ABSTRACT:

Genetic knockout mice studies suggested ATP-binding cassette transporter family G member 2 (ABCG2)/Abcg2 translocates nitrofurantoin at the mammary-blood barrier, resulting in drug accumulation in milk. The purpose of this study was to establish the role of Abcg2 in nitrofurantoin accumulation in rat milk using 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) as a "chemical knockout" equivalent. The inhibitory effect of GF120918 was verified in Madin-Darby canine kidney II cells stably expressing rat Abcg2 with Hoechst 33342 and nitrofurantoin flux in Transwells. Nitrofurantoin was infused (0.5 mg/kg) in the absence and presence of GF120918 (10 mg/kg in dimethyl sulfoxide) to Sprague-Dawley lactating female rats using a balanced crossover design. Administration of GF120918 increased nitrofurantoin concentration in serum (from 443 ± 51 to 650 ± 120 ng/ml) and decreased concentration in milk (from 18.1 ± 0.9 to 1.9 ± 1.2 μg/ml), resulting in corresponding mean values for milk to serum concentration ratio (M/S) of 41.4 ± 19.1 versus 3.04 ± 2.27 in the absence and presence of GF120918 (p < 0.05), respectively. There was a decrease in systemic clearance with GF120918 (2.8 ± 0.5 l/h/kg) compared with vehicle controls (4.1 ± 0.5 l/h/kg; p < 0.05). Western blot analysis revealed good expression of Abcg2 and no P-glycoprotein (P-gp) expression in mammary gland, whereas immunohistochemistry confirmed the apical expression of Abcg2 in lactating mammary gland epithelia. Nitrofurantoin active transport into rat milk can be inhibited by GF120918 resulting in a 10-fold lower M/S. Although GF120918 inhibits both Abcg2 and P-gp, the high expression of Abcg2 and the absence of detectable P-gp expression in lactating mammary gland validate an important role for Abcg2 in nitrofurantoin accumulation in rat milk. GF120918 is particularly useful as a rat chemical knockout model to establish ABCG2’s role in drug transfer into milk during breastfeeding.

Most xenobiotics are transferred into milk by passive diffusion, and the extent of their accumulation in milk [as measured by milk to serum concentration ratio (M/S)] can be predicted by in vitro measurements of drug binding and ionization in milk and serum, as well as lipid partitioning into milk (Fleishaker et al., 1987; Fleishaker and McNamara, 1988a). Such an approach successfully predicted the M/S for a number of drugs (Fleishaker and McNamara, 1988b); however, this approach was unable to estimate the large M/S observed for drugs like nitrofurantoin (Gerk et al., 2001a). In fact, the observed ratios for nitrofurantoin in the human and rat were 20 and 100 times greater (Gerk et al., 2001a; Oo et al., 2001), respectively, than could be accounted for by passive diffusion alone, clearly implicating an active transport process.

The transport of nitrofurantoin across a murine mammary epithelial cell culture model has been shown to be saturable and sodium-dependent in the basal-to-apical direction (Toddyywalla et al., 1997; Gerk et al., 2002). Merino et al. (2005) described that nitrofurantoin was efficiently transported by murine breast cancer resistance protein (Bcrp) 1 and human BCRP by using polarized cell lines. More importantly, they also showed that the milk-to-plasma ratio of nitrofurantoin was almost 80 times higher in wild-type compared with Bcrp1 knockout lactating female mice (Merino et al., 2005). Therefore, nitrofurantoin was identified as an excellent BCRP/Bcrp1 substrate that is translocated at the blood-mammary barrier, resulting in its accumulation in milk. A growing number of studies have shown the compensatory mechanism in genetic knockout mouse models. The most impressive evidence was from recent literature. In Bcrp (−/−) mice, several transporters, like Abca5, Abcb4, Abcc2, and Abcc4, were significantly up- or down-regulated, with the most pronounced differential expression.

