The Breast Cancer Resistance Protein (Bcrp1/Abcg2) Limits Fetal Distribution of Glyburide in the Pregnant Mouse – An OPRU Network and UW SCOR Study

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d) **Abbreviations:**

BCRP, breast cancer resistance protein; ABC, ATP-binding cassette; P-gp, P-glycoprotein; MRP2, multidrug resistance protein 2; MRP3, multidrug resistance protein 3; FTC, fumitremorgin C; GLB, glyburide; GLP, glipizide; MX, mitoxantrone; AUC, the area under concentration-time curve; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PEG 400, polyethylene glycol 400; gd, gestation day; OPRU, Obstetric-Fetal Pharmacology Research Unit; UW, University of Washington; SCOR, Specialized Center of Research; CYP, cytochrome P450; Ko143, 3-(6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydropyrazino[1’,2’:1,6]pyrido[3,4-b]indol-3-yl)-propionic acid tert-butyl ester.
Abstract

BCRP is most abundantly expressed in the apical membrane of placental syncytiotrophoblasts, suggesting that it may protect the fetus by impeding drug penetration across the placental barrier. Glyburide (GLB) is an antidiabetic drug routinely used to treat gestational diabetes. In this study, we first determined if GLB is a BCRP/Bcrp1 substrate. The intracellular $[^3]$H]-GLB concentrations in MDCK/BCRP cells were significantly lower than those in MDCK/vector cells. The addition of 10 µM FTC, a specific BCRP inhibitor, significantly increased the intracellular $[^3]$H]-GLB concentrations approximately 2-fold in MDCK/BCRP cells, but had no effect in MDCK/vector cells. Similar results were obtained using MDCKII parent and MDCKII/Bcrp1 cells. GLB was also shown to be a BCRP/Bcrp1 substrate in transwell transport experiments. We then examined if Bcrp1 limits fetal distribution of GLB in the pregnant mouse. GLB was administered by retro-orbital injection to the wild-type and Bcrp1$^{-/-}$ pregnant mice. The maternal plasma samples and fetuses were collected at various times (0.5 – 240 min) after drug administration. The GLB concentrations in the maternal plasma samples and homogenates of fetal tissues were determined by HPLC/MS. While the maternal plasma AUCs of GLB in the wild-type and Bcrp1$^{+/+}$ pregnant mice were comparable, the fetal AUC of GLB in the Bcrp1$^{-/-}$ pregnant mice was approximately 2 times greater than that in the wild-type pregnant mice. These results suggest that GLB is a BCRP/Bcrp1 substrate, and Bcrp1 significantly limits fetal distribution of GLB in the pregnant mouse, but has only a minor effect on the systemic clearance of the drug.
The breast cancer resistance protein (BCRP) is an ABC efflux transporter originally identified by its ability to confer multidrug resistance in cancer cells (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). BCRP mediates the efflux transport of a broad spectrum of substrates, including many drugs routinely administered to pregnant women to treat various diseases (Mao and Unadkat, 2005; Robey et al., 2007). BCRP is highly expressed in the apical membrane of placental syncytiotrophoblasts, in the epithelium of the small intestine and colon, in the liver canalicular membrane, and in the brain microvessel endothelium (Maliepaard et al., 2001). Given its tissue localization pattern, BCRP is expected to play an important role in absorption, distribution, and elimination of drugs. Indeed, the importance of BCRP in drug disposition has been demonstrated in numerous studies (Jonker et al., 2000; Kruijtzer et al., 2002; Merino et al., 2005).

Among human tissues, BCRP is most abundantly expressed in the placenta. In fact, the mRNA level of BCRP in human term placenta was found to be 10 times greater than that of P-gp (Ceckova et al., 2006). Although the precise physiological role of BCRP in the placenta is still unclear, existing data strongly suggest that BCRP plays an important role in protecting the fetus against environmental toxins, drugs, and metabolites by expelling them across the placental barrier. For example, Jonker et al. (Jonker et al., 2000) demonstrated that Bcrp1, the murine homolog of human BCRP, significantly reduced fetal distribution of topotecan. At 30 min after drug administration, the fetal/maternal plasma concentration ratio of topotecan was increased 2-fold in the pregnant mice treated with the BCRP inhibitor GF-120918, compared with that in the vehicle-treatment control animals. Most recently, rat Bcrp was also shown to facilitate the fetal-to-maternal transport of cimetidine, in a dually perfused rat placenta study (Staud et al., 2006).

Gestational diabetes affects up to 8% of all human pregnancy (Moore, 2004), and is associated with an increased risk of adverse pregnancy outcomes such as macrosomia, cesarean
delivery, and perinatal mortality (Beischer et al., 1996; Rochon et al., 2006; Sermer et al., 1998). Good control of the blood glucose levels is therefore necessary, and is often accomplished by dietary therapy with or without medication. Some of the oral hypoglycemic drugs, including some sulfonylureas, are avoided in pregnancy, because they readily cross the placenta and may lead to adverse reactions in the fetus. However, glyburide (GLB), a second generation sulfonylurea, has been compared to insulin for treatment of gestational diabetes in a randomized and controlled clinical trial. The GLB group was found to have similar maternal and fetal outcomes as in the insulin group, and GLB was not detected in umbilical cord serum samples (Langer et al., 2000).

Consistent with the clinical data, the ex vivo human placenta perfusion studies have demonstrated that GLB minimally crosses the placental barrier (Elliott et al., 1994; Kraemer et al., 2006), even though GLB is a highly lipophilic, low molecular weight drug. Since GLB has plasma protein binding of 99.8% and a short elimination half life, it has been suggested that the extensive protein binding and short elimination half life are the likely causes of the minimal transplacental transfer of GLB (Koren, 2001). Gedeon et al. recently showed that GLB could possibly be transported by BCRP and MRP3, but not by P-gp and MRP2 (Gedeon et al., 2006). Since P-gp, MRP2 and BCRP are the major efflux transporters in the apical membrane of placental syncytiotrophoblasts (Evseenko et al., 2006; Unadkat et al., 2004), and MRP3 is predominantly expressed in the fetal capillaries (St-Pierre et al., 2000), the possibility that BCRP plays a significant role in determining fetal exposure to GLB warrants further investigation.

