Pharm Res (2010) 27:2098–2105
DOI 10.1007/s11095-010-0208-5

RESEARCH PAPER

In Vivo Inhibition of BCRP/ABCG2 Mediated Transport of Nitrofurantoin by the Isoflavones Genistein and Daidzein: A Comparative Study in Bcrp1 $^{-/-}$ Mice

Gracia Merino • Miriam Perez • Rebeca Real • Estefania Egido • Julio G. Prieto • Ana I. Alvarez

Received: 29 January 2010 / Accepted: 28 June 2010 / Published online: 7 July 2010

ABSTRACT

Purpose The aim of this study was to determine *in vivo* inhibition by the isoflavones genistein and daidzein of nitrofurantoin (NTF), a well-known substrate of the ABC transporter BCRP/ABCG2.

Methods MDCKII cells and their human BCRP- and murine Bcrp1-transduced subclones were used to establish inhibition in transepithelial transport assays. Bcrp1 $^{-/-}$ and wild-type mice were coadministered with nitrofurantoin (20 mg/kg) and a mixture of genistein (100 mg/kg) and daidzein (100 mg/kg).

Results Transepithelial NFT transport was inhibited by the isoflavones. Plasma concentration of NTF at 30 min was 1.7-fold higher ($p \leq 0.05$) in wild-type mice after isoflavone administration. AUC values were not significantly different. BCRP/ABCG2-mediated secretion into milk was inhibited since milk/plasma ratios were lower in wild-type mice with isoflavones (7.1 ± 4.2 *vs* 4.2 ± 1.6, $p \leq 0.05$). NTF bile levels were significantly decreased by isoflavone administration in wild-type animals (8.8 ± 3.4 μg/ml with isoflavones *vs* 3.7 ± 3.3 μg/ml without isoflavones).

Conclusion Our data showed that *in vivo* interaction of high doses of soy isoflavones with BCRP substrates may affect plasma levels but the main effect occurs in specific target organs, in our case, liver and mammary glands.

KEY WORDS BCRP/ABCG2 · Bcrp1 $^{-/-}$ · isoflavones · MDCKII cells · nitrofurantoin

M. Perez · R. Real · J. G. Prieto · A. I. Alvarez (✉)
Departamento de Ciencias Biomedicas -Fisiologia,
Facultad de Veterinaria, Universidad de Leon
24071 Leon, Spain
e-mail: aialvf@unileon.es

G. Merino · M. Perez · E. Egido · A. I. Alvarez
Instituto de Sanidad Animal y Desarrollo Ganadero
Universidad de León
León, Spain

INTRODUCTION

BCRP, the protein encoded by the ABCG2 gene, may play a significant role in the disposition and pharmacological activity of a broad range of compounds and in the development of multidrug resistance in cancer (1,2). Consistent with this notion, a plethora of studies on the tissue distribution of ABCG2, its expression and activity, as well as its pharmacokinetic interaction, have been performed (1–3). BCRP/ABCG2 protein is expressed mainly in the apical membrane of cells in tissues with secretory functions (liver, kidney, intestine, breast), apical membranes of capillary vessels in the blood-brain barrier and apical membrane of trophoblasts (2,4). Studies on knock-out mice have indicated that Bcrp1 plays an important role in the milk transport of drugs and xenotoxins, chemotherapeutic agents such as topotecan, cimetidine, fluoroquinolones and vitamins (5–8). Nitrofurantoin, a nitrofuran-derivate antibacterial agent widely used as a urinary tract antibiotic prescribed for lactating women, is actively extruded into milk by BCRP/ABCG2 (9,10). The relevance of nitrofurantoin-BCRP/ABCG2 interaction is evidenced through reports such as the effect of pregnancy on nitrofurantoin disposition (11), nitrofurantoin fetal distribution (12), nitrofurantoin hepatobiliary excretion and sex difference (13), all of them performed on Bcrp1$^{-/-}$ mice, and also as a rat chemical knock-out model (10). Genistein and daidzein are isoflavones, polyphenolic compounds belonging to the flavonoid class; they are abundant in soybeans, represent the major active components in soy products, although the amount of each isoflavone can vary depending on the soy supplement, and appear as a mixture in red clover. High intake of soy has been associated with a variety of beneficial effects in several common diseases (14,15). A number of studies have demonstrated inhibition of drug transporters by flavonoids (16,17). The effect of flavonoids

on the pharmacokinetics of nitrofurantoin has been reported in rats by Wang and Morris (18); these authors showed that oral administration of chrysin (at the high dose of 200 mg/kg) can modify the pharmacokinetics profile of nitrofurantoin. Dihydroflavone is one of the most potent BCRP inhibitors (19). However, neither chrysin nor the flavonoid 7,8- benzoflavone significantly altered the pharmacokinetics of topotecan, a Bcrp1 substrate, in rats or mdr1a/1b$^{-/-}$ (20). A novel recent study on the effect of flavonoid chrysin on nitrofurantoin pharmacokinetics showed that this interaction occurs in rats but not in mice possibly due to the higher levels of BCRP in the small intestine in rats compared with mice (21). We obtained positive results regarding the role of exogenous isoflavones' administration in milk secretion of nitrofurantoin in ewes, but interindividual variation made the interpretation of results difficult (22).

