Expression and transport activity of breast cancer resistance protein (Bcrp/Abcg2) in dually perfused rat placenta and HRP-1 cell line

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Non-standard Abbreviations: ABC – ATP-binding cassette, BCRP/Bcrp – human/rodent breast cancer resistance protein; MDR – multidrug resistance; RT-PCR – reverse transcription polymerase chain reaction; P-gp – P-glycoprotein

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ABSTRACT

Breast cancer resistance protein (BCRP/ABCG2) is a member of the ABC transporter family that recognizes variety of chemically unrelated compounds. Its expression has been revealed in many mammal tissues including placenta. The purpose of this study was to describe its role in transplacental pharmacokinetics using rat placental HRP-1 cell line and dually perfused rat placenta. In HRP-1 cells, expression of Bcrp, but not of P-glycoprotein, was revealed at mRNA and protein levels. Cell accumulation studies confirmed Bcrp-dependent uptake of BODIPY FL prazosin. In the placental perfusion studies, a pharmacokinetic model was applied to distinguish between passive and Bcrp-mediated transplacental passage of cimetidine as a model substrate. Bcrp was shown to act in a concentration dependent manner and to hinder maternal-to-fetal transport of the drug. Fetal-to-maternal clearance of cimetidine was found to be 25 times higher than that in the opposite direction; this asymmetry was partly eliminated by BCRP inhibitors (2 µM fumitremorgin C or 2 µM GF120918) and completely abolished at high cimetidine concentrations (1000 µM). When fetal perfusate was recirculated, Bcrp was found to actively remove cimetidine from the fetal compartment to the maternal one even against concentration gradient and establish a two-fold maternal-to-fetal concentration ratio. Based on our results, we propose a two-level defensive role of Bcrp in the rat placenta: the transporter (i) reduces passage of its substrates from mother to fetus but also (ii) removes the drug already present in the fetal circulation.
INTRODUCTION

Placenta is an organ that brings maternal and fetal blood circulations to proximity, allowing mutual interchange of nutrients and waste products. On the other hand, placenta forms a barrier to protect fetus against harmful endo- and exogenous compounds from maternal circulation. As a barrier, the human and rodent placenta had for long been supposed to present only a mechanical obstruction formed by fetal endothelia, basal membranes and syncytiotrophoblast. However, over the last two decades, variety of metabolizing enzymes and drug efflux transporters of the ATP-binding cassette (ABC) transporter family have been localized in placental trophoblast (Marin et al., 2004; Syme et al., 2004). These proteins are believed to strengthen, in an active and capacity limited manner, placental barrier role and help in protecting the fetus.

Drug efflux transporters of the ABC family are membrane-embedded proteins that limit intracellular concentration of substrates by pumping them out of cell through an active, energy dependent mechanism (Schinkel and Jonker, 2003). The most intensively studied drug efflux transporters to date have been P-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2) and multidrug resistance-associated proteins 1 and 2 (ABCC1, ABCC2), all of which were found to be responsible for the phenomenon of multidrug resistance in cancer therapy (Fischer et al., 2005). In addition, due to their extensive distribution in non-tumorous tissues and wide substrate specificity, these proteins significantly affect body disposition of many clinically used drugs. With respect to expression, regulation, function and clinical relevance, the best described of placental ABC transporters to date is P-glycoprotein (Ceckova-Novotna et al., 2006).

Breast cancer resistance protein (BCRP) is the most recently described member of the ABC transporter superfamily (Doyle et al., 1998). Its expression has been assessed in many tissues and cells including blood-brain barrier, placenta, intestine, various tumors and “side
population” of stem cells (Staud and Pavek, 2005). Since its tissue distribution and substrate specificity overlap noticeably with that of P-glycoprotein (P-gp), it is generally believed that these transporters share similar role in protecting pharmacological sanctuaries such as brain and fetus.

Considerable levels of BCRP/Bcrp expression have been detected in placentas of various species. In humans, placental BCRP expression was found to be approximately 10-times higher than that of P-gp (Ceckova et al., 2006). Given the expression pattern and the broad range of substrates including exogenous (drugs, toxins) and endogenous (steroid conjugates, porphyrins) compounds (Staud and Pavek, 2005), it is reasonable to assume that BCRP may be an important component of the placental barrier. Kolwankar et al (Kolwankar et al., 2005) confirmed BCRP function in microvillous membrane vesicles of the human placenta. In addition, we have recently described BCRP expression, localization and function in an in vitro placental model, BeWo cell line (Ceckova et al., 2006). The only functional in vivo experiments proposing Bcrp activity in the placenta were performed by Jonker et al in transgenic mice (Jonker et al., 2000; Jonker et al., 2002). Nevertheless, transport activity of this efflux protein and its role in transplacental pharmacokinetics has not been fully evaluated to date.

Due to technical constraints and ethical issues, direct investigation of placental drug transfer under in vivo conditions in human is not feasible; therefore, several alternative experimental methods have been developed to assess potential risk that drugs in maternal circulation present to the fetus (Sastry, 1999). Among these, cell cultures and perfused placentas of various species are widely employed models for mechanistic studies to describe transplacental pharmacokinetics, including transport mechanisms and biotransformation. The dually perfused rat placenta, in particular, is a well established model that has been successfully employed to investigate placental physiology and pharmacology (Stulc et al.,
1995; Kertschanska et al., 2000). In our earlier studies, we employed this experimental model to evaluate functional activity of P-gp in the rat placenta (Pavek et al., 2001; Pavek et al., 2003).