Therefore, the objectives of the present study were to perform in vitro transport studies to confirm if GLB is a BCRP/Bcrp1 substrate and then systematically investigate the effect of Bcrp1 on fetal exposure to GLB in the wild-type and Bcrp1−/− pregnant mice.
Materials and Methods

Materials. Glyburide (GLB), glipizide (GLP), mitoxantrone (MX), and verapamil were purchased from Sigma (St. Louis, MO). [Cyclohexyl-2,3-3H(N)]-glyburide ([3H]-GLB) (40 – 70 Ci/mmol) was obtained from PerkinElmer (Waltham, MA). Fumitremorgin C (FTC) was obtained from NIH (Bethesda, MD). Ko143 was kindly provided by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Optima-grade methylene chloride, acetonitrile, methanol, and water were from purchased Fisher Scientific (Pittsburgh, PA). HPLC-grade n-hexane was obtained from Acros Organics USA (Morris Plains, NJ). Polyethylene glycol 400 (PEG 400) was obtained from Spectrum Laboratory Products, Inc. (Gardena, CA). Dulbecco’s modified Eagle’s phenol-free low glucose medium (DMEM), fetal bovine serum (FBS), geneticin (G418), phosphate buffered saline (PBS), and trypsin-EDTA solution were from Invitrogen (Carlsbad, CA). Eagle’s minimum essential medium (MEM), penicillin-streptomycin-glutamine solution, and serum-free Opti-MEM were purchased ATCC (Manassas, VA).

Cell culture. HEK cells stably transfected with pcDNA empty vector (HEK/vector) and cDNA coding for wild-type BCRP (HEK/BCRP) were obtained from Dr. Susan Bates (NCI, Bethesda, MD). The parent Madin-Darby canine kidney II (MDCKII) cells and the MDCKII cells stably transfected with cDNA coding for wild-type Bcrp1 (MDCKII/Bcrp1) were kindly provided by Dr. Alfred Schinkel (The Netherlands Cancer Institute). The MDCK cells stably transfected with pcDNA empty vector (MDCK/vector) and cDNA coding for wild-type BCRP (MDCK/BCRP) were developed in our laboratory (Zhang et al., 2005). Cell culture was performed essentially the same as previously described (Gupta et al., 2004; Zhang et al., 2005). In brief, all the cell lines, except the MDCKII parent and MDCKII/Bcrp1 cell lines, were grown and maintained in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml
streptomycin, and 0.5 mg/ml G418 at 37°C in a 5% CO2 incubator. The MDCKII parent and MDCKII/Bcrp1 cells were grown and maintained as other cells except that G418 was omitted in the culture medium. Cells were grown to 80 – 90% confluence and treated with trypsin-EDTA prior to harvesting for subculturing or efflux assays.

**Flow Cytometric Efflux Assay.** The flow cytometric efflux assay was essentially the same as previously described (Gupta et al., 2004; Zhang et al., 2005). Briefly, the HEK/vector and HEK/BCRP cells were harvested and suspended in incubation buffer (DMEM with 5 mM HEPES buffer) at a cell concentration of approximately 10^6 cells per reaction in 1 ml. In the accumulation phase, cells were co-incubated with 10 µM MX (a model BCRP substrate) and 10 or 100 µM GLB or 10 µM FTC at 37°C for 30 min. Cells were then washed once with ice-cold PBS and resuspended in 1 ml of incubation buffer, containing only GLB or FTC at their respective concentrations, and incubated for 1 h at 37°C to allow maximum efflux of MX (efflux phase). FTC is a specific BCRP inhibitor and was used as a control. Cells were then washed once and resuspended in 1 ml of ice-cold PBS. Intracellular MX fluorescence was measured within 1 h with a 488 nm argon laser and a 650 nm longpass filter in a BD FACSCAN flow cytometer. Ten thousand (10^4) events were collected for all the samples. Cell debris was eliminated by gating on forward versus side scatter. The concentration of DMSO as vehicle for FTC, GLB, and MX was limited to 0.2% (v/v). No effect of the vehicle on MX efflux activity was observed at this concentration. Cells in medium containing MX alone generated the control histograms. Cells in medium containing MX and GLB or FTC yielded the GLB or FTC histograms, respectively. The percentage of the medium fluorescence of the GLB or FTC histogram to the median fluorescence of the control histogram was used to express relative inhibition of BCRP-mediated MX efflux by GLB or FTC, respectively. The experiments were performed in triplicate. We
noted that the MX fluorescence in the incubation buffer did not quench over at least 3 h, and the
addition of GLB or FTC did not affect the MX fluorescence.

**Efflux of Radiolabeled Glyburide.** Direct efflux of $[^{3}H]$-GLB were performed to determine if
GLB is a BCRP substrate. Briefly, MDCK/vector and MDCK/BCRP cells were seeded at a cell
density of approximately $10^6$ per well in 6-well plates and grown for 48 h to form monolayers.
One hour before experiment, medium was replaced with Opti-MEM in the presence or absence
of 10 µM FTC. The cell monolayers were then washed once with ice-cold PBS and incubated at
37°C for 90 min with 0.5 or 5 nM $[^{3}H]$-GLB in the presence or absence of 10 µM FTC or 5 µM
Ko143 in serum-free Opti-MEM containing 20 µM verapamil or with no verapamil. Since
MDCK cells express a substantial amount of endogenous P-gp, verapamil was included in the
assay mixture to inhibit P-gp and therefore eliminate potential influence of endogenous P-gp on
$[^{3}H]$-GLB efflux. However, verapamil at 20 µM had no effect on BCRP activity (Zhang et al.,
2005). The cell monolayers were then washed three times with ice-cold PBS. Immediately after
wash, the cell monolayers were dissolved in 1 ml of 2% (w/v) SDS and 900 µl of the lysates
were subjected to counting in a scintillation counter. Counts were normalized to a protein
concentration that was measured by the Pierce BCA protein assay using the remaining lysates.
The intracellular $[^{3}H]$-GLB concentrations were calculated based on the radioactivity associated
with the cell monolayers and presented as pmoles of $[^{3}H]$-GLB per mg protein. The experiments
were performed in triplicate at 37°C in a humidified incubator. The efflux assays were also
performed in the MDCKII and MDCKII/Bcrp1 cells under the same experimental conditions.

**Transwell Transport of $[^{3}H]$-GLB.** Transwell transport assay was essentially the same as
described (Zhang et al., 2005). Briefly, transwell transport of $[^{3}H]$-GLB was performed on
microporous polycarbonate membrane filters (3.0-µm pore size, 24-mm diameter, Costar, Corning, NY, USA). The MDCK/vector and MDCK/BCRP cells were seeded onto filter membranes at a density of 1 × 10^6 cells per well and grown for 4 – 5 days with replacement of medium every day. To ensure that the cells were polarized and formed a tight junction, transport assays were conducted when the average transepithelial electrical resistance in each well reached above 200 Ω, measured with a Millicell-ERS (Millipore, Billerica, MA). One hour before experiment, medium was replaced in both compartments with Opti-MEM in the presence or absence of 10 µM FTC. The loading volume is 1.5 ml in the apical compartment and 2.5 ml in the basolateral compartment. The experiments were started by replacing the medium with fresh Opti-MEM containing 5 nM [³H]-GLB in the presence or absence of 10 µM FTC in the appropriate compartment. All experiments were performed in Opti-MEM containing 20 µM verapamil. Transport of GLB was tested in two directions (apical-to-basolateral, A→B and basolateral-to-apical, B→A), every 1 h up to 4 h, by taking aliquots (100 µl) from the opposite compartment. After each sampling, 100 µl of medium with or without 10 µM FTC were added back to the sampling compartment to keep the volume consistent. The amounts of GLB in the aliquots were measured by scintillation counting. The percentage of initially added radioactivity appearing in the opposite compartment was calculated. The efflux ratio R was calculated by dividing the basolateral-to-apical transport by the apical-to-basolateral transport at the end of 4 h. The apparent permeability (P_app) was calculated with the equation: \( P_{app} = \frac{dQ/dt}{A \cdot C_o} \), where A is membrane surface area (4.67 cm²), and C_o is donor concentration at time 0 (5 nM), and dQ/dt is the amount of drug transported per time (pmols/h). The studies were performed in triplicates at 37 °C in a humidified incubator.