The objective of this study was to clarify the proof-of-concept previously observed related to the specific role of BCRP inhibition by isoflavones and its consequences in drug excretion in milk. In addition, the role of soy isoflavones in transport pharmacokinetics and bile and milk secretion of the antibiotic NFT will be assessed. In order to achieve this goal, the effects of genistein and daidzein on human BCRP and murine Bcrp1-mediated transport of nitrofurantoin using polarized cell lines was studied. The *in vivo* interaction between genistein-daidzein and NFT was studied through nitrofurantoin concentration in plasma, bile and milk in Bcrp1$^{-/-}$ and wild-type mice.

MATERIAL AND METHODS

Reagents and Drugs

Nitrofurantoin was purchased from Sigma Chemical Co. (Steinheim, Germany), Genistein and daidzein were purchased from LC Laboratories (PKC Pharmaceuticals, Inc Woburn Ma USA), Isoflurane (Isovet®) from Schering-Plough (Madrid, Spain), and oxytocin (Oxiton®) from Ovejero (León, Spain). Ko143 has been previously described (23) and was kindly provided by Dr. A.H. Schinkel, Netherlands Cancer Institute (Amsterdam, The Netherlands). All the other chemicals were analytical grade and available from commercial sources.

Animals

Female lactating mice were housed and handled according to procedures approved by the Research Committee of Animal Use of the University of León (Spain) and carried out according to the "Principles of Laboratory Animal Care" and the European guidelines described in the EC Directive 86/609. The animals used in the experiments were Bcrp1$^{-/-}$ and wild-type mice (9-14 wk), all of >99% FVB genetic background. The Bcrp1$^{-/-}$ mice were kindly supplied by Dr. AH Schinkel from the Netherlands Cancer Institute. Animals were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle. They received a standard rodent diet (Panlab SA, Barcelona, Spain), and water was available *ad libitum*.

Cell Cultures

The polarized canine kidney cell line MDCK-II was used in the transport assays. Human BCRP- and murine Bcrp1-transduced MDCK-II subclones have been previously described (5,7). The MDCK-II cells and transduced subclones were kindly supplied by Dr. AH Schinkel from the Netherlands Cancer Institute. The cells were cultured in DMEM containing glutamax (2 mML-alanyl-L-glutamine) (Life Technologies, Inc.) and supplemented with penicillin (50 units/ml), streptomycin (50 µg/ml), and 10% (v/v) fetal calf serum (Life Technologies, Inc.) at 37 °C in the presence of 5% CO_2. The cells were trypsinized every 3 to 4 days for subculturing.

Transport Studies

Transport assays were carried out as described by Merino *et al.* (9), with minor modifications. Cells were seeded on microporous membrane filters (3.0 µm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY) at a density of 1.0×10^6 cells per well. Cells were grown for 3 days, and the medium was replaced daily. Transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore, Bedford, MA); wells registering a resistance of 200 ohms or greater, after correcting for the resistance measured in control blank wells, were used in the transport experiments. The measurement was repeated at the end of the experiment to check the integrity of the monolayer. Two hours before the start of the experiment, medium on both the apical and basolateral sides of the monolayer was replaced by 2 ml of Optimem medium (Life Technologies, Inc.), without serum, either with or without 1 µM Ko143, and the isoflavones at different concentrations: Gen (50 µM), Daid (50 µM), Gen(50 µM)-Daid (50 µM), or Gen(100 µM)-Daid(100 µM). The experiment was started ($t=0$) by replacing the medium in either the apical or basolateral compartment with fresh Optimem medium, either with or without Ko143, or the different concentrations of flavonoids, and 10 µM nitrofurantoin content. Aliquots of 100 µl were taken at $t=2$ and 4 h and stored at –20°C until HPLC analysis. The appearance of nitrofurantoin in the acceptor compartment was presented as the fraction of total nitrofurantoin added to the donor compartment at the beginning of the experiment. The

apparent permeability coefficient was calculated as follows: $P_{app} = (\Delta Q/\Delta t) \times [1/(A \times C_0)]$, where $\Delta Q/\Delta t$ is the rate of nitrofurantoin appearing in the receiver chamber, which was obtained from the slope of the regression line on the transport-time profile of nitrofurantoin across MDCKII cell monolayers, C_0 is the initial concentration of nitrofurantoin loaded in the donor chamber, and A is the cell monolayer surface area (4.71 cm^2).

