Breast Cancer Resistance Protein 1 Limits Fetal Distribution of Nitrofurantoin in the Pregnant Mouse

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ABSTRACT:
The efflux transporter, the breast cancer resistance protein (BCRP), is most abundantly expressed in the apical membrane of the placental syncytiotrophoblasts, indicating that it could play an important role in protecting the fetus by limiting xenobiotic/drug penetration across the placental barrier. In the present study, we examined whether Bcrp1, the murine homolog of human BCRP, limits fetal distribution of the model BCRP/Bcrp1 substrate, nitrofurantoin (NFT), in the pregnant mouse. NFT was administered i.v. to FVB wild-type and Bcrp1<sup>−/−</sup> pregnant mice. The maternal plasma samples and fetuses were collected at various times (5–60 min) after drug administration. The NFT concentrations in the maternal plasma samples and homogenates of fetal tissues were determined by a high-performance liquid chromatography/UV assay. Although the maternal plasma area under the concentration-time curve (AUC) of NFT in the Bcrp1<sup>−/−</sup> pregnant mice (97.4 ± 10.0 μg · min/ml plasma) was only slightly (but significantly) higher than that in the wild-type pregnant mice (78.4 ± 6.0 μg · min/ml plasma), the fetal AUC of NFT in the Bcrp1<sup>−/−</sup> pregnant mice (1493.0 ± 235.3 ng · min/g of fetus) was approximately 5 times greater than that in the wild-type pregnant mice (298.6 ± 77.4 ng · min/g of fetus). These results clearly suggest that Bcrp1 significantly limits fetal distribution of NFT in the pregnant mouse, but has only a minor effect on the systemic clearance of the drug.

Pregnant women often need to take medication to treat diseases including viral, fungal, or bacteria infections, epilepsy, hypertension, or pregnancy-induced conditions such as nausea and gestational diabetes (Glover et al., 2003). A major concern arising from the use of medication by pregnant women is the transfer of drugs across the placental barrier, leading to potential toxicity to the fetus.

An important determinant for drug distribution across the placental barrier is the expression of efflux transporters in the apical membrane of placental syncytiotrophoblasts (Unadkat et al., 2004; Evseenko et al., 2006a). Such efflux transporters include BCRP, P-glycoprotein, and multidrug resistance protein 2 that can transport xenobiotics/drugs from the fetal compartment to the maternal circulation, thus protecting the fetus from potential toxicity (Lankas et al., 1998; Jonker et al., 2000; Ceckova-Novotna et al., 2006; Evseenko et al., 2006a).

BCRP or MXR (gene symbol ABCG2) is a relatively recently identified ATP-binding cassette efflux drug transporter (Mao and Unadkat, 2005; Krishnamurthy and Schuetz, 2006). BCRP is highly expressed in various normal tissues important for drug disposition, such as the placenta, small intestine, and liver (Maliepaard et al., 2001). Functional characterization in recent years has revealed that BCRP can transport a broad spectrum of substrates, ranging from chemotherapeutic agents to organic anions (Doyle and Ross, 2003; Mao and Unadkat, 2005). Availability of such information on substrate specificity has greatly facilitated the characterization of BCRP with respect to its role in drug disposition (Jonker et al., 2000; Kruijtzner et al., 2002; Merino et al., 2005; Zhang et al., 2006).

Among normal tissues, BCRP is most abundantly expressed in the placenta (Maliepaard et al., 2001). In human term placenta, the mRNA level of BCRP was found to be even 10 times greater than that of P-glycoprotein (Ceckova et al., 2006). Moreover, many drugs including glyburide (Gedeon et al., 2006), cimetidine (Pavek et al., 2005), and nitrofurantoin (Merino et al., 2005) that are often taken by pregnant women to treat illnesses have been shown to be BCRP substrates. Thus, given the abundant expression in the placenta and a broad range of substrate specificity, it is reasonable to hypothesize that BCRP plays an important role in moderating the transfer of maternally administered drugs to the fetus. The transport function of BCRP/Bcrp1 in the placenta has been demonstrated using in vitro models or ex vivo perfused placenta. Kolwankar et al. (2005) demonstrated BCRP transport activity in membrane vesicles isolated from the human placenta. Expression and transport activity of BCRP have also been illustrated in BeWo cells, a model human placental cell line (Ceckova et al., 2006; Evseenko et al., 2006b; Wang et al., 2006b). Staud et al. (2006) examined the transport activity of Bcrp1 in perfused rat placenta using cimetidine as a model Bcrp1 substrate. Rat Bcrp1 was shown to significantly limit the maternal-to-fetal transport of cimetidine. When fetal perfusate was recirculated, rat Bcrp1 could actively transport cimetidine from the fetal to the maternal compartment against a concentration gradient. Several in vivo studies with respect to transport function of Bcrp1 in the placenta have been