**Animal Studies.** FVB wild-type mice and Bcrp1⁻/⁻ mice with FVB genetic background were
purchased from Taconic (Hudson, NY). Under a breeding license purchased from Taconic, male and female Bcrp1<sup>−/−</sup> mice were mated to generate Bcrp1<sup>−/−</sup> offsprings, which were used in the subsequent animal experiments. Pregnant and non-pregnant wild-type and Bcrp1<sup>−/−</sup> mice were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication 85 – 23, 1985. The animal studies were approved by the Institutional Animal Care and Use Committee at the University of Washington. The mice had free access to food (a standard diet) and water, and were maintained on a 12:12-h automatically timed light/dark cycle. Male mice, 8 to 10 weeks of age and weighing 20 to 30 g, were mated with female mice, 8 to 10 weeks of age and weighing 20 to 30 g. Female mice demonstrating sperm plugs were weighed and housed in new cages. Gestational age was calculated based on the estimated time of insemination (presence of the sperm plug). Progression of pregnancy in these female mice was regularly monitored by visual inspection and by measuring the increase in body weight. All experiments were performed with pregnant mice at gd 15 (term in mice is approximately 20 – 21 days), as we have previously shown that the expression of Bcrp1 in the placenta of the pregnant mouse peaks at gd 15 (Wang et al., 2006).

GLB was dissolved in 0.5% (v/v) DMSO, 10% (v/v) ethanol, 39.5% (v/v) saline and 50% (v/v) PEG 400 at a concentration of 0.5 mg/ml. Under anesthesia (isoflurane), the FVB wild-type or Bcrp1<sup>−/−</sup> pregnant mice were administered GLB by retro-orbital injection (1 mg/kg body weight). At various times (0.5, 5, 10, 20, 30, 40, 60, 120, 180, and 240 min) after drug administration, animals (n = 3 – 6 per time point) were sacrificed under anesthesia by cardiac puncture. Blood was collected in heparinized microcentrifuge tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 5,000 rpm at room temperature for 10 min. The harvested plasma was stored at -20°C until analysis. Fetuses were removed from pregnant mice, snap-frozen in liquid N<sub>2</sub>, and immediately stored at -80°C until use. The maternal plasma and fetal GLB
concentrations were determined by a validated HPLC/MS assay. The fetal GLB concentrations were determined from homogenates of fetal tissues.

**GLB HPLC/MS Assay.** The maternal plasma and fetal GLB concentrations were determined by a validated HPLC/MS assay (Naraharisetti et al., 2007). Briefly, a stock solution of GLB was prepared in pure acetonitrile at 0.25 mg/ml and was diluted to 2.5 or 25 µg/ml in methanol:water (1:1, v/v). Aliquots of the diluted solutions (≤ 200 µl) were further diluted in 25 ml of blank human plasma to yield the following calibrators for GLB: 5, 10, 25, 50, 75, 100, and 150 ng/ml. In initial experiments, no differences were observed between the calibration curves generated using the mouse plasma or the human plasma as blank. Therefore, human plasma was used as blank in all subsequent experiments because only a limited amount of mouse plasma was available. For preparation of quality control samples, an independent stock solution was prepared as above to yield the following concentrations in human plasma for GLB: 5, 25, and 100 ng/ml. A solution of the internal standard, GLP, was prepared in pure acetonitrile (200 µg/ml) and further diluted with methanol:water (1:1, v/v) to achieve a final concentration of 400 ng/ml. All the calibrators and quality control samples were stored in aliquots at -20°C until analysis.

To determine maternal plasma GLB concentrations, the maternal mouse plasma samples were processed as below. To every 100 µl of the mouse plasma sample, 20 µl of the internal standard solution (400 ng/ml) was added in a 13 × 100 mm borosilicate glass culture tube (VWR, West Chester, PA). Each mouse plasma sample (100 µl) was then acidified by adding 10 µl of 2 M HCl, followed by the addition of 1 ml of the solvent (n-hexane:methylene chloride, 1:1, v/v) for extraction of GLB and GLP. The samples were briefly vortexed, then gently shaken for 30 min, and centrifuged at 1,500 rpm for 5 min, all at room temperature. The upper organic phase was transferred to a disposable clean glass tube for each sample and dried under vacuum with
centrifugation (RC 1010, Jouan Inc., Winchester, VA). The dried residue for each sample was reconstituted in 100 µl of 20% (v/v) methanol. After gently vortexing, 15 µl of each reconstituted sample was injected for the HPLC/MS analysis. The mouse plasma samples with GLB concentrations outside the calibration range were diluted with blank mouse plasma.

To determine fetal GLB concentrations, the fetuses were processed as below. To generate calibration curves, blank fetuses were weighed and homogenized in ice-cold PBS using OMNI TH 115 tissue homogenizer (Omni International, Marietta, GA). GLB was added from a stock solution in acetonitrile to the blank fetus homogenate to achieve the intended concentrations (3 – 60 ng/g fetus) with less than 1% (v/v) acetonitrile. Approximately 16 ml of the fetus homogenate at a concentration of 0.5 g fetus/ml was needed to generate one calibration curve. For preparation of quality control samples, an independent GLB stock solution was prepared and diluted into the blank fetus homogenate as above to yield the following concentrations: 0.5, 2.5, and 10 ng/g fetus, all with less than 1% (v/v) acetonitrile. All calibrators and quality control samples used for determination of the fetal GLB concentrations were prepared on the day of HPLC/MS analysis. To every 500 µl of the fetal homogenate (approximately 0.25 g fetus), 20 µl of the internal standard solution (400 ng/ml) was added in a 13 × 100 mm borosilicate glass culture tube (VWR, West Chester, PA). Each fetus homogenate sample (500 µl) was then acidified by adding 50 µl of 2 M HCl, followed by the addition of 5 ml of the solvent (n-hexane:methylene chloride, 1:1, v/v) for extraction of GLB and GLP. The samples were mixed vigorously by vortexing for 1 min and centrifuged at 1,500 rpm for 5 min at room temperature. The upper organic phase was transferred to a disposable clean glass tube for each sample and dried under vacuum with centrifugation. The dried residue was reconstituted in 1 ml of 10% (v/v) methanol and loaded onto a conditioned and equilibrated cartridge (Strata Reversed Phase C8 Sorbent, Phenomenex, CA). The sorbent was then washed with 1 ml of 10% (v/v) methanol, followed by elution of GLB and GLP with 1 ml
of methanol. The elute was transferred to a disposable clean glass tube and dried under vacuum with centrifugation. The dried residue was reconstituted in 100 µl of 20% (v/v) methanol. After gentle vortexing, 15 µl of each reconstituted sample was injected for the HPLC/MS analysis.