The quotient Papp Basolateral-Apical (BL-AP) /Papp Apical-Basolateral (AP-BL) was used as Secretory/Absorptive ratio.

Plasma and Milk Levels of Nitrofurantoin

Nitrofurantoin and isoflavones (Gen-Daid) were administered intragastrically to wild and Bcrp1$^{-/-}$ lactating female mice by oral gavage feeding in 4-h-fasted mice, as a solution of 25% ethanol, 25% polyethylenglycol and 50% saline. The oral administration consisted of 100 µl of solution per 30 g body weight.

The mixture Gen(100 mg/kg)-Daid(100 mg/kg) was administered 5 min before NTF (20 mg/kg). The dose administered was 20 mg/kg of NTF in order to make sure of its secretion into milk after oral administration. Blood was collected by orbital bleeding after anesthesia with isoflurane at different time points (10, 30, 60 and 120 min). Two time-points were obtained from each animal. Heparinized blood samples were centrifuged immediately at 1000×g for 10 min. For milk experiments, pups of approximately 10 days old were separated from their mother approximately 4 h before the start of the experiment. Oxytocin (200 µl of 1 I.U./ml solution) was administered subcutaneously to lactating dams in order to stimulate milk secretion. Milk was collected at t=30 min from the mammary glands by gentle vacuum suction. At the end of the experiment mice were subsequently killed by cervical dislocation, and the bile in the gall-bladder was obtained and weighed (24). The samples collected from plasma, milk and bile were stored at –20°C until HPLC analysis. Between 4 and 7 animals were used for each experiment.

HPLC Analysis

The conditions for HPLC analysis of nitrofurantoin were modified based on a previously published method (9). Samples were thawed and kept protected from light in brown Eppendorf tubes during preparation. To each 50-µl aliquot of sample, 5 µl of a 12.5 µg/ml furazolidone solution was added as an internal standard in a 1.5-ml reaction tube. The mixture was vortexed vigorously, and 50 µl of methanol at –20°C was added for protein precipitation. Extraction was carried out by vigorously shaking the reaction tube for 60 s and incubating at –30°C for 15 min. The organic and water phases were separated by centrifugation at 16,000 g for 5 min at 4°C, and 50 µl of the organic phase was injected into the HPLC system.

Separation was performed at 30°C on a reversed-phase column (Nucleosil 120 C18, 10-µm particle size, 250×4 mm), preceded by a precolumn cartridge. The composition of the mobile phase was 25 mM potassium phosphate buffer, pH 3/acetonitrile (75:25). The flow rate of the mobile phase was set to 1.2 ml/min. UV absorbance was measured at 366 nm. Peak area ratios (nitrofurantoin/furazolidone) were used for comparison with the standard curve. Standard samples in the appropriate drug-free matrix were prepared at concentrations ranging from 1 to 120 µg/ml.

Statistical Analysis

Statistical analysis for significant differences was performed using the ANOVA LSD test. A probability of $p<0.05$ was considered to be statistically significant.

RESULTS

Effects of Genistein and Daidzein on *In Vitro* Transport of Nitrofurantoin

It is known that the flavonoids genistein and daidzein interact as inhibitors of BCRP-mediated efflux of mitoxantrone (19,25). However, the effects of genistein and daidzein on BCRP-mediated efflux of nitrofurantoin have not been evaluated. Therefore, we first looked at the effects of flavonoids on nitrofurantoin transport in both MDCK parental cells (MDCK-PAR) and MDCK cells expressing either murine Bcrp1 (MDCK-Bcrp1) or human BCRP (MDCK-BCRP).

As can be seen in Fig. 1, and reported by Merino *et al.* (9), the polarized transport of nitrofurantoin (10 µM) was observed in both MDCK-Bcrp1 and MDCK-BCRP cells in the absence of inhibitors. NTF is a good Bcrp1/BCRP substrate with secretory/absorptive ratios of 6.63±0.96 and 3.80±0.81 in MDCK-Bcrp1 and MDCK-BCRP, respectively, while the ratio was 0.57±0.06 in the parental cells. This ratio below 1 in the MDCK-II parental cell line suggests low endogenous basally directed transport (9). When the specific Bcrp1/BCRP inhibitor Ko143 (1 µM) was used, the Bcrp1/BCRP-mediated transport was completely inhibited, resulting in vectorial transport patterns similar to those of the parent MDCK-II cells.