The aim of the present study was to assess the effect of Bcrp on transplacental passage of its substrates. We investigated Bcrp activity both *in vitro*, using HRP-1 rat placental cell line, and *in situ* in dually perfused rat term placenta. Using these techniques, we describe Bcrp as an active component of the rat placental barrier that limits maternal-to-fetal and facilitates fetal-to-maternal transport of its substrates.
MATERIALS and METHODS

Reagents and chemicals

Cimetidine and radiolabelled [N-methyl-\(^3\)H]Cimetidine were purchased from Sigma-Aldrich (St. Louis, MO, USA) and from Amersham Biosciences (GE Healthcare Life Science, Little Chalfont, UK), respectively. BODIPY FL prazosin, a common BCRP and P-gp substrate, was obtained from Molecular Probes (Karlsruhe, Germany). Specific BCRP inhibitors, Ko143 and fumitremorgin C, were donated by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) or purchased from Alexis (Lausen, Switzerland), respectively. Specific P-gp inhibitors, PSC833 and cyclosporine, were kind gifts from Dr. Andrýsek (Ivax Pharmaceuticals, Opava, Czech Republic). Dual BCRP and P-gp inhibitor, GF120918, was from GSK (Greenford, England). All other compounds were reagent grade.

Cell cultures

For in vitro accumulation studies, HRP-1 rat trophoblast cells (Soares et al., 1987), received as a generous gift from Dr. Michael Soares at the University of Kansas Medical Center, were employed. They were maintained in RPMI-1640 culture medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 1 mM sodium pyruvate and 50 µM \(\beta\)-mercaptoethanol. Cells from passages 15-25 were used in experiments described herein.

Animals

All experiments were approved by the Ethical Committee of the Faculty of Pharmacy (Hradec Kralove, Charles University in Prague) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, 1996 and the European Convention for
the protection of vertebrate animals used for experimental and other scientific purposes, Strasbourg, 1986. Pregnant Wistar rats were purchased from Biotest Ltd. (Konárovice, Czech Republic) and were maintained in 12/12-h day/night standard conditions with water and pellets ad libitum. Experiments were performed on day 21 of gestation. Fasted rats were anesthetized with pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) in a dose of 40 mg/kg administered into the tail vein.

**RNA isolation and RT-PCR analysis**

Placentas and kidneys were collected on day 21 of gestation from 5 rats. Five randomly selected placentas from each animal were dissected free of maternal tissues and fetal membranes. Immediately after dissection, the organs were frozen in liquid nitrogen and stored at -70°C until analysis. RNA isolation and reverse transcription were performed as described previously (Novotna et al., 2004). Sequences of mRNAs for target genes were obtained from NCBI database; primers for *mdr1a*, *mdr1b* and *bcrp* genes were designed using the Vector NTI Suite software (Informax, Bethesda, MD, USA) and are given in Table 1.

RT-PCR analysis was performed on iCycler iQ (Bio-Rad, Hercules, CA, USA). cDNA was amplified with HotStart Taq polymerase under the following conditions: 3mM MgCl$_2$, 0.2mM dNTP, 0.03 U/µl polymerase, SybrGreen I in 1:100,000 dilution, 0.3 µM each primer; the temperature profile was 95°C for 14 min; 50 times: 95°C for 15s, 60°C for 20s, 72°C for 20s, 72°C for 5 min; melting curve program 72-95°C. Each sample of cDNA was amplified in duplicates. The PCR products were separated by electrophoresis on 2% agarose gel in the presence of ethidium bromide and visualized under ultraviolet light and compared with low molecular weight DNA ladder (25-766bp) (New England BioLabs, Herts, UK).
Western blot analysis

Cell membrane fractions of placenta tissues and whole cell lysates were prepared as described previously (Novotna et al., 2004; Ceckova et al., 2006). Protein contents were determined by BCA™ Protein Assay Detection Kit (Pierce, Rockford, IL, USA). Samples containing 100 µg of cell lysate protein or 40 µg of tissue cell membrane were subjected to electrophoresis on 8% SDS-polyacrylamide gels and subsequently electrotransferred onto Hybond-ECL membrane (GE Healthcare, Chalfont St. Giles, UK). After blocking in 5 % non-fat dry milk blocking buffer, the membranes were probed with mouse monoclonal anti-P-gp antibody C219 (Signet Laboratories, Dedham MA, USA) and rabbit polyclonal anti-ABCG2 antibody M-70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500 in 1 % blocking buffer; o/n at 4°C). Incubation with corresponding secondary horseradish peroxidase-conjugated antibody (anti-rabbit: 1:2000 in 1 % blocking buffer, anti-mouse 1:1000; 60 min at RT) was used for recognition of the primary antibodies. Immunoreactive proteins were visualized on FOMA® Blue Medical X-ray films (FOMA Bohemia, Hradec Kralove, Czech Republic) by ECL Advance Western blotting detection system (GE Healthcare, Chalfont St. Giles, UK).

Immunohistochemical localization of Bcrp in the rat term placenta

Preparation of rat placental tissue was performed as described previously (Pavek et al., 2003). The antigen (Bcrp) was unmasked by heating the specimens in sodium citrate buffer (pH 6.0) for two-times 8 minutes in a microwave oven at 750W. Slides were incubated with a polyclonal primary antibody for BCRP (M-70; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:10 in BSA (bovine serum albumin) for 15 to 18 h at 4°C. Subsequently, the slides were developed with a secondary antibody – goat anti-rabbit IgG conjugated to peroxidase-labelled polymer (DAKO EnVision™, Carpinteria, USA) for 30 minutes. The
reaction was visualized using diaminobenzidine (DAB substrate-chromogen solution, DAKO, Carpinteria, USA) and the sections were counterstained by hematoxylin. Slides were examined using computer image analysis (light microscope Nikon Eclipse E200, Japan; digital firewire camera PixeLINK PL-A642, Vitana Corp. Ottawa, Canada; LUCIA software, version 4.82, Laboratory Imaging, Prague, Czech Republic). Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Cellular uptake experiments