ABBREVIATIONS: BCRP (Bcrp), breast cancer resistance protein; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; gd, gestation day; IS, internal standard; AUC, area under the concentration-time curve.

performed, and in all of these studies fetal/maternal plasma concentration ratios were determined at only one time point after drug administration as a measure of fetal drug distribution (Jonker et al., 2000, 2002; Enokizono et al., 2007). For example, in the study using P-glycoprotein-deficient pregnant mice (Jonker et al., 2000), Jonker et al. showed that coadministration of a Bcrp1 inhibitor increased the fetal concentration of topotecan (a BCRP/Bcrp1 substrate) approximately 2-fold at 30 min after drug administration, compared with that in pregnant vehicle-treated control mice. However, detailed in vivo transplacental pharmacokinetics involving BCRP/Bcrp1 has not been investigated as yet.

Therefore, in the present study, we systematically investigated the effect of Bcrp1 on the pharmacokinetics of fetal distribution of a model BCRP/Bcrp1 substrate, nitrofurantoin, in wild-type and Bcrp1 /−− pregnant mice. Nitrofurantoin has been shown to be effectively transported by BCRP/Bcrp1, but not by P-gp and multidrug resistance protein 2 (Merino et al., 2005). Therefore, nitrofurantoin is a highly selective BCRP/Bcrp1 substrate and has been used as a probe to assess in vivo function of Bcrp1 (Merino et al., 2005; Wang and Morris, 2007). Our results clearly indicate that Bcrp1 significantly limits fetal exposure of nitrofurantoin in the pregnant mouse.

Materials and Methods

Chemicals. Nitrofurantoin (NFT) and furazolidone were purchased from Sigma-Aldrich (St. Louis, MO). Polyethylene 400 (PEG 400) was obtained from Spectrum Laboratory Products, Inc. (Gardena, CA). HPLC-grade acetonitrile and methylene chloride were from Fisher Scientific Co. (Morris Plains, NJ). PBS was from Invitrogen (Carlsbad, CA).

Animal Studies. FVB wild-type mice and the first generation of Bcrp1 /−− mice with an FVB genetic background were purchased from Taconic Farms (Hudson, NY). Under a breeding license purchased from Taconic Farms, male and female Bcrp1 /−− mice were mated to generate offspring, which were used in the subsequent animal experiments. The absence of the Bcrp1/Abcg2 gene in the offspring of Bcrp1 /−− mice was confirmed by genotyping (data not shown). Pregnant and nonpregnant wild-type and Bcrp1 /−− mice were cared for in accordance with the U.S. Public Health Service policy for the Care and Use of Laboratory Animals. 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 of 7 to 9 weeks of age were mated with female mice of the same age. Female mice that demonstrated sperm plugs were separated and housed in new cages. Gestational age was calculated on the basis of the estimated time of insemination (presence of sperm plug as gd 0). Progress of pregnancy was regularly monitored by visual inspection. All experiments were performed on gd 15, as we have previously shown that the placental expression of Bcrp1 in the pregnant mouse peaks at gd 15 (Wang et al., 2006a).

NFT was dissolved in 10% (v/v) ethanol, 40% (v/v) PBS, and 50% (v/v) PEG 400 at a concentration of 1.5 mg/ml. Under anesthesia (isoflurane), the FVB wild-type or Bcrp1 /−− pregnant mice (at gd 15) were administered NFT i.v. by retro-orbital injection (5 mg/kg b.wt.). At various times (5, 10, 20, 30, 40, 50, and 60 min) after drug administration (n = 3–5 per time point), animals were sacrificed under anesthesia by cardiac puncture. Blood was collected in heparinized microtainer tubes (BD, Franklin Lakes, NJ) and centrifuged, and plasma was stored at −20°C until use. The fetuses were removed from pregnant mice, rinsed with ice-cold PBS, and immediately stored at −80°C until analysis.

