure 2

Michaelis-Menten type kinetics of erythromycin uptake by TR-TBT 18d-1 cells

Initial uptake of $[^{14}\text{C}]$erythromycin (10 µM) by TR-TBT 18d-1 cells was measured in the presence of various concentrations of unlabeled erythromycin up to 5 mM (A). The fitting curve to the Michaelis-Menten equation is shown as a solid line, and non-saturable uptake estimated from the fitting is shown as a dotted line. Saturable $[^{14}\text{C}]$erythromycin uptake (●) was calculated by subtracting non-saturable uptake from total uptake (○). An Eadie-Hofstee plot is shown (B). Fitted saturable uptake is indicated by a dashed line. Data represent the mean ± S.E.M. of 4 determinations.

Figure 3

pH-dependency of $[^{14}\text{C}]$erythromycin uptake by TR-TBT 18d-1 cells and rat placental
brush-border membrane

Initial uptake of $[^{14}\text{C}]$erythromycin (10 µM) by TR-TBT 18d-1 cells was measured in the uptake buffer adjusted to pH 6.4 - 8.4 in the absence (○) and presence (●) of 5 mM erythromycin (A). Data represent the mean ± S.E.M. of 4 determinations. A significant difference from the corresponding control ($p < 0.05$) is indicated by an asterisk (*). $[^{14}\text{C}]$Erythromycin (10 µM) uptake by rat placental brush-border membrane vesicles was measured in the absence (○, pH_in7.4/ pH_out7.4) and presence (●, pH_in7.4/ pH_out8.4) of an outwardly directed pH gradient (B). Data represent the mean ± S.E.M. of 3 determinations.

Figure 4
Inhibitory effect of macrolide drugs on $[^{14}\text{C}]$erythromycin uptake by TR-TBT 18d-1 cells

Initial uptake of $[^{14}\text{C}]$erythromycin (10 µM) by TR-TBT 18d-1 cells was measured in the uptake buffer containing an inhibitor and 1% DMSO (A). Inhibitor concentrations were 1 mM except for clarithromycin (0.4 mM). Data represent the mean ± S.E.M. of 4 determinations and those are normalized by control uptake. A significant difference from the corresponding control ($p < 0.05$) is indicated by an asterisk (*).

Figure 5
Fetal-to-maternal clearance of $[^{14}\text{C}]$erythromycin in rat placenta

Fetal-to-maternal clearance of $[^{14}\text{C}]$erythromycin (0.2 µM, closed column) was measured by rat placental perfusion for 30 min. $[^{14}\text{C}]$Inulin (1.2 µM, open column) was used as a control of
paracellular transport. Clearance was estimated in terms of disappearance from the perfusion medium. Data represent the mean ± S.E.M. of 4-9 determinations. A significant difference from the corresponding control ($p < 0.05$) is indicated by an asterisk (*).

Figure 6

Effect of drug transporter inhibitors on $[^{14}C]$erythromycin efflux by TR-TBT 18d-1 cells and mRNA expression of NHEs

Initial efflux of $[^{14}C]$erythromycin (10 µM) by TR-TBT 18d-1 cells was measured for 1 min in the uptake buffer containing 1% DMSO (pH 7.4) in the absence and presence of a designated inhibitor (A). Inhibitor concentrations are shown in the figure. Data represent the mean ± S.E.M. of 4 determinations. A significant difference from the corresponding control ($p < 0.05$) is indicated by an asterisk (*). mRNA expression of NHEs in rat placenta and TR-TB 18d-1 cells was determined (B). Reaction mixture without reverse transcriptase, RT (-), was used as a negative control.
Figure 4

- Control
- Josamycin
- Spiramycin
- Azithromycin
- Clarithromycin
- Erythromycin

$[^{14}\text{C}]$Erythromycin Uptake (% of Control)
Figure 5

Fetus-to-mother Clearance (µL/min/g placenta)

Control + 5 mM Erythromycin

[^14C]Erythromycin [^14C]Inulin

* indicates significance.
Figure 6

A

Inhibitor
Concn. (μM)

<table>
<thead>
<tr>
<th></th>
<th>[14C]Erythromycin Efflux (%) of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>On ice</td>
<td></td>
</tr>
<tr>
<td>Erythromycin 5,000</td>
<td></td>
</tr>
<tr>
<td>EIPA 500</td>
<td></td>
</tr>
<tr>
<td>Bafilomycin 0.1</td>
<td></td>
</tr>
<tr>
<td>CsA 10</td>
<td></td>
</tr>
<tr>
<td>FTC 10</td>
<td></td>
</tr>
<tr>
<td>Probenecid 5,000</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates significant difference compared to control.
Figure 6

B

<table>
<thead>
<tr>
<th>RT</th>
<th>(+)</th>
<th>(-)</th>
<th>(+)</th>
<th>(-)</th>
<th>(+)</th>
<th>(-)</th>
<th>(+)</th>
<th>(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NHE1</td>
<td></td>
<td>NHE3</td>
<td></td>
<td>NHE1</td>
<td></td>
<td>NHE3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td></td>
<td>Placenta</td>
<td></td>
<td>TR-TBT 18d-1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>