HRP-1 cells were seeded on 24-well culture plates (1x10^5 per well) 2 days prior to experiment. Cell culture medium was removed and cells were washed twice with 500 µl pre-warmed PBS. Cells were then preincubated in OPTI-MEM medium with or without inhibitor (1 µM GF120918, 1 µM Ko143, 25 µM verapamil or 1 µM PSC833) at 37°C in 5 % CO₂ for 60 min before fluorescent substrate, BODIPY FL prazosin (500 nM), was added. Accumulation was allowed for 2 h at 37°C and was arrested by prompt cooling on ice and removal of medium. Cells were washed with ice-cold PBS, lyzed in 1% SDS and fluorescence was measured after 24 hours (Genios Plus, Tecan, Austria). Fluorescence of each well was related to protein content as assessed by BCA™ Protein Assay Detection Kit.

Dual perfusion of the rat placenta

The method of dually perfused rat placenta was used as described previously (Pavek et al., 2003). Briefly, one uterine horn was excised and submerged in heated Ringer saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying a selected placenta and connected with the peristaltic pump. Krebs’ perfusion liquid containing 1 % albumin was brought from the maternal reservoir at a rate of 1 ml/min. The uterine vein,
including the anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighboring ones by ligatures. The umbilical artery was catheterized using 24-gauge catheter connected to the fetal reservoir and perfused at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into pre-weighted glass vials to check a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments as described before (Pavek et al., 2001). At the end of experiment, placenta was perfused with radioactivity-free buffer for 10 min, excised from the uterine tissue, dissolved in tissue solubilizer (Solvable, PerkinElmer, Boston, MA) and its radioactivity measured in order to detect tissue-bound cimetidine.

Two types of perfusion systems were employed in this study:

1. For pharmacokinetic analysis of concentration-dependent transplacental passage of cimetidine, both maternal and fetal sides of the placenta were perfused in open-circuit systems, without recirculation of the perfusate. Cimetidine was added to the maternal (in maternal-to-fetal studies) or fetal (in fetal-to-maternal studies) reservoir immediately after successful surgery followed by approximately 10 min stabilization period before sample collection started (time 0). Fetal effluent was sampled into pre-weighted vials in 5-minute intervals and analyzed for [3H]cimetidine.

2. To investigate the capability of Bcrp to remove its substrate from fetal circulation, both maternal and fetal sides of the placenta were infused with equal concentrations of cimetidine and after 10 min stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (200 µl) were collected every 10 minutes from the maternal and fetal
reservoirs and [3H]cimetidine concentration was measured. This experimental setup assures steady cimetidine concentration on the maternal side of the placenta and enables investigations of maternal/fetal concentration ratio; any net transfer of the drug implies transport against a concentration gradient and is evidence for active transport.

**Effect of cimetidine inflow concentrations and BCRP inhibitors on transplacental clearance**

To investigate the effect of cimetidine concentrations on maternal-to-fetal and fetal-to-maternal clearances, cimetidine and [3H]cimetidine as a tracer were added to the maternal or fetal reservoir, respectively, in one of the following concentrations: 0.005, 0.1, 1, 30, 100 or 1000 µM. The inflowing cimetidine concentration was maintained constant for the duration of the experiment; transplacental clearances of cimetidine were calculated for every concentration from all measured intervals as described below.

To study the effect of BCRP and P-gp inhibitors, fumitremorgin C (2 µM), GF120918 (2 µM), cyclosporine (10 µM) or verapamil (25 µM) was added to the maternal or fetal reservoir in the 10th minute of perfusion. Subsequently, transplacental clearance of cimetidine in the period of 0-10 min (without inhibitor) was compared with that in 20-30 min (with inhibitor), leaving the mid-interval of 10-20 min as a stabilization period to achieve a new steady-state after addition of inhibitor. This experimental setup allows for direct observations of inhibitor effect in one animal, reducing possible interindividual variability.

**Pharmacokinetic analysis of efflux transport activity in the placenta**

Organ clearance concept was applied to mathematically describe maternal-to-fetal and fetal-to-maternal transport of cimetidine in open-circuit perfusion system. Averaged data from
the intervals of 0-10 minute (control) and 20-30 minute (inhibitor) of placental perfusions were used for the following calculations.

Total maternal-to-fetal transplacental clearance (ClTmf) of cimetidine normalized to placenta weight was calculated according to equation 1.

\[ Cl_{Tmf} = \frac{C_{fv} \cdot Q_f}{C_{ma} \cdot w_p} \]  

(1)

where \( C_{fv} \) is the concentration of cimetidine in the umbilical vein effluent, \( Q_f \) is the umbilical flow rate, \( C_{ma} \) is the concentration of cimetidine in the maternal reservoir and \( w_p \) is the wet weight of the placenta.