NFT HPLC/UV Assay. The maternal plasma and fetal NFT concentrations were determined by a validated HPLC/UV assay. In brief, for plasma samples, human plasma (used as blank) was spiked with a NFT stock solution in pure acetonitrile, and serial dilutions were prepared in human plasma with less than 1% (v/v) acetonitrile to generate NFT calibration curves (0.1–10 μg/ml). In initial experiments, no differences were observed between the calibration curves generated using mouse plasma or human plasma as a blank. Therefore, human plasma was used as a blank in all subsequent experiments because only a limited amount of mouse plasma was available. Quality control samples were prepared in human plasma with less than 1% (v/v) acetonitrile using a different NFT stock solution, and the concentrations were in the low (0.1 μg/ml), mid (5 μg/ml), and high (10 μg/ml) ranges of the calibration curves. All calibration and quality control samples were stored at −20°C in 350-μl aliquots until use. For fetus samples, to generate calibration curves, three blank fetuses from undosed animals were weighed, pooled, and homogenized in 3 ml of ice-cold PBS using an Omni TH homogenizer (Omni International, Marietta, GA). NFT stock solutions in pure acetonitrile were then added to the homogenates in appropriate volumes to achieve the intended concentrations (2–100 ng/g of fetus) with less than 1% v/v acetonitrile. Quality control samples were also prepared by independently spiking the prepared NFT stock solutions into blank fetus homogenates. The NFT concentrations of quality control samples ranged from the low (2 ng/g fetus) to the high (100 ng/g fetus) end of the calibration curves. All calibration and quality control samples used for determination of the fetal NFT concentrations were prepared on the day of HPLC analysis.

To determine NFT concentrations by HPLC/UV, the maternal mouse plasma and fetuses were processed as below. In every 200 μl of maternal plasma or 1 ml of fetus tissue homogenate in a 14-ml Falcon tube (Corning, NY), furazolidone was added as an internal standard (IS) to a final concentration of 1 μg/ml plasma or 25 ng/g of fetus. After gentle vortexing, 10 μl of 50% HCl was added to adjust the pH to approximately 3. Then, 2 or 5 ml of methylene chloride was added to each maternal plasma or fetus homogenate sample, respectively, for extraction of nitrofurantoin. The samples were then gently shaken for 30 min and centrifuged at 3000 rpm for 10 min at room temperature. The organic phase at the bottom of the tube was transferred to a clean glass tube for each sample, dried under N2 at 40°C in a Multivap analytical evaporator (Organomation Inc., Berlin, MA), and reconstituted in 100 μl of mobile phase for the HPLC/UV analysis. HPLC/UV was performed on a Waters Alliance 2695 liquid chromatograph with an autosampler (Milford, MA). The separation was achieved using a C18 reverse-phase column (Zorbax SB-C18, 4.6 mm × 150 mm, 5 μm; Agilent Technologies, Palo Alto, CA) equipped with a guard column (C18, 4.0 mm × 3.0 mm, 5 μm; Phenomenex, Torrance, CA). NFT and the IS were detected with a Waters 2996 photodiode array detector at wavelength 366 nm. The mobile phase was composed of 20% acetonitrile and 80% water. The flow rate was set at 1.2 ml/min. This assay was validated by measuring the NFT concentrations in calibration and quality control samples in triplicate on 3 different days. Calibration curves were constructed by linear regression of the peak area ratios (NFT/IS) plotted versus the NFT concentrations. The NFT concentrations in quality control and unknown samples were determined by inverse regression. When unknown samples were analyzed, calibration and quality controls were always run in triplicate and quality controls in duplicate in parallel with the unknown samples to ensure the accuracy of the assay.

Pharmacokinetic Data Analysis. For one-point sampling data (one blood sample from each mouse), we used Bailer’s approach (Bailer, 1988; Takemoto et al., 2006) to estimate the mean, S.D., and S.E. of the maternal plasma and fetal AUCs of NFT. Briefly, the mean E(AUC) and variance V(AUC) of AUC were calculated using the equations:

\[
E(AUC) = \sum_{i=1}^{n} \left( \frac{E(C_i) + E(C_{i-1})}{2} \right) \cdot (t_i - t_{i-1})/2
\]

\[
V(AUC) = \sum_{i=1}^{n} \left( \frac{V(C_i) + V(C_{i-1})}{2} \right) \cdot ((t_i - t_{i-1})/2)^2
\]

where \( n \) is the number of time points of the time course experiment and \( C_i, t_i \) are concentration and time, respectively. The time course was integrated by the trapezoidal formula without extrapolation. The S.D. of AUC was given as the square root of the variance of AUC. \( E(C_i) \) and \( V(C_i) \) are the mean and variance of the concentration at time \( t_i \), respectively, and are 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 the concentration data points at time \( t_i \). The variance of mean AUC is defined by

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

The S.E. of the AUC was then given as the square root of the variance of mean AUC.