ABBREVIATIONS: M/S, milk to serum concentration ratio; Bcrp/BCRP, breast cancer resistance protein; Abcg2/ABCG2, ATP-binding cassette transporter family G member 2; GF120918, 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; HPLC, high-performance liquid chromatography; MDCKII, Madin-Darby canine kidney II; DMSO, dimethyl sulfoxide; P-gp, P-glycoprotein; MDR/Mdr, multidrug resistance/-resistant.
observed with abcg5 and abcg8, which showed a 20- to 100-fold up-regulation in Bcrp (−/−) mice (Huls et al., 2008). Therefore, compensatory mechanisms in transport function in genetic knockout mice may complicate the interpretation of drug metabolism and disposition studies and active transport into milk.

In present study, we systematically evaluated the role of ATP-binding cassette transporter family G member 2 (Abcg2) (Bcrp) in nitrofurantoin accumulation in rat milk. GF120918 pretreatment was administered to generate a “chemical knockout” equivalent of Abcg2 in lactating rat, and nitrofurantoin was used as the prototypical Abcg2 substrate. The results obtained from these studies provide new insights into the utility of the rat model for ABCG2 substrates in drug active transport into milk and other drug metabolism and disposition studies.

Materials and Methods

Chemicals. Nitrofurantoin and furazolidone were purchased from Sigma-Aldrich (St. Louis, MO). GF120918 was a gift from GlaxoSmithKline (Research Triangle Park, NC). Hoechst 33342 was from Invitrogen (Carlsbad, CA). All the organic solvents [high-performance liquid chromatography (HPLC) grade] and polyethylene glycol 400 were purchased from Thermo Fisher Scientific (Waltham, MA), and all of the other chemicals were obtained from Sigma-Aldrich unless specified otherwise.

Animals. Five adult female lactating Sprague-Dawley rats (250–350 g) with 1- or 3-day-old pups were purchased from Harlan Laboratories (Indianapolis, IN). Animals were maintained under 12:12-h light-dark cycles and had access to food and water during the experiments. The rats were acclimatized for at least 1 week before the experiment. All the procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Expression and Functional Characterization of Rat Abcg2 in Madin-Darby Canine Kidney II Cells. Total RNA was isolated from a frozen liver of a Sprague-Dawley rat using RNAeasy kit (QIAGEN, Valencia, CA) and reverse-transcribed to cDNA using oligo(dT) primers and Thermoscript reverse-transcribed polymerase chain reaction kit (Invitrogen). Rat Abcg2 cDNA was amplified using forward (5′-CCGCTCGAGGCATAGATCCTAAAGAT-3′) and reverse (5′-CATGTCGAGGAGGTACTAATGTCT-3′) primers and Phusion DNA polymerase enzyme (New England Biolabs, Ipswich, MA). The polymerase chain reaction product was cloned into the pcDNA3.1 vector (Invitrogen) between XhoI and Bsal restriction sites. The Abcg2 sequence in this vector was confirmed (Davis Sequencing, Davis, CA), and it had 100% homology with the published rat Abcg2 sequence (GenBank accession number AB105817). Madin-Darby canine kidney II (MDCKII) cells were maintained in minimal essential medium with Earle’s salts (Mediatech, Manassas, VA) with 5% fetal bovine serum (Invitrogen) and 1× penicillin streptomycin (Invitrogen) at 37°C in 5% CO2. Cells were transfected with pcDNA3.1 or pcDNA3.1-rAbcg2 using Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. After 48 h, cells were selected with 800 μg/ml G418 (Invitrogen) for 10 days.