In fetal-to-maternal studies, the ability of the placenta to remove cimetidine from the fetal circulation was expressed as extraction ratio (ER) using equation 2 (Shargel and Yu, 1993):

\[ ER = \frac{C_{fa} - C_{fv}}{C_{fa}} \]  

(2)

where \( C_{fa} \) is the concentration of cimetidine in the fetal reservoir entering the perfused placenta via the umbilical artery. Total fetal-to-maternal clearance normalized to placenta weight (ClTfm) was calculated according to equation 3.

\[ Cl_{Tfm} = \frac{ER \cdot Q_f}{w_p} \]  

(3)

To distinguish between passive and active components of the transplacental movement, the following concept was applied (Fig. 1). Assuming the total transplacental passage of cimetidine being a function of passive diffusion and efflux activity of BCRP, the total transplacental clearance in maternal-to-fetal (ClTmf) and fetal-to-maternal (ClTfm) direction is described by equations 4 and 5, respectively:

\[ Cl_{Tmf} = Cl_{pd} - Cl_{efflux} \]  

(4)
\[ Cl_{Tfm} = Cl_{pd} + Cl_{\text{efflux}} \] (5)

where \( Cl_{pd} \) is clearance of passive diffusion and \( Cl_{\text{efflux}} \) expresses the efflux activity of the transporter. Since \( Cl_{\text{efflux}} \) is a capacity-limited (non-linear) process, it can be expressed in terms of Michaelis-Menten kinetics:

\[ Cl_{\text{efflux}} = \frac{V_{\text{max}}}{K_m + C_{ma(fa)}} \] (6)

where \( V_{\text{max}} \) is the maximal velocity of the transport, \( K_m \) is the concentration at which half the maximal velocity is reached and \( C_{ma(fa)} \) is substrate concentration in maternal (\( C_{ma} \)) or fetal (\( C_{fa} \)) circulation.

In maternal-to-fetal studies, adding \( Cl_{\text{efflux}} \) into (4) yields the following equation which was used to fit clearance vs inflow concentration data:

\[ Cl_{Tmf} = Cl_{pd} - \frac{V_{\text{max}}}{K_m + C_{ma}} \] (7)

In analogy, when the effect of fetal inflow concentrations on fetal-to-maternal clearance was investigated, data were fitted by the following equation:

\[ Cl_{Tfm} = Cl_{pd} + \frac{V_{\text{max}}}{K_m + C_{fa}} \] (8)

Radioactivity remaining in the placental tissue after perfusion was less than 0.4 ± 0.06 % of the infused dose in both maternal-to-fetal and fetal-to-maternal studies, regardless of total cimetidine concentration. Therefore, it was ignored in pharmacokinetic modeling. Data were fitted using reciprocal weighting and the numerical module of SAAM II (SAAM Institute, Seattle, WA, USA).
Statistical analysis

For each group of placental perfusion experiments, the number of animals was \( n \geq 4 \). Cellular uptake studies are based on \( n = 4 \). One-way ANOVA followed by Bonferroni test or Student’s \( t \)-test were used where appropriate to assess statistical significance. Differences of \( p < 0.05 \) were considered statistically significant.
RESULTS

Expression of Bcrp and P-gp in rat placenta and HRP-1 cells

RT-PCR and Western blotting were employed to investigate the expression of Bcrp and P-gp in the rat placenta and HRP-1 cell line and compared with that in kidney as a positive control (Tanaka et al., 2005). The bands corresponding to 329, 331 and 188 bp for mdr1a, mdr1b and Bcrp, respectively, were visualized under the ultraviolet light (Fig. 2A). Expression of Bcrp was detected in rat kidney, placenta and HRP-1 cell line samples. In contrast, expression of both mdr1a and mdr1b (coding for P-gp) was detected only in rat kidney and placenta but not in HRP-1 cell line. Similarly, application of polyclonal anti-Abcg2 antibody M-70 revealed significant levels of Bcrp in the rat placenta, kidneys and HRP-1 cells. Using C219 monoclonal antibody we confirmed the expression of P-gp in the rat placenta and kidneys only while no signal for P-gp was detected in HRP-1 cell lysate (Fig. 2B).

Immunohistochemical localization of Bcrp in the rat term placenta

Localization of Bcrp expression in the rat term placenta was investigated by immunohistochemistry at the light microscopy level. Four different placentas (gestation day 21) were used for the experiments. The rat placenta is composed of two morphologically different zones, the junctional zone (maternal blood spaces separated by trophoblastic trabeculae that do not contain fetal blood vessels) and the labyrinth zone (maternal blood separated from fetal blood vessels by trophoblast cells). Bcrp was detected in the inner layers of the syncytiotrophoblast (layers II and III) of the labyrinth zone only (Fig. 3). No Bcrp staining was visible in either layer I or in the fetal capillaries.
Bcrp efflux activity in HRP-1 cell line

To investigate Bcrp and/or P-gp activity in HRP-1 placental cells, BODIPY FL prazosin (500 nM) as a common substrate of BCRP and P-gp was added to the cells and the effect of Bcrp and/or P-gp inhibitors was observed. In agreement with gene expression data, only BCRP specific inhibitor, Ko143 (1 µM), and dual BCRP and P-gp inhibitor, GF120918 (1 µM), increased BODIPY FL prazosin accumulation by more than 100% (p < 0.05). In contrary, P-gp specific inhibitors, PSC833 (1 µM) and verapamil (25 µM) did not affect BODIPY FL prazosin accumulation suggesting undetectable activity of P-gp in the HRP-1 cell line (Fig. 4).

Consistency of perfusion experiments

To determine a steady-state period suitable for inhibitor studies in both maternal-to-fetal and fetal-to-maternal experiments, placenta was first perfused with 0.1 µM cimetidine for 50 minutes. If no inhibitor was added, we observed steady clearances for the whole period. When a BCRP inhibitor was added to the maternal perfusate in the 10th minute of perfusion, it took about 5-10 minutes to achieve a new steady-state (data not shown). Therefore, to evaluate the effect of inhibitor on cimetidine transplacental passage, samples from 0-10 min interval of perfusion were averaged and compared to those collected in 20-30 min interval.