Our results showed that the BL-to-AP transport of NTF (10 µM) was significantly inhibited by the presence of isoflavones. The BL-AP/AP-BL ratios in presence of Gen (50 µM)-Daid(50 µM) decreased significantly with respect to

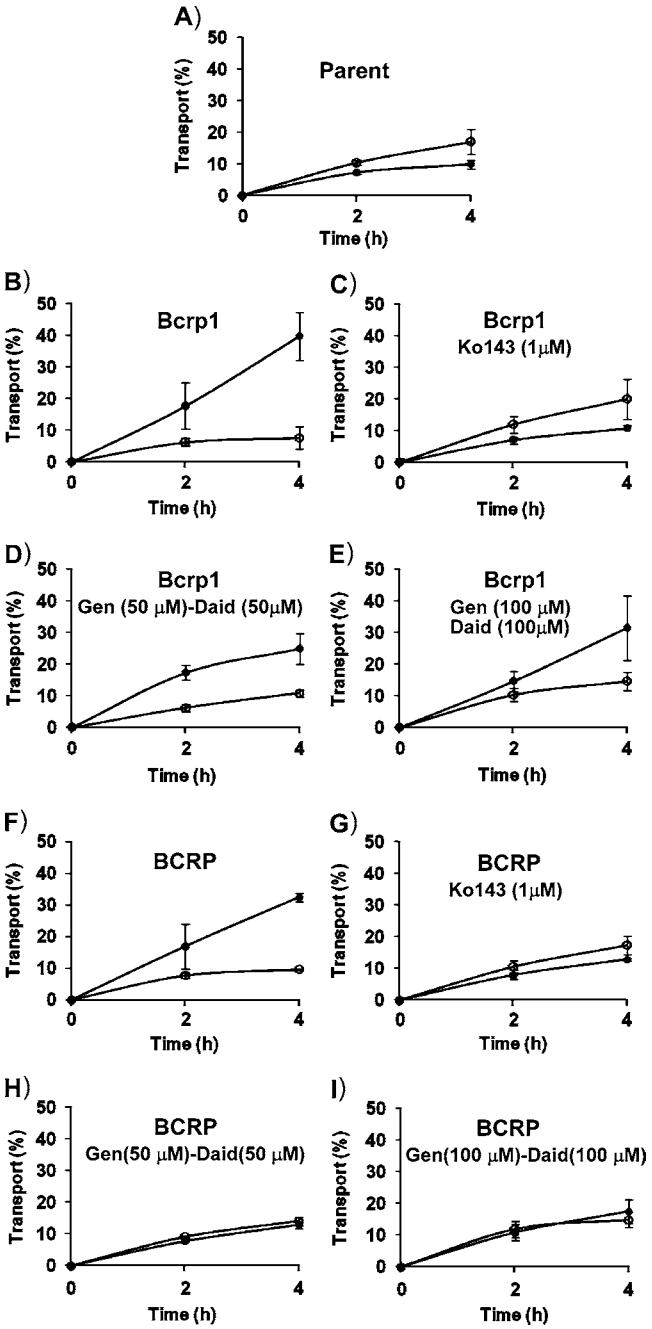

Fig. 1 Transwell transport of nitrofurantoin. Transepithelial transport of 10 μM nitrofurantoin in MDCKII (parent) (A), MDCKII-Bcrp1 (B) and MDCKII-BCRP (F), monolayers. The experiment was started with the addition of nitrofurantoin to one compartment (basolateral or apical). After 2 and 4 h, the percentage of drug in the opposite compartment was measured by HPLC and plotted. BCRP inhibitors, Ko143 (C and G), Gen (50 μM)-Daid (50 μM) (D and H) and Gen (100 μM)-Daid (100 μM) (E and I) were present as indicated. Results are the means; error bars (sometimes smaller than the symbols) indicate the standard deviations ($n=3$). ●, transport from the basolateral to apical compartment; ○, transport from the apical to basolateral compartment.

experiments without isoflavones (2.30±0.39 in MDCK-Bcrp1, 0.91±0.03 MDCK-BCRP). The same significant effects were shown when Gen (50 μM) (BL-AP/AP-BL ratios of 2.55±0.27 in MDCK-Bcrp1, 0.91±0.03 in MDCK-BCRP) and Gen(100 μM)-Daid(100 μM) (BL-AP/AP-BL ratios of 2.11±0.35 in MDCK-Bcrp1, 0.80±0.70 in MDCK-BCRP) were added. No effects were seen in the parental cell line. These results suggest that isoflavones genistein and daidzein indeed inhibit both human BCRP and murine Bcrp1-mediated transport of nitrofurantoin in MDCKII cells.

