Expression and Transport Activity of Breast Cancer Resistance Protein (Bcrp/Abcg2) in Dually Perfused Rat Placenta and HRP-1 Cell Line

Frantisek Staud, Zuzana Vackova, Katerina Pospechova, Petr Pavek, Martina Ceckova, Antonin Libra, Lenka Cygalova, Petr Nachtigal, and Zdenek Fendrich

Departments of Pharmacology and Toxicology (F.S., Z.V., P.P., M.C., A.L., L.C., Z.F.) and Biomedical Sciences (K.P., P.N.), Faculty of Pharmacy in Hradec Kralove, Charles University, Prague, Czech Republic

Received March 22, 2006; accepted June 28, 2006

ABSTRACT

Breast cancer resistance protein (BCRP/ABCG2) is a member of the ATP-binding cassette transporter family that recognizes a 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 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 fumitremorgin C (2 μM) or N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918; 2 μM) and 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 compartment even against a concentration gradient and to establish a 2-fold maternal-to-fetal concentration ratio. Based on our results, we propose a two-level defensive role of Bcrp in the rat placenta in which the transporter 1) reduces passage of its substrates from mother to fetus but also 2) removes the drug already present in the fetal circulation.

Placenta is an organ that brings maternal and fetal blood circulations into proximity, allowing mutual interchange of nutrients and waste products. Conversely, placenta forms a barrier to protect the 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 past two decades, a 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 thought 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 (Pgp; ABCB1), breast cancer resistance protein (BCRP; ABCG2) and multidrug resistance-associated proteins 1 and 2 (ABCC1 and ABCC2), all of which were found to be responsible for the phenomenon of multidrug resistance in cancer therapy (Fischer et al., 2005). In addition, because of their extensive distribution in nontumorous 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).

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). Because its tissue distribution and substrate specificity overlap noticeably with that of P-gp, it is generally thought that these transporters share a similar rule 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 and toxins) and endogenous (steroid conjugates, and 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. (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. (2001, 2002) in transgenic mice. Nevertheless, transport activity of this efflux protein and its role in transplacental pharmacokinetics has not been fully evaluated to date.

Because of 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 used 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 used to investigate placental physiology and pharmacology (Stulc et al., 1995; Kertschanska et al., 2000). In our previous studies, we used this experimental model to evaluate functional activity of P-gp in the rat placenta (Pavek et al., 2001, 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 radiolabeled \([N\text{-methyl-}^3\text{H}]\) cimetidine were purchased from Sigma-Aldrich (St. Louis, MO) and from GE Healthcare (Little Chalfont, Buckinghamshire, UK), respectively. BODIPY FL prazosin, a common BCRP and P-gp substrate, was obtained from Invitrogen (Carlsbad, CA). Specific BCRP inhibitors Ko143 and furmitremorin C were donated by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) or purchased from Alexis Corporation (Laussanne, Switzerland), respectively. Specific P-gp inhibitors, PSC833 and cyclosporine, were gifts kindly provided by Dr. Andrysek (Ivax Pharmaceuticals, Opava, Czech Republic). Dual BCRP and P-gp inhibitor GF120918 was from GlaxoSmithKline (Greenford, UK). 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 (University of Kansas Medical Center, Kansas City, KS) were used. They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Cells from passages 15 to 25 were used in experiments described herein.

#### Animals.
All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) 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, Abbott Park, IL) in a dose of 40 mg/kg administered into the tail vein.

#### RNA Isolation and Reverse Transcription-Polymerase Chain Reaction Analysis.
Placentas and kidneys were collected on day 21 of gestation from five 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 National Center for Biotechnology Information data base; primers for mdr1a, mdr1b, and bcrp genes were designed using the Vector NTI Suite software (Informax, Bethesda, MD) and are given in Table 1.

RT-PCR analysis was performed on iCycler iQ (Bio-Rad, Hercules, CA). cDNA was amplified with HotStarTaq polymerase under the following conditions: 3 mM MgCl₂, 0.2 mM dNTP, 0.03 U/μl poly-

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sequence 5′−3′</th>
<th>Product Length</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>mdr1a</td>
<td>AF257746</td>
<td>ctg ctc aag tga aag ggg cta ca (f)</td>
<td>329 bp</td>
<td>2526–2854</td>
</tr>
<tr>
<td>mdr1b</td>
<td>AY082609</td>
<td>agc att tct gta tgg tat ctg caa gc (r)</td>
<td>331 bp</td>
<td>2489–2819</td>
</tr>
<tr>
<td>Bcrp</td>
<td>NM181381</td>
<td>cct ctg gag tga tga ata gag (f)</td>
<td>188 bp</td>
<td>1340–1527</td>
</tr>
</tbody>
</table>
merase, SYBR Green I in 1:100,000 dilution, and 0.3 μM of each primer; the temperature profile was 95°C for 14 min; 50 times; 95°C for 15 s, 60°C for 20 s, 72°C for 20 s, and 72°C for 5 min; and melting curve program was 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 (25–766 bp) (New England BioLabs, Hertsfordshire, UK).