Effect of BCRP and P-gp inhibitors on transplacental passage of cimetidine

To test interactions of cimetidine with placental Bcrp or P-gp, dual and/or specific inhibitors of these transporters were added to maternal reservoir. Addition of BCRP inhibitors, GF120918 (2 µM) or fumitremorgin C (2 µM), caused significant change in transplacental clearance of cimetidine (Fig. 5). Interaction of cimetidine with human P-gp has recently been ruled out using MDR1-transfected MDCKII and LLC-PK1 cell lines (Pavek et
al., 2005). To exclude any confounding effects of rat P-gp in transplacental passage of cimetidine, P-gp inhibitors, cyclosporine (10 µM) or verapamil (25 µM), were tested. Although these compounds increased maternal-to-fetal clearance of rhodamine123, a P-gp substrate, in our previous study (Pavek et al., 2003), they did not interfere with transport of cimetidine. Therefore, transplacental passage of cimetidine does not seem to be affected by P-gp and cimetidine can be used as a marker compound to functionally analyze efflux activity of Bcrp in the rat placenta. Inhibitory effect observed after addition of GF120918 may be ascribed to Bcrp blockade only, although we cannot exclude possible contribution of other, yet unidentified GF-sensitive cimetidine transporter.

Effect of inflow cimetidine concentrations on transplacental clearance

Cimetidine was infused to maternal or fetal side of the placenta in one of the following concentrations: 0.005, 0.1, 1, 30, 100, or 1000 µM. In both maternal-to-fetal and fetal-to-maternal transport studies, increase in cimetidine concentration caused significant change in transplacental clearance, confirming non-linearity of the process and involvement of a capacity-limited mechanism (Figs. 6 and 7). Furthermore, addition of a BCRP inhibitor significantly affected clearances at lower cimetidine concentrations while at concentrations above 30 µM, inhibitor was rather ineffective.

Fitting experimental data with equations 7 and 8 provided pharmacokinetic parameters describing passive and Bcrp-mediated transplacental passage of cimetidine (Table 2). It is evident, that passive movement across the placenta (described by Cl_{pd}) is independent of direction and of inhibitor used. On the other hand, capacity limited clearance (Cl_{efflux}) is a concentration- and inhibitor-dependent parameter. At substrate concentrations largely exceeding the Michaelis-Menten constant (C>>K_m) the transporter is saturated, the non-linear fraction of equations 7 and 8 approaches zero and both equations are reduced to linear
processes only \((\text{Cl}_T = \text{Cl}_{\text{pd}})\); under these conditions, transplacental pharmacokinetics is beyond any quantifiable effect of efflux transporter and is governed exclusively by passive diffusion. This has been experimentally achieved in both maternal-to-fetal and fetal-to-maternal studies when cimetidine concentration was increased to 1000 µM. Furthermore, since addition of inhibitor caused no change in transplacental clearance of 1000 µM cimetidine (see Figs. 6 and 7), it is reasonable to assume that at high substrate concentrations, drug-drug interactions will have no effect on penetration of BCRP substrates through placenta.

Comparing maternal-to-fetal and fetal-to-maternal clearances revealed great asymmetry in favor of fetal-to-maternal direction. This was most evident at low cimetidine concentrations (0.005 µM), where fetal-to-maternal clearance was almost 25 times higher \((p < 0.05)\) than that in the opposite direction (Fig. 8). At a concentration of 1000 µM, however, both maternal-to-fetal and fetal-to-maternal clearances of cimetidine reached identical values of 0.042 ml/min/g confirming saturation of Bcrp and limited role of its efflux activity. This experimental value corresponds well with the calculated clearance of passive diffusion \((\text{Cl}_{\text{pd}} \approx 0.041 - 0.043\ \text{ml/min/g})\), see Table 2.

**Bcrp transports cimetidine from fetus to mother against concentration gradient**

To investigate the potential of Bcrp to remove its substrate from fetal circulation, cimetidine was added to both maternal and fetal reservoirs at equal concentrations of 0.005 µM or 1000 µM and fetal perfusate was recirculated. At a low drug concentration (0.005 µM), cimetidine in the fetal circulation steadily decreased and stabilized after about 40 min of perfusion. Fetal to maternal concentration ratio reached a value of 0.49 towards the end of the experiment. Decrease in fetal cimetidine was blocked by co-infused BCRP inhibitors (GF120918 or fumitremorgin C), see Fig. 9A. At a high cimetidine concentration (1000 µM), maternal and fetal concentrations remained unchanged throughout the perfusion period with
fetal/maternal concentration ratio staying close to 1 (Fig. 9B). These findings demonstrate the potency of placental Bcrp to remove, in a capacity-limited manner, its substrate from fetal compartment and to establish a concentration gradient between maternal and fetal circulations.
DISCUSSION

Detailed knowledge of transplacental kinetics of drugs is essential with respect to fetal safety, fetal medication and drug-drug interactions during pregnancy. Apart from physical-chemical properties, placental passage of many drugs is controlled by interactions with biotransformation enzymes and/or efflux transporters. It is widely believed that enzymes and drug efflux transporters form an active component of the placental barrier that helps protect fetus against maternal toxins (Marin et al., 2004). In addition, it seems plausible, that these proteins may, to at least some extent, actively metabolize/transport compounds already present in the fetal circulation. Using rat placental perfusion, we have previously demonstrated that 11β-hydroxysteroid dehydrogenase type 2 metabolizes both maternal and fetal corticosterone with a comparable potency (Staud et al., 2006). Similarly, P-gp has been confirmed to favor fetal-to-maternal transport of its substrates in perfused rat (Pavek et al., 2003) or human placenta (Molsa et al., 2005; Sudhakaran et al., 2005). In the present study, we focused on functional analysis of the latest of ABC drug efflux transporters, Bcrp, using dually perfused rat term placenta and rat placenta-derived cell line, HRP-1.