HPLC/MS was performed on an Agilent series 1100 HPLC equipped with an auto-sampler and interfaced with an Agilent series 1100 MSD single Quadrupole Mass Spectrometer (Agilent Technologies, Foster City, CA). Separation of GLB and GLP was achieved using an Agilent Zorbax XDB-C8 analytical column (2.1 × 50 mm, 5 µm) equipped with a Phenomenex Security Guard C18 guard column (2.0 × 4 mm) with gradient elution. The mobile phase was composed of methanol and water at pH 6, both containing 0.5 mM ammonium formate. The flow rate was set at 0.25 ml/min. At time 0, the mobile phase was 20% (v/v) methanol and 80% (v/v) water. The concentration of methanol was increased linearly to 45% (v/v) over 1 min, held for 3 min, then increased linearly to 75% (v/v) over 1.5 min, held for 1 min, then further increased linearly to 90% (v/v) over 10 s, held for 3 min, and finally returned to the initial composition of 20% (v/v) methanol and 80% (v/v) water. The column was pre-equilibrated for 5.4 min, and the total run time was 15 min. The following parameters were set for optimum detection sensitivity: fragmentation voltage 50 V, capillary voltage 5,000 V, drying gas temperature 350°C, drying gas flow rate 10 l/min, and nebulizer pressure 25 psig (172 kPa). Mass spectrometric data were collected between 3.5 and 9.0 min. At all other times, column elute was diverted to waste.

**Pharmacokinetic Data Analysis.** For one-point sampling data (one blood sample from each mouse), we used the Bailer’s approach (Bailer, 1988; Takemoto et al., 2006) to estimate the mean and standard error (S.E.) of the maternal plasma and fetal AUCs, and other standard pharmacokinetic parameters such as MRT (mean residence time), CL (clearance), and Vss (steady-state volume of distribution) with the following equations:
\[ E(\text{AUC}) = \sum_{i=2}^{m} (E(C_i) + E(C_{i-1})) \cdot (t_i - t_{i-1}) / 2 \]

\[ V(\text{AUC}) = \sum_{i=2}^{m} (V(C_i) + V(C_{i-1})) \cdot ((t_i - t_{i-1}) / 2)^2 \]

\[ E(\text{AUMC}) = \sum_{i=2}^{m} (t_i \cdot E(C_i) + t_{i-1}E(C_{i-1})) \cdot (t_i - t_{i-1}) / 2 \]

\[ V(\text{AUMC}) = \sum_{i=2}^{m} (t_i^2 \cdot V(C_i) + t_{i-1}^2 \cdot V(C_{i-1})) \cdot ((t_i - t_{i-1}) / 2)^2 \]

\[ E(\text{MRT}) = E(\text{AUMC}) / E(\text{AUC}) \]

\[ V(\text{MRT}) = \frac{V(\text{AUMC})}{E(\text{AUC})^2} + \frac{V(\text{AUC}) \cdot E(\text{AUMC})^2}{E(\text{AUC})^4} \]

\[ E(\text{CL}) = D / E(\text{AUC}) \]

\[ V(\text{CL}) = D^2 \cdot V(\text{AUC}) / E(\text{AUC})^4 \]

\[ E(\text{V}_{ss}) = E(\text{CL}) \cdot E(\text{MRT}) \]

\[ V(\text{V}_{ss}) = E(\text{MRT})^2 \cdot V(\text{CL}) + E(\text{CL})^2 \cdot V(\text{MRT}) \]

Where \( m \) is the number of time points of the time course experiment. \( C_i \) and \( t_i \) are concentration and time, respectively. The time course was integrated by the trapezoidal formula without extrapolation. \( E(C_i) \) and \( V(C_i) \) are the mean and variance of the concentration at time \( t_i \), respectively, and defined by:

\[ E(C_i) = \frac{1}{n_i} \sum_{j=1}^{n_i} C_{ij} \]

\[ V(C_i) = \frac{1}{n_i - 1} \sum_{j=1}^{n_i} (C_{ij} - E(C_i))^2 \]

where \( n_i \) is the number of concentration data points at time \( t_i \). The variance of mean AUC, AUMC, MRT, CL, or \( V_{ss} \) is defined by:
\[
V(\text{AUC}) = \sum_{i=2}^{m} \left( V(C_i)/n_i + V(C_{i-1})/n_{i-1} \right) \times \left( (t_i - t_{i-1})/2 \right)^2
\]

\[
V(\text{AUMC}) = \sum_{i=2}^{m} \left( t_i^2 \cdot V(C_i)/n_i + t_{i-1}^2 \cdot V(C_{i-1})/n_{i-1} \right) \times \left( (t_i - t_{i-1})/2 \right)^2
\]

\[
\frac{V(\text{MRT})}{E(\text{AUC})^2} = \frac{V(\text{AUMC}) \cdot V(\text{AUC})}{E(\text{AUC})^4} + \frac{V(\text{AUC}) \cdot E(\text{AUMC})^2}{E(\text{AUC})^4}
\]

\[
\frac{V(\text{CL})}{D^2} = \frac{V(\text{AUC})}{E(\text{AUC})^4}
\]

\[
\frac{V(\text{Vss})}{E(\text{MRT})^2 \cdot V(\text{CL}) + E(\text{CL})^2 \cdot V(\text{MRT})}
\]

The S.E. of each parameter was then given as the square root of the variance of mean AUC, AUMC, MRT, CL, or Vss.

The normal hypothesis test was performed using the following equation to assess the statistically significant difference of each parameter between two animal groups:

\[
Z_0 = \frac{\bar{\phi}_1 - \bar{\phi}_2}{\sqrt{SE_1^2 + SE_2^2}}
\]

where \(\bar{\phi}_1\) and \(\bar{\phi}_2\) are means of a pharmacokinetic parameter, and \(SE_1\) and \(SE_2\) are the standard errors of the pharmacokinetic parameter, for groups 1 and 2, respectively. If \(Z_0 > 1.96\) and \(p < 0.05\) (95\% confidence interval), the difference of the pharmacokinetic parameter between the groups 1 and 2 was considered to be statistically significant.