Effects of Genistein and Daidzein on Nitrofurantoin Plasma and Milk Levels in Bcrp1$^{-/-}$ and Wild-Type Mice

In order to attempt to establish a potential correlation between *in vitro* and *in vivo* results, we determined inhibition by isoflavones (genistein and daidzein) of NTF disposition mediated by the Bcrp1 protein in wild-type mice compared with Bcrp1 knock-out mice. Results showed that plasma concentrations of NTF were significantly higher in wild-type mice after isoflavone administration at 30 min (Table I). Plasma concentration at 30 min of NTF was unmodified in Bcrp1$^{-/-}$ in both experimental conditions, and their values were in the range obtained from wild-type after isoflavone administration. AUC values did not show significant differences after isoflavone treatment.

Regarding milk experiments (Fig. 2), despite the significantly higher plasma level, the concentration of NFT into milk in wild-type mice after the dose of isoflavones was significantly lower than the control wild-type mice group (55.3±21.1 μg/ml *vs* 93.5±19.8 μg/ml $p\leq0.05$). Significant inhibition of active transport of nitrofurantoin in milk was reflected in milk/plasma ratios that were significantly diminished in wild-type lactating females with isoflavones: 4.2±1.6 (NTF+Gen-Daid) *vs* 7.1±4.2 (NTF, control), $p\leq0.05$. (Fig. 2). NTF excretion into milk in knockout was very low, as was expected, since Bcrp1 is the main mechanism involved in secretion into milk (9,10), with no differences between the two experimental groups

Table I Effect of Genistein and Daidzein on Plasma Nitrofurantoin Concentration in Female Lactating Wild-Type and Bcrp1$^{-/-}$

Time (min)	Wild-type	Wild-type Gen-Daid	Bcrp1$^{-/-}$	Bcrp1$^{-/-}$ Gen-Daid
10	17.9±3.5	16.1±3.5	26.4±7.7	18.3±2.5
30	10.4±2.1	17.6±7.7*	22.4±13.9*	21.4±7.0*
60	9.3±2.2	9.1±2.8	15.3±2.4*	17.8±7.2
120	6.6±0.5	6.5±0.4	9.9±2.6*	10.5±2.2*
$AUC_{(0\text{-}120)}$	19.1±2.0	21.4±3.4	32.4±7.5*	32.1±7.2*

Plasma samples were obtained after oral administration of nitrofurantoin (20 mg /kg) with or without isoflavones (Genistein 100 mg/kg-Daidzein 100 mg/kg). Values of nitrofurantoin concentration (μg/ml) and AUC (μg/ml h) are means±SD.

* $p<0.05$ significantly different from wild-type without isoflavones

(Fig. 2). These results indicate that isoflavones interact with nitrofurantoin *in vivo*.

In addition, Bcrp1 plays a predominant role in the hepatobiliary excretion of NFT (13,18). Inhibition by isoflavones of bile excretion of NTF was shown since NTF levels in bile were significantly decreased by isoflavone administration: 8.8±3.4 μg/ml in wild-type (NTF, control) *vs* 3.7±3.3 μg/ml in wild-type mice (NTF+Gen-Daid), $p \leq 0.05$ (Fig. 3). In Bcrp1$^{-/-}$ mice, as expected, the levels of NTF were very low (0.64±0.19 μg/ml in the control group and 0.46±0.44 μg/ml after isoflavone administration) without significant differences.