To date, only few studies have reported on BCRP activity in placenta and these are mainly based on in vitro models. Very recently, Kolwankar et al (Kolwankar et al., 2005) employed placental microvillous membrane vesicles to confirm BCRP function in the human placenta. Subsequently, we have described BCRP expression, localization and function in an in vitro placental model, BeWo cell line (Ceckova et al., 2006). In the present study, we used rat placental HRP-1 cell line derived from placental labyrinth region at midgestation (Soares et al., 1987). This cell line has previously been employed to study several aspects of placental physiology (Soares et al., 1989; Shi et al., 1997; Morris Buus and Boockfor, 2004), metabolism (Xu et al., 2005) or nutrient transport (Novak et al., 2001; Zhou et al., 2003). To our knowledge, however, no studies have been performed so far to investigate expression
and/or activity of ABC drug efflux transporters in this \textit{in vitro} placental model. Bcrp expression was revealed at both mRNA and protein levels. Surprisingly, we did not detect any expression of \textit{mdr1a} or \textit{mdr1b} genes coding for rat P-gp, neither did we find any signal by Western blotting. Consistent with gene and protein expression results, uptake studies revealed only Bcrp activity, while P-gp did not affect cell accumulation of BODIPY FL prazosin. These data are similar to what has previously been observed in human choriocarcinoma cell line BeWo (Atkinson et al., 2003; Ceckova et al., 2006; Evseenko et al., 2006) where only BCRP was found to be functionally expressed, while P-gp activity was negligible. Lack of expression and function of P-gp in the HRP-1 cell line makes this model inappropriate to investigate the transplacental transport of P-gp substrates. On the other hand, it may well serve as a tool to study Bcrp role in transplacental pharmacokinetics as its efflux activity will not interfere with that of P-gp.

The only functional \textit{in vivo} studies on Bcrp activity in the placenta so far have been performed in transgenic mice (Jonker et al., 2000; Jonker et al., 2002); however, detailed evaluation of BCRP role in transplacental pharmacokinetics is still lacking. In the present study, cimetidine was employed to comprehensively describe the role of Bcrp in maternal-to-fetal and fetal-to-maternal transport. Cimetidine was chosen as a model substrate for its convenient properties: it is a BCRP substrate which is not recognized by human P-gp (Pavek et al., 2005), it weakly binds to plasma proteins, and its biotransformation by placental enzymes is negligible (Schenker et al., 1987). In addition, cimetidine passive diffusion through biological membranes is delayed by its physical-chemical properties as shown in transepithelial passage (Pavek et al., 2005) or placental transport (Ching et al., 1987; Schenker et al., 1987); this seems to be an important feature to study substrate interactions with an efflux transporter (Eytan et al., 1996; Lentz et al., 2000).
The localization of Bcrp on the apical, maternal-facing membrane of the rat placenta closely resembles that of P-gp described in our earlier studies (Pavek et al., 2003; Novotna et al., 2004). This finding suggests that Bcrp is, like P-gp, important in preventing entry of potential toxins into the fetal compartment. This assumption has been functionally validated in the present study by means of rat perfused placenta: at low cimetidine concentrations (0.005 µM), maternal-to-fetal clearance was 25-fold lower than clearance in the opposite direction. These data confirm that Bcrp causes asymmetry in transplacental clearances in rats by returning substrates coming from maternal side and facilitating transport of drugs from fetus to mother. Interestingly, pharmacokinetic modeling revealed Michaelis-Menten constant for fetal-to-maternal direction to be 20 times higher than that for maternal-to-fetal direction. We assume this difference is caused by polarized localization of Bcrp on the maternal side of the placenta; as a result, a compound administered to the fetal circulation needs to pass through fetal tissues to reach the transporter. This suggests that much higher cimetidine concentrations are needed to saturate Bcrp transporter during fetal-to-maternal passage than in the opposite direction.

To investigate the potential of Bcrp to remove drugs already present in the fetal compartment, both maternal and fetal sides of the placenta were perfused with equal concentrations of cimetidine and fetal perfusate was re-circulated. After short equilibration period, we observed significant decrease in fetal cimetidine concentrations, confirming that Bcrp can actively remove its substrate from the fetal compartment. Since decrease in cimetidine concentration continued even at later intervals (dropping by more than 50% within 60 minutes of perfusion), it is evident that Bcrp in rats can pump this compound from fetus to mother even against concentration gradient.

Interestingly, several studies on cimetidine placental transfer were published two decades ago with intriguing results. When studied in sheep, large cimetidine gradient between mother
and fetus was observed (Mihaly et al., 1983). In a follow-up study, the authors suggested that an active transporter from the fetal to the maternal circulation might be responsible for this discrepancy (Ching et al., 1985). In contrast, when investigated in the dually perfused human placenta, two papers concluded that transport of cimetidine was very slow and occurred by passive diffusion with lack of saturation kinetics (Ching et al., 1987; Schenker et al., 1987). These contrasting findings might be explained by interspecies differences; however, one has to realize that these studies were performed before efflux transporters were discovered and described, with limited range of cimetidine concentrations, and without the option to use appropriate inhibitors. Therefore, possible role of a drug efflux transporter in the transplacental pharmacokinetics of cimetidine could not have been taken in account. Our present findings suggest that Bcrp is the transporter responsible for limited maternal-to-fetal passage and large maternal/fetal concentration ratio of cimetidine in rats. However, BCRP activity in perfused placentas of other species must be elucidated before a final conclusion is drawn.