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

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

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

**Results**

The NFT assay was linear \((r = 0.99)\) over the calibration range of NFT concentrations, 0.1 to 10 \( \mu \)g/ml plasma and 2 to 100 ng/g of fetus for the maternal plasma and fetus, respectively. The percentage of differences between the spiked and measured concentrations was always less than 20%, indicating good accuracy of the calibration curves. The interday and intraday variations were consistent with coefficients of variation less than 15 to 20% across the calibration ranges. The retention times for NFT and IS were approximately 4.3 and 5.6 min, respectively. Three fetuses from each pregnant mouse were randomly selected and pooled to form one fetus group. Each pregnant mouse usually had more than six fetuses. We found that the fetal NFT concentrations of two different fetus groups from the same pregnant mouse were very similar (data not shown). Thus, the average fetal NFT concentration of two different fetus groups from the same pregnant mouse was used as the fetal NFT concentration of this mouse.

Overall, after i.v. administration of NFT, the maternal plasma concentration-time profiles of the wild-type and Bcrp1\(^{-/-}\) pregnant mice were comparable (Fig. 1). However, at 30 and 40 min after drug administration, the maternal plasma NFT concentrations in the Bcrp1\(^{-/-}\) pregnant mice were slightly, but significantly, higher than those in the wild-type pregnant mice (Fig. 1). The fetal NFT concentrations in the wild-type and Bcrp1\(^{-/-}\) pregnant mice were low at early time points (5–10 min) and then reached the maximum at time points 20 to 40 min (Fig. 2). The fetal NFT concentrations in the wild-type pregnant mice peaked earlier than those in the Bcrp1\(^{-/-}\) pregnant mice (10–20 min in the wild-type mice versus 30–40 min in the Bcrp1\(^{-/-}\) mice) (Fig. 2). At 5 and 60 min, the fetal NFT concentrations in the wild-type pregnant mice were under the detection limit and thus considered as 0. In contrast, NFT was readily detectable in the fetuses of the Bcrp1\(^{-/-}\) pregnant mice (Fig. 2). Moreover, the fetal NFT concentrations in the Bcrp1\(^{-/-}\) pregnant mice were generally greater than those in the wild-type pregnant mice at all time points (Fig. 2). In particular, at 20, 30, 40, 50, and 60 min, the mean fetal NFT concentrations in the Bcrp1\(^{-/-}\) pregnant mice were at least 3 times greater than those in the wild-type pregnant mice. For example, at 40 and 50 min, the fetal NFT concentrations in the Bcrp1\(^{-/-}\) pregnant mice were 42.4 ± 8.0 and 23.2 ± 5.3 ng/g of fetus, respectively, which were significantly higher than those in the wild-type pregnant mice (1.9 ± 1.6 and 5.6 ± 6.5 ng/g of fetus at 40 and 50 min, respectively). At 60 min, the fetal NFT concentrations in the Bcrp1\(^{-/-}\) pregnant mice were 23.1 ± 9.4 ng/g of fetus; however, NFT in the fetuses of the wild-type pregnant mice could not be detected (Fig. 2).