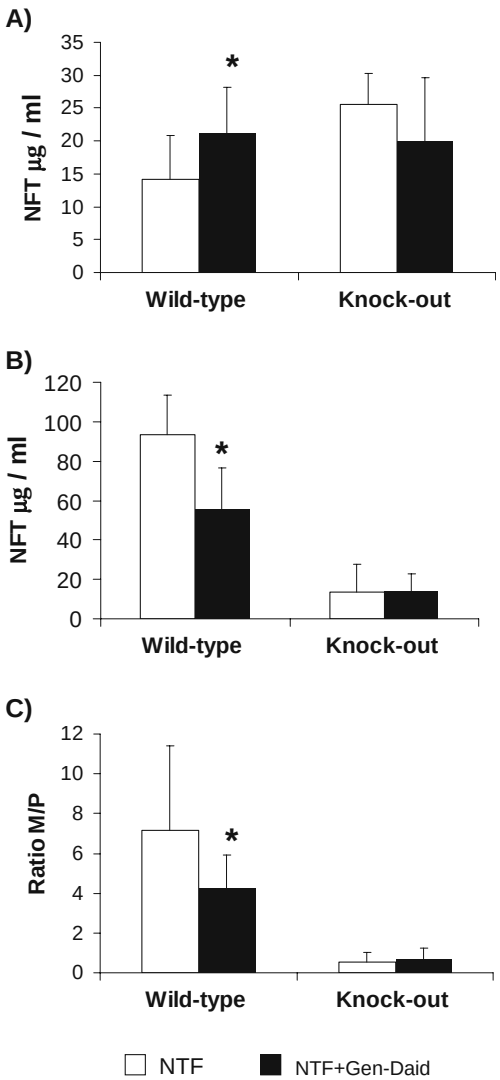

Fig. 2 Plasma (**A**) and milk concentration (**B**) and milk-to-plasma ratio (**C**) of nitrofurantoin in wild-type and Bcrp1$^{-/-}$ lactating female mice. Nitrofurantoin (20 mg/kg) was administered p.o. to mice 5 min after Gen (100 mg/kg)-Daid (100 mg/kg) oral administration. Milk and plasma were collected after 30 min and analyzed by HPLC. Each bar represents the mean ± SD of 3-4 experiments. * denotes a significant $p \leq 0.05$ difference between control and isoflavone administration in wild-type mice.

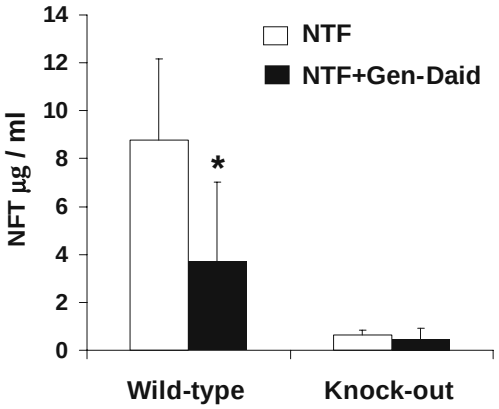

Fig. 3 Bile concentration of nitrofurantoin in wild-type and Bcrp1$^{-/-}$ lactating female mice. Bile was collected after 30 min and analyzed by HPLC. Each bar represents the mean ± SD of 3-4 experiments. * denotes a significant $p \leq 0.05$ difference between control and isoflavone administration in wild-type mice.

These results accurately support the *in vivo* modulation of Bcrp1 by isoflavones such as genistein and daidzein when the drug, such as nitrofurantoin, is a good substrate of this transporter.

DISCUSSION

The growing interest in flavonoids has resulted in a dramatic increase in the consumption of flavonoid-containing products in the general population for health maintenance and disease prevention, but evidence from animal and human studies has indicated that flavonoids may interact with many clinically important drugs, causing favourable or adverse pharmacokinetic interactions. Some flavonoid-drug interactions are mediated by major efflux drug transporters (17).

In spite of the results showing *in vitro* interaction of flavonoids with efflux transporters (19,25), there is a disconnection between *in vitro* and *in vivo* drug inhibition (20,26,27). In this respect, it is important to consider the potential species differences with regard to flavonoid-BCRP interaction (20,21), and the fact that this interaction could depend on the substrate (28), as well as the effects that these compounds may have on other transporters and enzymes that may be important in the disposition of a drug (29). Mouse Bcrp1 shares only 81% amino acid identity with human BCRP (30), and even a single amino acid mutation at position 482 of BCRP was shown to significantly alter BCRP substrate and antagonist specificity (2). In corroboration of this, results from the inhibition of nitrofurantoin transport by chrysin administration have shown that the inhibition profile, for the same transporter, is different in mice and rats (21). Even our *in vitro* data also reveal a species difference regarding the effect of genistein and daidzein on nitrofurantoin transport. In this case, inhibition is higher in cells transduced with human

BCRP than the ones with mouse Bcrp1. This species differential inhibition interaction has been previously reported by Wang and Morris (18) with NTF and chrysin.

Xu *et al.* (31) add a new element of discussion since efflux transporters Mrp2 and Bcrp1 are shown to compensate for each other and enable the intestinal excretion of flavonoids (naringenin) and presumably other BCRP substrates such as topotecan. The existence of a compensatory mechanism in Bcrp1$^{-/-}$ pregnant mice has also been argued by Zhang *et al.* (11) to explain the low AUC values for nitrofurantoin.

Our *in vitro* results show that isoflavones' main BCRP-mediated inhibition occurs in cells transduced with human BCRP where concentrations of 50 μM of genistein and 50 μM daidzein completely inhibited nitrofurantoin transport. Notwithstanding these *in vitro* results, the *in vivo* inhibition in mice of nitrofurantoin mediated by genistein and daidzein is very significant, and presumably *in vivo* inhibition of BCRP-mediated NFT transport in humans could occur with high doses of isoflavones.