Regarding BCRP expression in human tissues, relatively high mRNA levels were observed in placenta, liver and small intestine with lower expression in the kidney, heart and brain (Doyle et al., 1998). In rodents, on the other hand, different mRNA distribution pattern was indicated by Tanaka et al (Tanaka et al., 2005) who found high expression levels in kidney, small and large intestine while lower levels were found in other tissues including brain and placenta. Based on these observations, the authors suggested limited importance of placental Bcrp in rodents (Tanaka et al., 2005). In contrast, functional role of placental Bcrp has been proposed in mice by Jonker et al (Jonker et al., 2000; Jonker et al., 2002) and thoroughly assessed in the rat placenta in our study. Therefore, mRNA expression levels do not have to necessarily correlate with transport potency of the protein since there is a number of other factors that will determine its functional activity, such as posttranscriptional/
posttranslational modifications in protein expression as well as strategic localization of BCRP along the maternal interface.

In conclusion, functional expression of Bcrp in the rat placenta and rat placental HRP-1 cell line was confirmed in this study. A pharmacokinetic model was applied to distinguish between passive and Bcrp-mediated placental transport of cimetidine as a model substrate. We provide evidence for striking asymmetry between maternal-to-fetal and fetal-to-maternal transport of cimetidine; this difference is partly lowered by addition of BCRP inhibitors and completely abolished at high substrate concentrations. In addition, using closed perfusion system on the fetal side of the placenta, we are the first to demonstrate that Bcrp, despite being localized on the maternal-facing side, actively removes cimetidine from the fetal circulation against concentration gradient. Based on our findings, we propose a two-level defensive role of placental BCRP: the transporter (i) reduces passage of its substrates from mother to fetus but also (ii) removes the drug already present in the fetal circulation even against concentration gradient. Given the broad range of BCRP substrates, this transporter seems to be an important component of the rat placental barrier playing a significant role in protection and detoxication of the fetus.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Schematic depiction of pharmacokinetic analysis applied in this study to evaluate efflux transporter activity in the placenta. This model assumes two processes being involved in the transplacental passage: (i) passive diffusion governed by Fick’s law (depending mainly on drug’s physical-chemical properties, concentration gradient, protein binding and membrane area and thickness) here described as *Clearance of passive diffusion (Cl* <sub>pd</sub>) and (ii) saturable efflux process governed by the rules of Michaelis-Menten non-linear kinetics here described as *Capacity limited clearance (Cl* <sub>efflux</sub>). Depending on the direction of substrate movement, these two events add up (in fetal-to-maternal direction) or subtract (in maternal-to-fetal direction) to obtain the value of total transplacental clearance (Cl<sub>T</sub>). If substrate concentration largely exceeds the Michaelis-Menten constant (C>>K<sub>m</sub>), then total placental clearance equals to clearance of passive diffusion. Cl<sub>Tmf</sub> is total transplacental clearance in maternal-to-fetal direction; Cl<sub>Tfm</sub> is total transplacental clearance in fetal-to-maternal direction.

Figure 2. A - mRNA expression of the rat breast cancer resistance protein (Bcrp) and P-glycoprotein (Mdr1a and Mdr1b) in rat kidney, placenta and HRP-1 rat placental cell line. B - Western blot immunoanalysis of protein expression of the rat breast cancer resistance protein (Bcrp) and P-glycoprotein (P-gp) in the rat placenta and kidney and in the HRP-1 rat trophoblast cell line. Kidneys and placentas were randomly sampled from 5 female rats and independently processed as described in Experimental Procedures; two representative samples are shown for each tissue.
Figure 3. Immunohistochemical detection of Bcrp in the labyrinth of rat term placenta. Immunohistochemical staining was performed with the monoclonal antibody M-70 (1:10 dilution). (A) Strong reactivity for Bcrp is visible in the inner layers (II and III) of syncytiotrophoblast. Fetal capillaries or layer I of syncytiotrophoblast do not reveal any positivity. For negative control (B) the slides were treated in the same manner, except non-immune isotype-matched immunoglobulins were substituted for the primary antibody to Bcrp. F – fetal capillaries, M – maternal blood, arrowhead shows nucleus of endothelial cell of the fetal capillary, arrows point to layer I and layers II + III of syncytiotrophoblast (hematoxylin counterstained, scale bars – 30µm).

Figure 4. Uptake of common BCRP and P-gp substrate, BODIPY FL prazosin, by rat placental HRP-1 cells. BODIPY FL prazosin was added to cells without inhibitor (control) or in the presence of BCRP or P-gp inhibitors. Both GF120918 and Ko143 (BCRP inhibitors) significantly increased accumulation of BODIPY FL prazosin in the cells. P-gp selective inhibitors (PSC 833 and verapamil) had no effect on BODIPY FL prazosin accumulation. Data are presented as means ± SD of four experiments. One-way ANOVA followed by Bonferroni test was used; ** p < 0.05 compared with control.

Figure 5. Effect of Bcrp and P-gp inhibitors on maternal-to-fetal clearance of cimetidine. Cimetidine and [3H]cimetidine tracer were added to the maternal compartment in a concentration of 0.1 µM, its radioactivity was measured in fetal venous outflow, and total transplacental clearance was calculated by eq. 1. Inhibitor was added to the maternal perfusate in the 10th min. Only BCRP inhibitors, GF120918 and fumitremorgin C, affected transplacental clearance of cimetidine while P-gp inhibitors, cyclosporine and verapamil, had
no significant effect. Data are presented as means ± SD of six experiments. One-way ANOVA followed by Bonferroni test was used; ** p < 0.05 compared with control.