**Statistical Analysis.** Data from *in vitro* studies were analyzed for statistical significance using Student’s *t*-test, and differences with \(p\) values of < 0.05 were considered statistically significant. Data from *in vivo* studies were analyzed for statistical significance using one-way ANOVA analysis followed by Student’s *t*-test with the Bonferroni correction assuming a significant level of 0.05.
Results

Inhibition of BCRP-mediated MX efflux by GLB. We have previously shown that, relative to the expression of BCRP, the HEK cells express little endogenous P-gp, MRP1, or MRP2. Therefore, we performed efflux assays in HEK cells to minimize the potential influence of endogenous efflux transporters on BCRP-mediated MX efflux. We observed that the levels of intracellular MX fluorescence in HEK/vector cells were much greater than those in HEK/BCRP cells (Fig. 1A). The addition of 10 µM FTC, a specific BCRP inhibitor, increased the intracellular MX fluorescence in HEK/BCRP cells approximately 2-fold, but had no effect on that in HEK/vector cells (Fig. 1). These results confirm that MX is effluxed out of the cells by BCRP. We then examined if GLB is a BCRP inhibitor. GLB at 10 and 100 µM increased the intracellular MX fluorescence in HEK/BCRP cells approximately 1.1- and 1.7-fold, respectively, but had no significant effect on the intracellular MX fluorescence in HEK/vector cells (Fig. 1). These data demonstrate that GLB is a BCRP inhibitor.

Efflux of [3H]-GLB by BCRP/Bcrp1. GLB is a BCRP inhibitor, indicating that it is possibly a BCRP substrate. To determine if GLB is a BCRP substrate, direct efflux of [3H]-GLB in the MDCK/vector and MDCK/BCRP cells was measured. Since the MDCK/BCRP cells stably express a high level of BCRP and readily form a tightly attached cell monolayer which is optimal for measuring drug efflux, we used the MDCK cells, rather than the HEK cells, in the direct [3H]-GLB efflux assay. When 5 nM [3H]-GLB in the presence or absence of 20 µM verapamil was used, intracellular [3H]-GLB concentrations in the MDCK/BCRP cells were approximately 50% of those in the MDCK/vector cells (Fig. 2A and 2B). The addition of 10 µM FTC significantly increased the intracellular [3H]-GLB concentrations in the MDCK/BCRP cells approximately 2-fold, but had no effect on those in the MDCK/vector cells (Fig. 2A and 2B).
Similar results were obtained using 0.5 nM $[^3\text{H}]-\text{GLB}$ (Fig. 2E and 2F). These results clearly indicate that GLB is a BCRP substrate. We next determined if GLB is a substrate for Bcrp1, the murine homolog of human BCRP. When 5 nM $[^3\text{H}]-\text{GLB}$ was used, intracellular $[^3\text{H}]-\text{GLB}$ concentrations in the MDCKII/Bcrp1 cells were approximately 50% of those in the MDCKII parent cells (Fig. 2C and 2D). The addition of 10 µM FTC significantly increased the intracellular $[^3\text{H}]-\text{GLB}$ concentrations in the MDCKII/Bcrp1 cells approximately 2-fold, but had no effect on those in the MDCKII cells (Fig. 2C and 2D). Similar results were obtained using 0.5 nM $[^3\text{H}]-\text{GLB}$ in the presence of 20 µM verapamil (Fig. 2H). These results suggest that GLB is also a Bcrp1 substrate.

When 0.5 nM $[^3\text{H}]-\text{GLB}$ in the absence of 20 µM verapamil was applied, we noted that the addition of 10 µM FTC increased the intracellular $[^3\text{H}]-\text{GLB}$ concentrations in MDCK/BCRP or MDCKII/Bcrp1 cells to significantly higher levels than those in control cells (MDCK/vector or MDCKII) with no FTC added (Fig. 2E and 2G). This is also the case for MDCKII/Bcrp1 and MDCKII cells with 5 nM $[^3\text{H}]-\text{GLB}$ (Fig. 2C). However, the addition of 20 µM verapamil abolished such differences (Fig. 2D, 2F, and 2H). This is likely due to inhibition of $[^3\text{H}]-\text{GLB}$ efflux mediated by endogenous P-gp by verapamil, and P-gp may be differentially expressed with relatively higher levels in MDCKII or MDCK/vector cells than in MDCKII/Bcrp1 or MDCK/BCRP cells, respectively. Previous studies have indeed suggested that GLB is likely a P-gp substrate (Golstein et al., 1999). To confirm if the observed $[^3\text{H}]-\text{GLB}$ efflux is indeed mediated by BCRP/Bcrp1, we also performed efflux studies using 0.5 nM $[^3\text{H}]-\text{GLB}$ containing 20 µM verapamil in the presence and absence of 5 µM Ko143, a highly selective BCRP inhibitor, and similar results as shown in Fig. 2F and Fig. 2H were obtained (data not shown).

**Transwell Transport of $[^3\text{H}]-\text{GLB}$.** Further evidence that GLB is a BCRP/Bcrp1 substrate was
obtained from Transwell transport studies. Since GLB is likely also a P-gp substrate (Golstein et al., 1999), we included 20 µM verapamil in all Transwell transport experiments to inhibit endogenous P-gp. As shown in Fig. 3, the B→A and A→B transport of [3H]-GLB in MDCK/vector cells was comparable (Fig. 3B), and the addition of 10 µM FTC had no effect on both the B→A and A→B transport (Fig. 3D). In contrast, the B→A transport of [3H]-GLB in MDCK/BCRP cells was significantly greater than the A→B transport with an efflux ratio of 2.73 (Fig. 3A). The addition of 10 µM FTC completely abolished the difference between the B→A and A→B transport of [3H]-GLB (Fig. 3C). These results clearly indicate that BCRP mediates the apically directed transport of [3H]-GLB in MDCK/BCRP cells. Similar results were obtained to indicate that [3H]-GLB is also a Bcrp1 substrate with an efflux ratio of 3.3 (Fig. 3E, 3F, 3G, and 3H). Thus, GLB was transported by BCRP/Bcrp1 as effectively as other BCRP substrates such as dipyridamole (Zhang et al., 2005) and cimetidine (Pavek et al., 2005). [3H]-GLB readily crossed the MDCK cell monolayers by passive diffusion with \( P_{app} (A→B) \) values at approximately \( 1 \times 10^{-3} \) ml/(cm²·h).

**GLB HPLC/MS Assay.** For maternal mouse plasma, the assay was linear \((r = 0.99)\) over the calibration range of 5 – 150 ng GLB/ml maternal plasma. The accuracy of the assay was 0.6, 5, and 2%, and the precision was 16, 9, and 12%, at the low (5 ng/ml), mid (25 ng/ml), and high ends (100 ng/ml) of the calibration range, respectively. For the fetus, the assay was linear \((r = 0.98)\) over the calibration range 0.5 to 10 ng GLB/g fetus. The accuracy of the assay was 3.5, 3.5, and 1.2%, and the precision was 15, 15, and 7.4%, at the low (0.5 ng/g fetus), mid (2.5 ng/g fetus) and high ends (10 ng/g fetus) of the calibration range, respectively. The interday and intraday variations of the assay over the calibration ranges for both the maternal plasma and fetus samples were less than 20%. The retention times for GLB and GLP were approximately 8.2 min and 6.4
min, respectively. Each pregnant mouse usually had 6 or more fetuses. We found that the GLB concentrations in different fetuses from the same pregnant mouse were very similar (data not shown). Thus, the GLB concentration in one fetus randomly selected from a pregnant mouse was used as the fetal GLB concentration of that mouse.