Inhibition of Rat Abcg2 by GF120918. To assess the utility of GF120918 as a chemical inhibitor of rat Abcg2, we first incubated MDCKII-rat Abcg2 cells with Hoechst 33342 in the presence and absence of 1 μM GF120918. In brief, cells were incubated with dye-containing medium at 37°C for 45 min. GF120918 was added at the indicated concentrations 10 min before the dye incubation. All of the flow cytometry assays were done with 10 μM Hoechst 33342 (Leggas et al., 2006). Second, the effect of GF120918 on nitrofurantoin directional transport was evaluated using MDCKII cells expressing rat Abcg2 or empty vector (pcDNA3.1). Cells were seeded on microporous membrane filters (3.0-μm pore size; 24-mm diameter; Transwell 3414; Corning, NY) at a density of 1.0 × 106 cells/well. Cells were grown for 4 days to achieve transpethelial electrical resistance >200 Ω·cm², and medium was replaced every other day. Before the experiment, the medium at both the apical and basolateral side of the monolayer was replaced with 2 ml of OptiMEM medium (Invitrogen) without serum and either 1 μM GF120918 or vehicle only [0.1% dimethyl sulfoxide (DMSO)] with 10 μM nitrofurantoin containing 0.2 μCi/ml [3H]nitrofurantoin. Cells were incubated at 37°C in 5% CO2. To ensure the tightness of each monolayer, 50-μl aliquots were collected to assess the paracellular flux of [3H]nitrofurantoin into the opposite compartment. We required that layers restrictedannocil transport to <1% of the total radioactivity per hour. For nitrofurantoin transport, 140-μl aliquots were taken at 0.5, 1, 2, and 3 h. Samples were stored at −80°C until the time of analysis by HPLC assay.

In Vivo Studies. The jugular and femoral veins of Sprague-Dawley lactating female rats were cannulated under ketamine/xylazine anesthesia on days 10 to 12 postpartum. After a day of recovery, each dam (n = 5) was randomized to receive an i.v. infusion of nitrofurantoin in polyethylene glycol 400 (0.5 mg/0.25 ml/h) for 5 h either with pretreatment of the inhibitor (GF120918, 10 mg/kg in DMSO) or with equivalent volume (approximately 0.15 ml of DMSO) of vehicle administered i.v. over 10 min and crossed over on the second day to complete both phases (Gerk et al., 2001b; Edwards et al., 2005). One milliliter of normal saline was given hourly to prevent dehydration during infusion. The dams were separated from the pups before the infusion. Blood samples were drawn hourly for the last 4 h of the infusion. The blood samples were protected from light, allowed to clot, centrifuged to harvest serum, and frozen at −80°C until analysis. Milk samples were obtained by manual manipulation under light anesthesia (ketamine) at the end of the infusion, protected from light, and also frozen at −80°C until analysis. Mammary gland and liver samples were harvested from a separate group of lactating rats on days 16 to 18 postpartum, frozen in liquid nitrogen, and stored at −80°C until analysis.

Nitrofurantoin HPLC Analysis. To each aliquot (70 μl) of serum, 15 μl of 5-μg/ml furazolidone HCl was added as an internal standard and was precipitated with 85 μl of dry ice-cold methanol, vortexed for 5 min, and then centrifuged at 16,000g for 10 min at 4°C. Fifty microliters of the supernatant plus 50 μl of mobile phase was mixed for 10 s and injected onto the HPLC. To each aliquot of milk, 25 μl of furazolidone (25 μg/ml) HCl was added as an internal standard to 50 μl of milk sample and was precipitated with 200 μl of cold methanol, vortexed for 5 min, and then centrifuged at 16,000g for 10 min at 4°C. Then, 50 μl of the supernatant was mixed with 50 μl of mobile phase and injected onto the HPLC. The HPLC system consists of a Lichrosorb 5 RP18 125 × 4.0-mm column (Phenomenex, Torrance, CA) and eluted with 20% acetonitrile/80% 25 mM potassium phosphate buffer, pH 3.0, at 1.0 ml/min. UV absorption was measured at 366 nm. Peak height ratios (nitrofurantoin/furazolidone) were used for comparison with the standard curve. The standard curve range for nitrofurantoin in serum was 62.5 to 4000 ng/ml. The milk samples were diluted if the concentration was greater than the linear range of the standard curve (0.725–29 μg/ml). Aliquots of nitrofurantoin in OptiMEM medium were directly injected onto the HPLC. The standard curve range was 7.8 to 2000 ng/ml. All the standard curves showed an intraday and interday variability of <10% and r2 > 0.99.