**Figure 6.** Transport of cimetidine across the dually perfused rat placenta in maternal-to-fetal direction. Cimetidine with [3H]cimetidine tracer were added to the maternal reservoir, its radioactivity was measured in fetal venous outflow, and total transplacental clearance was calculated by eq. 1. Changes of clearance with increasing cimetidine concentration confirm non-linearity of the event and involvement of a saturable mechanism. Inhibitor (2 µM GF120918) was added to block Bcrp activity. At the highest cimetidine concentration tested (1000 µM), clearance reached the value of 0.042 ml/min/g and inhibitor activity was negligible. Experimental values are presented as means ± SD of at least four experiments; the lines represent the best fit of these data to eq. 7. Note the sigmoid shape of the lines with the lower plateau delineating combined effect of passive clearance and efflux activity of Bcrp and the upper plateau representing clearance of passive diffusion alone. Asterisks indicate significant (p < 0.05) effect of inhibitor.

- cimetidine without inhibitor, □ – cimetidine with GF120918

**Figure 7.** Transport of cimetidine across the dually perfused rat placenta in fetal-to-maternal direction. Cimetidine with [3H]cimetidine tracer were added to the fetal reservoir, its radioactivity was measured in fetal venous outflow, and total transplacental clearance was calculated by eq. 3. Changes of clearance with increasing cimetidine concentration confirm non-linearity of the event and involvement of a saturable mechanism. Inhibitor (2 µM GF120918) was added to block Bcrp activity. As in maternal-to-fetal transport, at the highest cimetidine concentration tested (1000 µM), clearances reached the value of 0.042 ml/min/g and inhibitor activity was negligible. Experimental values are presented as means ± SD of at
least four experiments; the lines represent the best fit of these data to eq. 8. Note the sigmoid shape of the lines with the upper plateau delineating combined effect of passive clearance and efflux activity of Bcrp and the lower plateau representing clearance of passive diffusion alone. Asterisks indicate significant (p < 0.05) effect of inhibitor.

■ – cimetidine without inhibitor, □ – cimetidine with GF120918

**Figure 8.** Ratio of clearances between fetal-to-maternal (fm) and maternal-to-fetal (mf) directions. Cimetidine with [3H]cimetidine tracer were added to the maternal or fetal compartment, its radioactivity was measured in fetal venous outflow, and total transplacental clearances were calculated by eq. 1 or 3, respectively. At low substrate concentrations (0.005 µM), Bcrp efflux activity caused almost 25-times higher clearance in fetal-to-maternal direction. At high substrate concentrations (1000 µM), however, this ratio equalized. Numbers in parenthesis show ratio of fm to mf clearance.

**Figure 9.** Elimination of cimetidine from the fetal circulation by placental Bcrp. Cimetidine and [3H]cimetidine tracer were added to both maternal (closed symbol) and fetal (open symbols) circulations at equal concentrations. Fetal perfusate (10 ml) was recirculated and sampled for 60 minutes. At low cimetidine concentrations of 0.005 µM (A), fetal cimetidine decreased from 0.005 µM down to 0.0024 µM and stabilized after 40 min of perfusion. This decrease was inhibited by both BCRP inhibitors, GF120918 (2 µM) and fumitremorgin C (2 µM). At high cimetidine concentrations of 1000 µM (B) no decrease in fetal compartment was observed suggesting saturation of the transporter. Data are presented as means ± SD of three experiments.

◆ – maternal cimetidine concentration

□ - fetal cimetidine concentration with GF120918
○ - fetal cimetidine concentration with fumitremorgin C

△ – fetal cimetidine concentration without inhibitor
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<thead>
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| mdr1b | AY082609 | cgc ttc taa tgt taa agg ggc tat g (f)  
                       agec att tct gta tgg tat ctc caa gc (r) | 331 | 2489-2819 |
| Bcrp  | NM181381 | cca ctg gaa tgc aaa ata gag (f)  
                       cct cat agg tag taa gtc aga cac a (r) | 188 | 1340-1527 |
Table 2. Pharmacokinetic parameters of transplacental passage of cimetidine

<table>
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<th>Fetal-to-maternal transport</th>
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<td>inhibitor</td>
</tr>
<tr>
<td>Cl_{pd} (ml/min/g)</td>
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<td>0.043</td>
</tr>
<tr>
<td>V_{max} (nmol/min/g)</td>
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<tr>
<td>K_{m} (µM)</td>
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<td>0.028</td>
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</table>

Pharmacokinetic parameters were obtained by fitting experimental data with equations 7 and 8. Cl_{pd} – transplacental clearance occurring by passive diffusion; V_{max} – maximal velocity of Bcrp mediated transport; K_{m} - concentration of cimetidine at which half the maximal velocity is reached. GF120918 (2 µM) added to the maternal compartment was used as an inhibitor.
Fig. 1

Total transplacental clearance: \( Cl_T = Cl_{pd} \pm Cl_{efflux} \)

\[
Cl_{Tmf} = Cl_{pd} - \frac{V_{max}}{K_m + C_{ma}} \\
Cl_{Tfm} = Cl_{pd} + \frac{V_{max}}{K_m + C_{fa}}
\]

(If \( C >> K_m \) then \( Cl_T = Cl_{pd} \))
Fig. 4

Fluorescence units/mg protein

Control
GF120918 (1 μM)
Ko143 (1 μM)
PSC833 (1 μM)
Verapamil (25 μM)
Fig. 5

Maternal-to-fetal cimetidine clearance (ml/min/g)

Control  GF120918 (2 μM)  Fumitremorgin C (2 μM)  Cyclosporine (10 μM)  Verapamil (25 μM)
Fig. 6

Maternal-to-fetal cimetidine clearance (ml/min/g)

Cimetidine concentration (µM)
Fig. 8

Total transplacental cimetidine clearance (ml/min/g)

<table>
<thead>
<tr>
<th></th>
<th>0.005 µM</th>
<th>1000 µM</th>
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[fm/mf = 24.6] [fm/mf = 1.0]