Because the NFT concentrations in the maternal circulation will affect fetal distribution of the drug, to determine whether Bcrp1 does play a role in limiting fetal distribution of NFT in the pregnant mouse, we normalized the fetal NFT concentrations to the respective maternal plasma NFT concentrations. The fetal/maternal plasma NFT concentration ratios were considered as 0 for the wild-type pregnant mice at 5 and 60 min at which time NFT in the fetuses of these mice was not detectable (Fig. 2). At all other time points, the Bcrp1\(^{-/-}\) pregnant mice demonstrated generally higher fetal/maternal plasma NFT concentration ratios than the wild-type pregnant mice (Fig. 3). In particular, at 40 and 50 min, the ratios in the Bcrp1\(^{-/-}\) pregnant mice were significantly increased approximately 9- and 4-fold, respectively, compared with those in the wild-type pregnant mice (Fig. 3). These results suggest that the fetal exposure of NFT in the Bcrp1\(^{-/-}\) pregnant mice was increased compared with that in the wild-type pregnant mice. AUC is a common index of drug exposure over a certain time period after drug administration. Therefore, we estimated the maternal plasma and fetal AUCs (5–60 min) in the wild-type and Bcrp1\(^{-/-}\) pregnant mice to further evaluate the role Bcrp1 plays in fetal distribution of NFT. We used Bailer’s approach to estimate AUCs for the one-point sampling data we obtained in the animal
wild-type pregnant mice (Table 1), suggesting that Bcrp1 significantly limits fetal distribution of NFT in the pregnant mouse. Note that NFT cannot be detected in the fetus of the wild-type pregnant mice at 60 min but was readily detectable in the fetus of the Bcrp1<sup>−/−</sup> pregnant mice. Thus, if the fetal AUCs were calculated beyond 60 min, the difference in the fetal/maternal plasma AUC ratio between the wild-type and Bcrp1<sup>−/−</sup> pregnant mice would be even greater. These results clearly suggest that Bcrp1 significantly limits fetal NFT exposure in the pregnant mouse. To the best of our knowledge, this is the first study in which fetal drug exposure was determined by measuring fetal and maternal plasma AUCs. Our data also suggest that the use of fetal/maternal plasma concentration ratios at only one time point after drug administration as a measure of fetal drug distribution may not precisely reflect the extent of in vivo fetal drug exposure, as the ratio varies at different time points. For example, at 40 and 50 min, the fetal/maternal plasma NFT concentration ratios in the Bcrp1<sup>−/−</sup> pregnant mice were approximately 8 and 4 times greater than those in the wild-type pregnant mice, respectively (Fig. 3). However, at earlier time points, the ratios were not significantly different between the wild-type and Bcrp1<sup>−/−</sup> pregnant mice.

Bcrp1 in the placenta may function by limiting the transfer of NFT from the maternal circulation to the fetus and/or by removing the drug already present in the fetus back to the maternal circulation against a concentration gradient. This explanation is consistent with the data obtained in the recent ex vivo rat placenta perfusion study for cimetidine (Staud et al., 2006). Perry and Leblanc (1967) studied the transfer of NFT across the human placenta by measuring the maternal and cord serum concentrations of NFT after i.v. administration of the drug to women in labor. They found that NFT passed across the human placenta to only a small extent and hence proposed that the minimal transfer of NFT across the human placenta was probably due to the relatively rapid disappearance of the drug from the maternal circulation. At the time Perry and Leblanc performed their study, there was no such concept regarding efflux transporters in the placenta, and it was therefore unlikely for them to consider the contribution of these transporters in determining fetal drug exposure. The data of the present study clearly suggest that BCRP is very likely the transporter that limits fetal exposure of NFT in humans. Future studies in human subjects are needed to confirm this hypothesis. We also note that the fetal NFT concentrations in the wild-type pregnant mice peaked earlier than those in the Bcrp1<sup>−/−</sup> pregnant mice (Fig. 2). Thus, the fetal clearance of NFT seems to be delayed in the Bcrp1<sup>−/−</sup> pregnant mice compared with that in the wild-type pregnant mice. It has been shown that Bcrp1 is also significantly expressed in fetal tissues (Kalabis et al., 2007). Thus, the delay of fetal clearance of NFT in the Bcrp1<sup>−/−</sup> pregnant mice is likely to be caused, at least in part, by the lack of Bcrp1 expression in the fetal drug-clearing organs.

In summary, our data show that Bcrp1 significantly limits fetal distribution of NFT in the pregnant mouse but has only a minor effect on the systemic clearance of the drug. Such findings may have significant clinical relevance. NFT is commonly used to treat urinary tract infection in pregnant women (Le et al., 2004). Although animal
Studies have not shown side effects of fetal exposure of this drug (Prytherch et al., 1984), and human studies with a relatively small amount of data did not show evidence of harmful effects of NFT to the fetus (Perry et al., 1967; Ben David et al., 1995), adverse effects related to the fetal exposure of NFT have recently been suggested. For example, there is evidence that links craniosynostosis to fetal exposure to NFT and drugs with similar chemical structures (Gardner et al., 1998; Källén and Robert-Gnansia, 2005). Thus, the fetuses of pregnant women with BCRP natural variants, such as Q141K, that display lower transport activity (Imai et al., 2002) may have an increased risk for potential NFT toxicity. To prevent overexposure of NFT to the fetus, use of NFT should be contraindicated in such pregnant women. In addition, if a BCRP inhibitor happens to be coadministered to pregnant women with NFT, fetal exposure to NFT could possibly be increased because of a drug-drug interaction on BCRP through inhibition of the transporter. Finally, because BCRP can transport many drugs that are routinely administered to pregnant women, we expect that BCRP could play a significant role in protecting the fetus from the potential toxicity of these drugs.

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References


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