Western Blot Analysis for P-Glycoprotein and Abcg2. Crude membrane fractions were prepared from lactating mammary gland and kidney. In brief, the tissue samples were homogenized in Dounce buffer (Tris buffer, pH 7.6, at 4°C, 0.5 M magnesium chloride) with protease inhibitors (Complete Mini EDTA-free tablets; Roche Diagnostics), and the tonicity was restored to 150 mM with sodium chloride. After centrifugation at 12000 g for 3 min at 4°C, the supernatant, and removed, and EDTA was added to a final concentration of 5 mM. The sample was then centrifuged further at 100,000g for 1 h at 4°C to pellet the crude membrane fractions. Pellets were resuspended in resuspension buffer (0.2 M mannitol, 0.07 M sucrose, 50 μM Tris HCl, 1 μM EDTA). Rat Abcg2- and pcDNA3.1 (empty vector)-MDCKII cell pellets were resuspended in radioimmunoprecipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, protease inhibitor mixture), rotated for 30 min at 4°C, and then centrifuged at 13,000 rpm for 20 min at 4°C. Supernatants were collected for immunoblot. Protein concentrations were measured using the BCA Protein Assay kit (Pierce, Rockford, IL). PNPase F (New England Biolabs) was used for deglycosylation of rat Abcg2 following the manufacturer’s instructions. In brief, 60 μg (lactating mammary gland),
100 μg (kidney), and 7 μg (pcDNA3.1-expressed MDCKII cell and Abcg2-expressed MDCKII cell) of membrane fractions were incubated with 10X denaturing buffer supplied and PNGase F (4 μl) at 37°C for 2.5 h. Then the samples were mixed with 2X SDS-loading buffer and reducing buffer at 37°C for 30 min as the non-PNGase treatment group. Western blotting was performed as described previously (Edwards et al., 2005). P-glycoprotein (P-gp) expression was determined using the C219 (mouse anti-human P-gp; 1:100) monoclonal antibody, which was obtained from Kamiya Biomedical (Seattle, WA). Antibody binding was detected using a horseradish peroxide-linked IgG (H+L) (goat anti-rabbit) and IgG (H+L) (rabbit anti-mouse) antibodies (1:20,000) obtained from Pierce. The bands were visualized using a SuperSignal Pico Chemiluminescent substrate detection kit (Pierce).

Immunohistochemistry for Abcg2 Localization in Lactating Mammary Gland. Six-micrometer sections of paraffin blocks were cut using microtomy. After deparaffinization and rehydration, endogenous peroxidase was blocked with H2O2 in methanol for 20 min. The sections were blocked using an avidin blocking kit (Vector Laboratories, Burlingame, CA), and Abcg2 was determined using the PC-138 (1:1000) antibody, which was obtained from Kamiya Biomedical (Seattle, WA). Antibody binding was visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride dehydrate-H2O2 solution to develop a brown color and counterstained with hematoxylin, dehydrated, and coverslipped.

Pharmacokinetic Calculations and Statistical Analysis. Systemic clearance was calculated using the following formula:

\[
Cl_s = \frac{\text{Infusion Rats}}{M/S}
\]

where \( Cl_s \) is the average concentration of nitrofurantoin in serum from 3 to 5 h. M/S was calculated by:

\[
\frac{M}{S} = \frac{C_m}{C_s}
\]

where \( C_m \) is concentration of nitrofurantoin in milk at 5 h.

All the data are expressed as mean ± S.D. Analyses were performed to estimate intergroup differences using the one-way analysis of variance. A \( p \) value of <0.05 was considered statistically significant.