Our results clearly show that soy isoflavones, genistein and daidzein, through Bcrp1 inhibition, significantly diminished the excretion of nitrofurantoin into milk. The BCRP/ABCG2 function in drug excretion and in drug transfer into milk has been demonstrated specifically with nitrofurantoin. Bcrp1 plays a major role in the secretion of nitrofurantoin into milk, as indicated by large differences in milk-to-plasma ratios between wild-type and Bcrp1$^{-/-}$ mice; in fact, in the absence of Bcrp1, the only remaining component of the transport is passive diffusion (9). Our results, in agreement with those previously published, showed a large difference in the concentration of nitrofurantoin into milk between wild-type and Bcrp1$^{-/-}$ (9-fold higher in the wild-type mice), but we also observed a significant difference between wild-type animals with or without isoflavones, the excretion of NTF into milk being reduced two-fold. These results agree with our preliminary results obtained from lactating sheep (22,32), thus confirming that when a drug is a specific substrate of BCRP, milk secretion can be modulated by these isoflavones.

Lactating mice have a high expression of BCRP in the mammary gland, as do other mammalian species (man, sheep, cow) (6,32), but BCRP expression elsewhere in the mouse body (liver, kidney, small intestine) is not altered during pregnancy and lactation (13). The decreased content of nitrofurantoin in bile in the presence of daidzein and genistein highlights the magnitude of such isoflavone inhibition. Bcrp1 expression in the liver of female mice has been shown to be significantly lower than in male mice, and the role of Bcrp1 in hepatobiliary excretion of NTF in female wild-type mice was almost completely diminished compared to male wild-type mice (13). However, we observed a significant decrease in NTF levels in bile in the lactating wild-type group with isoflavones: 3.7±3.2 μg/ml *vs* 8.9±3.3 μg/ml in lactating wild-type control. This indicates that even with relatively low BCRP levels, isoflavones can effectively inhibit BCRP transport (Fig. 3).

The absence of variations in plasma, bile and milk levels in Bcrp1$^{-/-}$ in the presence of isoflavones underlines the specific participation of the Bcrp1 protein in inhibition.

Reports on nitrofurantoin-Bcrp1 interaction have highlighted the important contribution of Bcrp1 to NFT intestinal elimination, but intestinal degradation and drug enterohepatic circulation could be important contributors to plasma levels (9). Therefore, inhibiting Bcrp1 by genistein and daidzein might also possibly interfere with nitrofurantoin enterohepatic circulation.

Both isoflavones, genistein and daidzein, act as Bcrp inhibitors *in vitro* and *in vivo* (33), although *in vivo* they are extensively metabolized to glucuronide and sulphate forms (34). In fact, in rats considerable conjugation activity of genistein has been shown to result in a very low amount in blood circulation as unconjugated compound (35), resulting in low bioavailability (36). Presumably, therefore, part of the nitrofurantoin-Bcrp1 interaction could involve conjugated forms (34).

The effect of other transporters or enzymes on this reported interaction should not be discounted. Nitrofurantoin is a selective substrate for BCRP/Bcrp1, but not for P-gp and MRP2 (9,11). CYP1A mediates the metabolism of nitrofurantoin, although this does not represent a major metabolic pathway (18). Isoflavones such as genistein and daizdein do not induce CYPs in either the transcriptional step or through post-transcriptional mRNA (37). On the other hand, the lack of variation in nitrofurantoin levels in knockout mice after isoflavone administration supports the specificity of the interaction with Bcrp1.

Our results reveal that high doses of the main isoflavones present in soy (genistein and daidzein) may be able to modify the disposition of BCRP/ABCG2 substrates. For clinical implications of our findings, physiological levels of isoflavones in diet should be taken into account. It has been shown that plasma levels that might be reached from a dose of 50 mg of either daidzein or genistein, as typically used in intervention studies (ranging from 37–128 mg per person per day), yield a peak plasma concentration of ~2 μmol/L at ~6 h (14,38). The glycosides are not present in plasma, and most of the isoflavones are conjugated as sulfates or glucuronides (38). Many *in vitro* studies report inhibition concentrations for BCRP interaction at this concentration range (19,25). However, *in vivo* correlation of these data is very controversial (20,26,27) and points to the possibility that high doses are needed to show an *in vivo* interaction. The plasma free-fraction of isoflavones is relatively low, and it can be speculated that concentrations of isoflavones were lower than IC_{50}, although it is known that conjugated forms are also active in BCRP interaction (39), and active deconjugation could occur in the relevant tissues (25). Recently, IC_{50} values of 0.24 μM and