**Effect of Bcrp1 on Fetal GLB Exposure.** Building upon the fact that GLB is a substrate of BCRP/Bcrp1, we next examined the effect of Bcrp1 on fetal GLB distribution in the pregnant mouse. After administration of GLB by retro-orbital injection, the maternal plasma concentration-time profiles of wild-type and Bcrp1−/− pregnant mice were comparable (Fig. 4). One-way ANOVA analysis followed by Student’s *t*-test with the Bonferroni correction indicated that there was an overall significant difference (p < 0.05) in maternal plasma GLB concentrations between the wild-type and Bcrp1−/− pregnant mice, but there was no significant difference at any given time points (Fig. 4). The maternal plasma AUC<sub>0.5 – 240 min</sub> of GLB in the Bcrp1−/− pregnant mice was approximately 14% but significantly lower than that in the wild-type pregnant mice (Table 1). Other pharmacokinetic parameters such as MRT, CL, and Vss were also estimated using data shown in Fig. 4 (Table 1). The maternal plasma CL of GLB in the Bcrp1−/− pregnant mice was approximately 17% but significantly greater than that in the wild-type pregnant mice.

The fetal GLB concentrations in the wild-type and Bcrp1−/− pregnant mice were low at early time points up to 30 min and reached the maximum at approximately 60 min (Fig. 5). One-way ANOVA analysis followed by Student’s *t*-test with the Bonferroni correction revealed that there was an overall significant difference (p < 0.001) in fetal GLB concentrations between the wild-type and Bcrp1−/− pregnant mice (Fig. 5), and when making comparisons within a given time point, we found that at 180 min, the mean fetal GLB concentrations in the Bcrp1−/− pregnant mice were significantly greater (2 times) than those in the wild-type pregnant mice. As a result, the
fetal/maternal plasma GLB concentration ratios in the Bcrp1<sup>−/−</sup> pregnant mice were generally greater than those in the wild-type pregnant mice (Fig. 6). At 60, 180, and 240 min, the ratios in the Bcrp1<sup>−/−</sup> pregnant mice were significantly increased approximately 2.6-, 3.9-, and 3.1-fold, respectively, compared with those in the wild-type pregnant mice (Fig. 6).

We then estimated fetal AUCs (0.5 – 240 min) to further evaluate the role Bcrp1 plays in fetal distribution of GLB. As shown in Table 1, while the maternal plasma AUC<sub>0.5 – 240 min</sub> of GLB in the Bcrp1<sup>−/−</sup> pregnant mice was only slightly lower than that in the wild-type pregnant mice, the fetal AUC<sub>0.5 – 240 min</sub> of GLB in the Bcrp1<sup>−/−</sup> pregnant mice was approximately 2 times greater than that in the wild-type pregnant mice. Thus, the fetal/maternal plasma AUC ratio in the Bcrp1<sup>−/−</sup> pregnant mice was approximately 2.5 times greater than that in the wild-type pregnant mice.
Discussion

In the present study, we first showed that GLB is a BCRP inhibitor (Fig. 1). GLB seems to be a general inhibitor for ABC transporters, as GLB can also inhibit P-gp (Golstein et al., 1999) and MRPs (Payen et al., 2001). Further analyses suggest that GLB can be transported by BCRP/Bcrp1 at nM concentrations (Figs. 2 and 3). The mean unbound $C_{\text{max}}$ of GLB following a 3 mg oral Glynase® tablet taken with breakfast or a 5 mg oral Micronase® tablet given to healthy adults is approximately 0.5 nM (Jonsson et al., 1994). Therefore, BCRP/Bcrp1 can transport GLB at clinically relevant concentrations. These results are consistent with the data reported by Gedeon et al. (Gedeon et al., 2006). These authors demonstrated a significant increase in intracellular $[^3H]$-GLB accumulation in BCRP-overexpressing cells by the addition of the BCRP inhibitor novobiocin, indicating that $[^3H]$-GLB was transported by BCRP. The data of the present study provide further supportive evidence to suggest that GLB is a BCRP substrate. Gedeon et al. also showed that GLB was likely transported by MRP3. Unlike BCRP which is mainly expressed in the apical membrane of the placental syncytiotrophoblasts, MRP3 is predominantly expressed in the fetal capillaries (St-Pierre et al., 2000). Therefore, MRP3 seems unlikely to play a role in limiting drug penetration across the placental barrier. Based on in vitro data reported in this study and by others, and given its abundant expression in the placenta, we have hypothesized that BCRP plays an important role in impeding GLB penetration across the placental barrier.

To test the above hypothesis, we next examined the role of Bcrp1 in determining the pharmacokinetics and fetal distribution of GLB in the pregnant mouse. We measured the maternal plasma and fetal AUCs of GLB in the wild-type and Bcrp1−/− pregnant mice after administration by retro-orbital injection. At 240 min after drug administration, GLB was nearly completely eliminated from the systemic circulation in the wild-type and Bcrp1−/− pregnant mice (Fig. 4). The maternal plasma AUCs$_{0.5–240 \text{ min}}$ of GLB in the wild-type and Bcrp1−/− pregnant
mice were comparable (Table 1), suggesting that Bcrp1 has only a minor effect on the systemic clearance of GLB in the pregnant mouse. GLB has been shown to be extensively metabolized by CYP enzymes with negligible renal excretion in humans (Naritomi et al., 2004; van Giersbergen et al., 2002). Therefore, contribution of BCRP expressed in the maternal organs to the systemic clearance of GLB is not major. Likewise, our data indicate that CYP enzyme-mediated metabolism is likely also a major determinant of the systemic clearance of GLB in the pregnant mouse. We will test this hypothesis in future work. On the other hand, the maternal plasma AUC<sub>0.5 – 240 min</sub> of GLB in the Bcrp1<sup>−/−</sup> pregnant mice was slightly lower than that in the wild-type pregnant mice (Table 1), suggesting that Bcrp1 may serve, at least to certain extent, as a tissue barrier for the distribution of GLB into the maternal organs, because more GLB in the maternal tissues could lead to less GLB present in the maternal circulation. This is consistent with the fact that Bcrp1 is highly expressed in various mouse tissues, such as the liver, small intestine, kidney, and brain (Cisternino et al., 2004; Jonker et al., 2002).