Results

Functional Characterization of Rat Abcg2 Expressed in MDCKII Cells and GF120918 as an Inhibitor of Rat Abcg2. The full-length rat Abcg2 complementary DNA was expressed in MDCKII cells. The functional activity of Abcg2 was characterized, and high-activity clones were selected using fluorescence-activated cells sorting based on the ability to limit the accumulation of the prototypical human and mouse ABCG2 substrate, Hoechst 33342, in MDCKII-empty cells. These MDCKII-empty cells showed a 7.5-fold increase in apical-directed transport and a 55% decrease in basolateral transport of nitrofurantoin (Fig. 1C), which was largely reversed by GF120918 treatment (Fig. 1D). These results show the creation of a MDCKII-rat Abcg2 clone.
were different sizes in the mammary gland (72 kDa), rat Abcg2/H11011 in MDCKII cell line (Fig. 2, B and C); however, the native Abcg2 bands detected in lactating mammary gland, kidney, and Abcg2-expressing in lactating mammary gland (Fig. 2A). Abcg2 expression was down-regulated during lactation (Alcorn et al., 2002), and protein expression was not detectable in the lactating rat mammary gland. P-gp seems to be transcriptionally expressed in the lactating rat. P-gp inhibition of rat Abcg2 to its role in drug transfer into rat milk. GF120918 was originally developed as a second-generation P-gp inhibitor (Tan et al., 2000). It belongs to the family of acridone carboxamide derivatives (Warram et al., 2002; Boumendjel et al., 2007) and has an IC₅₀ value of ~30 to 300 nM on the cellular accumulation of phosphine technetium complexes by human P-gp in SF9 cells (Luker et al., 1997). GF120918 is also a potent ABCG2 inhibitor with IC₅₀ values of ~300 nM on efflux or accumulation in cell lines transfected with human ABCG2 (Ahmed-Belkacem et al., 2005; Pan et al., 2007). Based on our results, GF120918 (1 μM) seemed to effectively inhibit the efflux of Hoechst 33342 and nitrofurantoin from rat Abcg2-expressing MDCKII cell line, thus confirming that GF120918 is an effective in vitro inhibitor of rat Abcg2.

After a 10-mg/kg dose, GF120918 significantly increased the serum concentrations of nitrofurantoin by decreasing its systemic clearance. Previous investigators have noted the systemic clearance of nitrofurantoin to be mediated, in part, by Abcg2-dependent processes (Merino et al., 2005; Wang and Morris, 2007). After p.o. and i.v. administration of nitrofurantoin, the area under the concentration–time curve in Bcrp1 knockout mice was 4- and 2-fold higher, respectively, than in wild-type mice (Merino et al., 2005) because of reduced first-pass and hepatobiliary excretion of nitrofurantoin. Zhang et al. (2007) have also shown that Bcrp1 limited the fetal distribution of nitrofurantoin and had a minor effect on the systemic clearance of the drug in the pregnant mouse.

In previous work in our laboratory, the P-gp substrate nelfinavir was dosed to lactating rats with GF120918 (Edwards et al., 2005). GF120918 had no effect on the ratio of drug concentration in milk to plasma but did change the brain to plasma ratio. Nelfinavir, like other human immunodeficiency virus protease inhibitors, is a well established substrate of P-gp (Kim et al., 1998; Lee et al., 1998; Washington et al., 1998). These protease inhibitors have been shown to be inhibitors but not substrates of Abcg2 (Gupta et al., 2004). This evidence suggests that P-gp does not play a role in drug transfer into rat milk. By contrast, nitrofurantoin has not been shown to be a substrate of other major efflux transporters, such as P-gp, Mrp1, and Mrp2 (Merino et al., 2005; Wang and Morris, 2007). Hence, the effect of GF120918 on the clearance and M/S of nitrofurantoin is not mediated by P-gp but largely the result of Abcg2 inhibition. However, it should be noted that a study of membrane transporters in MDCKII-human multidrug-resistant (MDR) 1 cells using GF120918 to inhibit digoxin and loperamide efflux implicated a possible basolateral transporter(s) that could be inhibited by GF120918 (Acharya et al., 2008). The identity or function of basolateral transporter(s) on lactating mammary epithelial cells and their potential impact on M/S has not been established.