Western Blot Analysis. Cell membrane fractions of placenta tissues and whole-cell lysates were prepared as described previously (Novotna et al., 2004; Cekcova et al., 2006). Protein contents were determined by BCA Protein Assay detection kit (Pierce Chemical, Rockford, IL). 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 electo transferred onto Hybond-ECL membrane (GE Healthcare). 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) and rabbit polyclonal anti-ABCg2 antibody M-70 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:500 in 1% blocking buffer overnight 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 room temperature) 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).

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, two times for 8 min each in a microwave oven at 750 W. Slides were incubated with a polyclonal primary antibody for BCRP (M-70; Santa Cruz Biotechnology, Inc.) diluted 1:10 in bovine serum albumin for 15 to 18 h at 4°C. Subsequently, the slides were developed with the secondary antibody goat anti-rabbit IgG conjugated to peroxidase-labeled polymer (DAKO EnVision, DAKO North America Inc., Carpinteria, CA) for 30 min. The reaction was visualized using diaminobenzidine (diaminobenzidine substrate-chromogen solution; DAKO North America Inc.), and the sections were counterstained by hematoxylin. Slides were examined using computer image analysis (Nikon Eclipse E200 light microscope; Nikon, Tokyo, Japan; PixelINK PL-A642 digital firewire camera; Vitana Corp., Ottawa, ON, Canada; and 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 (1 × 10^4/well) 2 days before the experiments. Cell culture medium was removed, and cells were washed twice with 500 μl of prewarmed 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 μM) 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 and lysed in 1% SDS, and fluorescence was measured after 24 h (Genios Plus; Tecan, Salzburg, 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). In brief, one uterine horn was excised and submerged in heated Ringer’s 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 fetuses 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 preweighed 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 previously (Pavek et al., 2001). At the end of experiment, placenta was perfused with radioactivity-free buffer for 10 min, excised from the uterine tissue, and dissolved in tissue solubilizer (Solvable; PerkinElmer Life and Analytical Sciences, Boston, MA), and its radioactivity was measured to detect tissue-bound cimetidine.

Two types of perfusion systems were used in this study. 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 an approximately 10-min stabilization period before sample collection started (time 0). Fetal effluent was sampled into preweighed vials in 5-min intervals and analyzed for [3H]cimetidine.

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 min from the maternal and fetal reservoirs, and [3H]cimetidine concentration was measured. This experimental setup ensures steady cimetidine concentration on the fetal side of the placenta and enables investigation 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, 2 μM fumitremorgin, 2 μM GF120918, 10 μM cyclosporine, or 25 μM verapamil was added to the maternal or fetal reservoir in the 10th min of perfusion. Subsequently, transplacental clearance of cimetidine in the period of 0 to 10 min (without inhibitor) was compared with that in 20 to 30 min (with inhibitor), leaving the mid-interval of 10 to 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 to 10 min (control) and 20 to 30 min (inhibitor) of placental perfusions were used for the following calculations. Total maternal-to-fetal transplacental clearance (ClRTM) of cimetidine normalized to placenta weight was calculated according to eq. 1.

\[
Cl_{RTM} = \frac{C_{Om} \cdot Q_{m}}{C_{Om} \cdot W_p}
\]
where $C_{fa}$ 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 eq. 2 (Shargel and Yu, 1993):

$$ER = \frac{(C_{fa} - C_{fa})}{C_{fa}}$$

(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 ($Cl_{Tfm}$) was calculated according to eq. 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 $Cl_{Tmf}$ and $Cl_{Tfm}$ are described by eqs. 4 and 5, respectively:

$$Cl_{Tmf} = Cl_{pd} - Cl_{efflux}$$

(4)

$$Cl_{Tfm} = Cl_{pd} + Cl_{efflux}$$

(5)

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

$$Cl_{efflux} = \frac{V_{max}}{K_m + C_{mat(fa)}}$$

(6)

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

In maternal-to-fetal studies, adding $Cl_{efflux}$ into eq. 4 yields the following equation, which was used to fit clearance versus inflow concentration data:

$$Cl_{Tmf} = Cl_{pd} - \frac{V_{max}}{K_m + C_{ma}}$$

(7)

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

$$Cl_{Tfm} = Cl_{pd} + \frac{V_{max}}{K_m + C_{fa}}$$

(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).

**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’s 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 used 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. Likewise, 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, whereas 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 placentas (gestation day 21) were used for the experiments. The rat placenta is composed of two morphologically different regions, 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...
Two representative samples are shown for each tissue. /H9262

BCRP and P-gp inhibitor GF120918 (1 μM) caused significant increase in transplacental clearance of cimetidine (Fig. 5). Interaction of cimetidine with human P-gp has recently been ruled out using MDR1-transfected Madin-Darby canine kidney II 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 GF120918-sensitive cimetidine transporter.

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 Madin-Darby canine kidney II 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 GF120918-sensitive cimetidine transporter.