We note that the fetal GLB concentrations in both the wild-type and Bcrp1<sup>−/−</sup> pregnant mice were at low levels up to 30 min and then peaked at 60 min (Fig. 5). This delay in reaching peak concentrations indicates that the partitioning of GLB into the fetus takes some time, even though the placenta is a well-perfused organ. Also, the fetal GLB concentrations (generally < 10 ng/g fetus) were much lower than the maternal plasma GLB concentrations (0.1 – 10 µg/ml maternal plasma) (Figs. 4 and 5), suggesting that the total amount of GLB accumulated in the fetus only accounts for a small fraction of the total amount of GLB in the body. Since Bcrp1 is highly expressed in the maternal drug-clearing organs of mice (e.g., the small intestine, liver, and kidney) (Jonker et al., 2002; Wang et al., 2006), the findings that the lack of Bcrp1 did not increase the systemic exposure of GLB further suggests that maternal GLB disposition is not significantly affected by Bcrp1 after administration of the drug by retro-orbital injection. As to
whether intestinal Bcrp1 affects the absorption of GLB is yet to be determined.

However, when the fetal AUCs were compared, the fetal/maternal plasma AUC ratio of the Bcrp1−/− pregnant mice was approximately 2.5 times greater than that of the wild-type pregnant mice (Table 1), suggesting that Bcrp1 significantly limits fetal GLB exposure in the pregnant mouse. To the best of our knowledge, this is the first in vivo animal study which clearly demonstrated the role of Bcrp1 in protecting the fetus by limiting fetal distribution of GLB. This study provides detailed transplacental pharmacokinetics of GLB involving Bcrp1 in the pregnant mouse. Such study is particularly valuable, as similar studies of direct placental drug transport in humans are not feasible due to ethical reasons. Bcrp1 may function by limiting the transfer of GLB from the maternal circulation to the fetal compartment. Since GLB is a highly lipophilic drug, it is expected that a certain amount of GLB may cross the placenta by passive diffusion based on its substantial apparent membrane permeability (Fig. 3). Hence, Bcrp1 could also remove the drug already present in the fetus back to the maternal circulation against a concentration gradient. This explanation is supported by the data obtained in the ex vivo perfusion study using rat placenta for cimetidine (Staud et al., 2006).

We anticipate that BCRP in human placenta plays a similar role in transporting GLB as mouse Bcrp1. Recently, Kraemer et al. (Kraemer et al., 2006) performed ex vivo perfusion studies of human placenta to quantify placental transfer of GLB. Unlike Elliott et al. (Elliott et al., 1994), these authors have excluded albumin from the perfusion buffer so that net transfer of GLB could be measured without the effect of high protein binding. Substantial fetal-to-maternal transfer of GLB against a concentration gradient was observed. Furthermore, such a transfer of GLB was not blocked by the addition of verapamil, a P-gp inhibitor (Kraemer et al., 2006). Based on these data, the authors concluded that GLB is effluxed by a transporter other than P-gp or P-gp is not a major player. We have previously shown that verapamil is not an effective
inhibitor for BCRP (Zhang et al., 2005). Thus, the data available from \textit{in vitro} transport, \textit{ex vivo} perfusion, and \textit{in vivo} animal studies all point to the unique role of BCRP/Bcrp1 in limiting fetal distribution of GLB. Previous studies showed that the cellular accumulation of \textit{[3H]}-GLB was low and substantially increased by the addition of P-gp inhibitors such as cyclosporine A and verapamil only in P-gp-expressing cells (Golstein et al., 1999), suggesting that GLB is likely also a P-gp substrate. However, the recent study (Gedeon et al., 2006) indicated that GLB is not a substrate of P-gp. The reason for this discrepancy remains to be investigated. Therefore, at present, the possibility that P-gp also contributes to placental transport of GLB cannot be excluded, based on the following reasons. First, verapamil is a relatively weak P-gp inhibitor. Second, term placentas were used in the perfusion study of Kraemer et al. It is well known that P-gp expression in human term placenta is drastically decreased compared with that in early pregnancy (Mathias et al., 2005), thus P-gp-mediated GLB transport may not have been detectable in the perfusion study using term placenta. Nevertheless, since it has been shown that the BCRP mRNA level was 10 times greater than that of P-gp in human term placenta (Ceckova et al., 2006), it is reasonable to assume that BCRP likely plays a much greater role than P-gp in placental transfer of GLB in human term placenta. Other factors such as plasma protein binding may also play a role in determining fetal GLB exposure \textit{in vivo} (Nanovskaya et al., 2006).

In summary, our data show that Bcrp1 significantly limits fetal distribution of GLB in the pregnant mouse, but has only a minor effect on the systemic clearance of the drug. Such findings may have significant clinical relevance for the treatment of gestational diabetes with GLB. For example, the fetuses of pregnant women carrying the BCRP natural variant Q141K which displays lower transport activity (Imai et al., 2002) may be at increased risk for the GLB toxicity. If a BCRP inhibitor happens to be co-administered to pregnant women with GLB, fetal exposure to GLB could possibly be increased due to drug-drug interaction on BCRP through inhibition of
the transporter. These data also suggest that, to limit fetal drug exposure, drugs that are actively
effluxed by human placenta may be preferred. On the other hand, if the fetus is the target of drug
therapy, drugs that bypass the efflux transporters in human placenta may be optimal.
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References


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Footnotes:

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Figure Legends

Figure 1. Effect of GLB on MX Efflux in HEK cells. Intracellular MX fluorescence in the presence or absence of 10 μM FTC, 10 μM or 100 μM GLB was measured as described in “Materials and Methods”. All efflux experiments were performed with 10 μM MX. The solid and open bars represent the intracellular MX levels in the HEK/vector and HEK/BCRP cells, respectively. A, the intracellular MX levels were presented as the absolute fluorescence values. B, the intracellular MX levels in the absence of FTC or GLB in both cell lines were set as controls (100%). The relative intracellular MX levels in the presence of FTC or GLB normalized to the respective controls are shown. Data shown are mean ± S.D. from three independent experiments. The differences as indicated are statistically significant: * p < 0.05 or ** p < 0.01 by the two-sample Student’s t-test.

Figure 2. Efflux of [3H]-GLB by BCRP/Bcrp1. The efflux assays were performed with 5 nM [3H]-GLB (A, B, C, and D) or 0.5 nM [3H]-GLB (E, F, G, and H) with or without 20 μM verapamil in the presence and absence of 10 μM FTC as described in “Materials and Methods”. The solid and open bars represent the intracellular [3H]-GLB concentrations in the presence and absence of FTC, respectively. The intracellular [3H]-GLB concentrations in MDCK/vector or MDCK/BCRP cells are shown in A, B, E, and F. The intracellular [3H]-GLB concentrations in MDCKII parent and MDCKII/Bcrp1 cells are shown in C, D, G, and H. Data shown are mean ± S.D. from three independent experiments. The differences as indicated are statistically significant: * p < 0.05 or ** p < 0.01 by the two-sample Student’s t-test.