Consistent with mammary gland expression in other species (Merino et al., 2005), our Western blotting results show Abcg2 is highly expressed in the lactating rat. P-gp seems to be transcriptionally down-regulated during lactation (Alcorn et al., 2002), and protein expression was not detectable in the lactating rat mammary gland (Fig. 2A). All the native Abcg2 bands were reduced to approximately 60 kDa after deglycosylation. This observation is consistent with the...
result previous work suggesting that ABCG2 contains N-linked oligosaccharides, probably at both the putative glycosylation sites (Litman et al., 2002). The native Abcg2 bands in kidney and rat Abcg2-MDCKII cell line had a larger size (approximately 80 kDa) than the mammary gland (approximately 70 kDa), suggesting rat Abcg2 may have tissue- or cell-specific patterns of glycosylation.

Consistent with the Western blot analysis, immunohistochemistry clearly indicated that Abcg2 is strongly expressed on the apical side of epithelial cells in the lactating mammary gland. Apical expression also confirms the role of Abcg2 in actively transporting nitrofurantoin out of the epithelial cells and into rat milk.

The rat is a frequently used animal model in drug development and may be a better animal model under certain experimental paradigms (e.g., multiple samples, crossover study design). Furthermore, drugs identified as accumulating in rat milk by active transport are consistent with human literature (McNamara et al., 1991, 1992). Hence, evidence gathered to date suggests that the rat is a good model for identifying which drugs might undergo active transport into human milk.

As noted in the introduction, genetic knockout models are useful tools to show transporter function with the assumption that the only difference from wild-type is the abolishment of expression of the intended protein. However, knockout models have been found to undergo additional changes that might further complicate interpretation. For example, brain microvessels from Mdr1a-deficient mice express 3 times more Abcg2 RNA than microvessels of wild-type mice (Cisternino et al., 2004). Although the accumulation of Abcg2 substrates mitoxantrone and prazosin was significantly increased in the presence of GF120918 in both wild-type and P-gp-deficient mice, there was significant difference between them (Cisternino et al., 2004). Whether this increased expression and activity of Abcg2 is a compensatory mechanism for the lack of Mdr1a at the mouse blood-brain barrier remains to be elucidated. There are other compensatory functions that also may be present in genetic knockout animals. In Mrp2 (−/−) mice, Mrp4 mRNA and protein expression in liver and kidney were increased approximately 6- and 2-fold, respectively (Chu et al., 2006). In Mrp2-deficient rats, Mrp3 was significantly up-regulated in the liver (approximately 6-fold) and kidney (approximately 3.5-fold) compared with wild-type controls. Likewise, the expression of the Ugt1 enzyme was increased in the liver and kidney of Mrp2-deficient rats by approximately 3.5- and 5.5-fold (Johnson et al., 2006). Therefore, alterations in drug disposition using knockout
The inhibition of Abcg2-associated nitrofurantoin accumulation by GF120918 in the lactating rat (chemical knockout) provides support for the use of this alternative to the genetic knockout model for establishing the role of Abcg2 in drug active transport into milk. GF120918 has been tested in phase I clinical trials and was confirmed to have little toxicity (Planting et al., 2005; Kuppens et al., 2007). Various studies have shown that GF120918 can be tolerated in humans and animals at concentrations sufficient to inhibit ABCG2. Hence, GF120918 is suitable for in vivo animal and perhaps even clinical studies (Mao and Unadkat, 2005).

In summary, Abcg2, but not P-gp, is expressed in lactating mammary epithelium and seems to be responsible for nitrofurantoin active transport into rat milk. The rat seems to be a good animal model for identifying those drugs that would accumulate in human milk. GF120918 seems to be an effective in vivo inhibitor (chemical knockout) tool for confirming the role of Abcg2 in milk transfer. These data are especially valuable for the design of studies of ABCG2 substrates during breastfeeding using the rat as a model.

References