Effect of Inflow Cimetidine Concentrations on Transplacental Clearance. Cimetidine was infused to maternal or fetal side of the placenta at 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 nonlinearity 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, whereas at concentrations above 30 μM, inhibitor was rather ineffective.

Fitting experimental data with eqs. 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 $Cl_{pm}$) is independent of direction and of inhibitor used. Conversely, $Cl_{mmax}$ is a concentration- and inhibitor-dependent parameter. At substrate concentrations largely exceeding the Michaelis-Menten constant ($C \gg K_m$), the transporter is saturated, the nonlinear fraction of eqs. 7 and 8 approaches zero, and both equations are reduced to linear processes only [total transplacental clearance ($Cl_T = Cl_pm$); 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 \( \mu \text{M} \). Furthermore, because addition of inhibitor caused no change in transplacental clearance of 1000 \( \mu \text{M} \) cimetidine (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 \( \mu \text{M} \)), 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 \( \mu \text{M} \), 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 (\( \text{Cl}_{\text{pd}} = 0.041–0.043 \) ml/min/g; Table 2).

**Bcrp Transports Cimetidine from Fetus to Mother against a 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 or 1000 \( \mu \text{M} \) and perfused. At a low drug concentration (0.005 \( \mu \text{M} \)), cimetidine in the fetal circulation steadily decreased and stabilized after approximately 40 min of perfusion. Fetal-to-maternal concentration ratio reached a value of 0.49 toward the end of the experiment. Decrease in fetal cimetidine was blocked by co-infused BCRP inhibitors (GF120918 or fumitremorgin C; Fig. 9A). At a high cimetidine concentration (1000 \( \mu \text{M} \)), maternal and fetal perfusates were recirculated. At a low drug concentration (0.005 \( \mu \text{M} \)), cimetidine in the fetal circulation steadily decreased and stabilized after approximately 40 min of perfusion. Fetal-to-maternal concentration ratio reached a value of 0.49 toward the end of the experiment. Decrease in fetal cimetidine was blocked by co-infused BCRP inhibitors (GF120918 or fumitremorgin C; Fig. 9A). At a high cimetidine concentration (1000 \( \mu \text{M} \)), maternal and fetal concentrations remained unchanged throughout the perfusion period with fetal/maternal concentration ratio staying close to 1.
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 thought 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). Likewise, 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 a few studies have reported on BCRP activity in placenta, and these are mainly based on in vitro models. Very recently, Kolwankar et al. (2005) used 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, the 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 mid-gestation (Soares et al., 1987). This cell line has previously been used 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 in vitro placental model. Bcrp expression was revealed at both mRNA and protein levels. Surprisingly, we did not detect any expression of mdr1a or 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, whereas P-gp did not affect cell accumulation of BODIPY FL prazosin. These data are similar to what has previously been observed in human chorionic carcinoma cell line BeWo (Atkinson et al., 2003; Ceckova et al., 2006; Evseenko et al., 2006) where only BCRP was found to be functionally expressed, whereas 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. In contrast, it may well serve as a tool to study Bcrp role in transplacental pharmacokinetics, because its efflux activity will not interfere with that of P-gp.

The only functional in vivo studies on Bcrp activity in the placenta so far have been performed in transgenic mice (Jonker et al., 2000, 2002); however, detailed evaluation of BCRP role in transplacental pharmacokinetics is still lacking. In the present study, cimetidine was used 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 that 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 previous 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. Cimetidine with [3H]cimetidine tracer was added to the maternal or fetal compartment, and its radioactivity was measured in fetal venous outflow. Total transplacental clearances were calculated by eq. 1 or 3, respectively (see Materials and Methods). 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 parentheses show ratio of fm-to-mf clearance.

![Fig. 8. Ratio of clearances between fetal-to-maternal (fm) and maternal-to-fetal (mf) directions. Cimetidine with [3H]cimetidine tracer was added to the maternal or fetal compartment, and its radioactivity was measured in fetal venous outflow. Total transplacental clearances were calculated by eq. 1 or 3, respectively (see Materials and Methods). 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 parentheses show ratio of fm-to-mf clearance.](image-url)
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 recirculated. After short equilibration period, we observed significant decrease in cimetidine concentrations, confirming that Bcrp can actively remove its substrate from the fetal compartment. Because decrease in cimetidine concentration continued even at later intervals (dropping by more than 50% within 60 min of perfusion), it is evident that Bcrp in rats can pump this compound from fetus to mother even against a 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, however, a different mRNA distribution pattern was indicated by Tanaka et al. (2005). They found high expression levels in kidney and small and large intestine, and 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. (2000, 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, because there are a number of other factors that determine its functional activity, such as post-transcriptional/post-translational 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 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 in which the transporter 1) reduces passage of its
substrates from mother to fetus but also 2) removes the drug already present in the fetal circulation even against a concentration gradient. Given the broad range of BCRP substrates, this transporter seems to be an important component of the placental barrier playing a significant role in protection and detoxication of the fetus.

Acknowledgments

We thank Anežka Kunova and Dana Součkova for skillful assistance in perfusion experiments.

References