Figure 3. Transwell transport of [3H]-GLB. Transwell transport of [3H]-GLB across the MDCK/BCRP (A and C), MDCK/vector (B and D), MDCKII/Bcrp1 (E and G), and MDCKII
parent (F and H) cell monolayers in the presence (A, C, E, and G) or absence (B, D, F, and H) of 10 µM FTC was determined as described in “Materials and Methods”. The experiments were started by adding 5 nM [³H]-GLB to one compartment. Verapamil (20 µM) was included in all the experiments. The radioactivity appearing in the opposite compartment was measured, and the molar amounts of [³H]-GLB were calculated. The percentage of the amount of initially applied [³H]-GLB appearing in the opposite compartment was plotted. Data shown are mean ± SD from three determinations in one representative experiment. Similar results were obtained from three independent experiments. Translocation from the basolateral compartment to the apical compartment (solid triangles) and from the apical compartment to the basolateral compartment (solid squares) is shown. R values are the efflux ratios of the basolateral-to-apical transport to the apical-to-basolateral transport at the end of the experiments (4 h). The differences between the basolateral-to-apical transport and the apical-to-basolateral transport as indicated are statistically significant: *** p < 0.005 by the two-sample Student’s t-test.

**Figure 4. The maternal plasma GLB concentration-time profiles.** Wild-type (solid squares) and Bcrp1⁻/⁻ (open squares) pregnant mice at gd 15 were administered GLB (1 mg/kg body weight) by retro-orbital injection. At various time points (0.5 – 240 min) after drug administration, maternal plasma samples were collected. The GLB concentrations in the maternal plasma samples were determined by HPLC/MS as described in “Materials and Methods”. Shown are mean ± S.D. (n = 3 – 6 per time point). Statistically significant differences in the maternal plasma GLB concentrations between the two animal groups were analyzed using one-way ANOVA analysis followed by Student’s t-test with the Bonferroni correction assuming a significant level of 0.05.
Figure 5: The fetal GLB concentration-time profiles. Wild-type (solid squares) and Bcrp1<sup>−/−</sup> (open squares) pregnant mice at gd 15 were administered GLB (1 mg/kg body weight) by retro-orbital injection. At various time points (0.5 – 240 min) after drug administration, fetuses were collected. The GLB concentrations in the fetuses were determined by HPLC/MS as described in “Materials and Methods”. Shown are mean ± S.D. (n = 3 – 6 per time point). At 180 min, the fetal GLB concentrations in the Bcrp1<sup>−/−</sup> pregnant mice were significantly greater than those in the wild-type pregnant mice, as indicated with *, by one-way ANOVA analysis followed by Student’s t-test with the Bonferroni correction assuming a significant level of 0.05.

Figure 6: The fetal/maternal plasma GLB concentration ratios. The fetal/maternal plasma GLB concentration ratios [(ng/g fetus)/(µg/ml maternal plasma)] in the wild-type (solid bars) and Bcrp1<sup>−/−</sup> (open bars) pregnant mice are presented. Shown are mean ± S.D. (n = 3 – 6 per time point). At 60, 180, and 240 min, the fetal/maternal plasma GLB concentration ratios in the Bcrp1<sup>−/−</sup> pregnant mice were significantly greater than those in the wild-type pregnant mice, as indicated with *, by one-way ANOVA analysis followed by Student’s t-test with the Bonferroni correction assuming a significant level of 0.05.
Table 1. The maternal plasma and fetal AUCs (0.5 – 240 min) of GLB in the wild-type and Bcrp1<sup>−/−</sup> pregnant mice after intravenous administration of the drug (1 mg/kg body weight).

<table>
<thead>
<tr>
<th></th>
<th>Wild-type pregnant</th>
<th>Bcrp1&lt;sup&gt;−/−&lt;/sup&gt; pregnant</th>
<th>Z&lt;sub&gt;0&lt;/sub&gt;</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Maternal plasma AUC</td>
<td>55.8 ± 2.7</td>
<td>47.9 ± 2.3</td>
<td>2.27</td>
<td>&lt; 0.05</td>
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</tbody>
</table>
<sub>(µg • min/ml plasma)</sub>
| Fetal AUC              | 575.6 ± 44.6       | 1108.2 ± 62.3                 | 6.95        | < 0.01|
<sub>(ng • min/g fetus)</sub>
| Fetal/maternal plasma AUC ratio | 10.3         | 23.2                         |             |       |
| Maternal plasma CL     | 17.9 ± 0.9         | 20.9 ± 1.0                    | 2.26        | < 0.05|
<sub>(ml/min)</sub>
| Maternal Vss           | 37.9 ± 127.8       | 22.0 ± 76.1                   | 0.11        | > 0.05|
<sub>(ml)</sub>
| Maternal MRT           | 2.1 ± 7.1          | 1.1 ± 3.6                     | 0.13        | > 0.05|

The maternal and fetal AUCs (0.5 – 240 min) as well as the maternal plasma MRT, CL, and Vss values were estimated using the Bailer’s approach as described in “Materials and Methods”. Shown as mean ± S.E. (n = 3 – 6 per time point). The fetal/maternal plasma AUC ratios [((ng • min/g fetus)/(µg • min/ml plasma))] are also presented. Z<sub>0</sub> and p values were calculated to assess the statistically significant differences of the pharmacokinetic parameters between the wild-type and Bcrp1<sup>−/−</sup> pregnant mice groups.
Figure 1

A

Intracellular MX Level (Fluorescence)

No inhibitor, 10 µM FTC, 10 µM GLB, 100 µM GLB

B

Intracellular MX level (% of control)

No inhibitor, 10 µM FTC, 10 µM GLB, 100 µM GLB
Figure 2

A  B

5 nM GLB without verapamil

C  D

5 nM GLB with verapamil

E  F

0.5 nM GLB without verapamil

G  H

0.5 nM GLB with verapamil
Figure 3

A. MDCK/BCRP without FTC

B. MDCK/Vector without FTC

C. MDCK/BCRP with FTC

D. MDCK/Vector with FTC

E. MDCKII/Bcrp1 without FTC

F. MDCKII without FTC

G. MDCKII/Bcrp1 with FTC

H. MDCKII with FTC
**Figure 4**

Maternal plasma GLB concentration (µg/ml)

- **Wild-type**
- **Bcrp1⁻/⁻**

Time (min)

0 50 100 150 200 250 300
Figure 5

Graph showing the fetal GLB concentration (ng/g fetus) over time (min) for Wild-type and Bcrp1-/- mice. The graph demonstrates a significant difference (*) between the two groups at certain time points.
Figure 6

Fetal/maternal plasma GLB concentration ratio (ng/g fetus)/(µg/ml) vs. Time (min)

- Wild-type
- Bcrp1-/-