2.4 μM for genistein and daidzein respectively, have been reported and that the inhibitory potency of daidzein was decreased 100-fold by 7-glucuronidation but virtually unaffected by 4'-sulfation (39). Enokizono (33) reported that a tissue-to-plasma concentration of genistein and daidzein of 1.5 nM in mice at steady state can interact *in vivo* with Bcrp1 in brain, testis, epididymus and fetus. In humans after intake of soy milk and soy supplements, isoflavones (genistein and daidzein) reach exposure levels in breast tissue at which potential health effects may occur (40). All this suggests that protein unbound fractions of genistein and daidzein aglycones and metabolites reach target tissues where they might be clearly active and affect Bcrp1 function. Considering dose translation from mice to humans based on body surface area (BSA) (41), our doses are approximately equivalent to 16 mg/kg in humans. Using body weight to scale up, our doses give an equivalent dose in humans of 14000 mg (200 mg/kg, 100 mg/kg genistein plus 100 mg/kg daidzein). Experimental works reporting aglycone and total plasma concentrations in mice and humans (42,43) indicate that aglycone concentrations are in the same range for both species after ingestion of similar doses of isoflavones. In any case, one should consider that there are significant interspecies differences in isoflavone metabolism (44). For instance, genistein is metabolized more rapidly in mice than in humans (45). In addition, UDP-glucuronosyltransferases(UGTs)-efflux transporters coupling in both the intestine and the liver may result in enterohepatic circulation and enteric recycling, leading to longer duration, and thus accumulation of flavonoids in the body, in particular with repeated intake. Therefore, despite low oral bioavailabilities, flavonoids and some of their bioactive phase II conjugates may accumulate an adequate amount in the body to produce their pharmacological activities (34).

Current research clearly shows that the soy isoflavones genistein and daidzein, through Bcrp1 inhibition, significantly decrease the excretion of nitrofurantoin into milk, thereby opening up new perspectives for future research. BCRP/ABCG2 is expressed in the mammary gland and is induced in lactation. The mammary gland is a target organ of isoflavones, and their metabolites and isoflavones may interact with BCRP/ABCG2. Another of the most outstanding consequences of the interaction between BCRP/ABCG2 and isoflavones is related to the presence of drug residues in milk, since secretion can be modulated in a feasible manner. In addition, from a therapeutic point of view, soy presence in the diet of lactating females together with the administration of nitrofurantoin in infectious processes must be considered.

ACKNOWLEDGEMENTS

The authors thank Dr. A.H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands), who provided MDCK cells and their transduced cell lines, and Bcrp1 knockout mice. We wish to thank Dr. Juan Carlos Espín (CEBAS-CSIC, Murcia, Spain) for assistance in the revision of the manuscript. We are grateful to Prof. James McCue for assistance in language editing. This study was partially supported by the Ministry of Science and Technology (Spain) with the research projects AGL2006-13186, AGL2009-11730 and Ramon y Cajal grant to (GM), Junta de Castilla y León (Research grant LE028A08 to AIA, Research group of Excellence grant GR132 to JGP, predoctoral fellowship to EE) and Diputación de León (predoctoral fellowship to RR). The group is a member of the Network for Cooperative Research on Membrane Transport Proteins (REIT) (GrantBFU2007-30688-E/BFI).

REFERENCES

1. Ishikawa T, Nakagawa H. Human ABC transporter ABCG2 in cancer chemotherapy and pharmacogenomics. J Exp Ther Oncol. 2009;8(1):5–24.
2. Robey RW, To KK, Polgar O, Dohse M, Fetsch P, Dean M, *et al.* ABCG2: a perspective. Adv Drug Deliv Rev. 2009;61:3–13.
3. Hardwick LJA, Velamakanni S, van Veen HW. The emerging pharmacotherapeutic significance of the breast cancer resistance protein (ABCG2). Brit J Pharmacol. 2007;151:163–74.
4. Robey RW, Polgar O, Deeken J, To KKW, Bates SE. Breast Cancer Resistance Protein. In: You G, Morris ME, editors. Drug Transporters: molecular characterization and role in drug disposition. New Jersey: Wiley; 2007. p. 319–58.
5. Van Herwaarden AE, Wagenaar E, Karnekamp B, Merino G, Jonker JW, Schinkel AH. Breast cancer resistance protein (Bcrp1/Abcg2) reduce systemic exposure of the dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into breast milk. Carcinogenesis. 2006;27:123–30.
6. Jonker JW, Merino G, Musters S, van Herwaarden AE, Bolscher E, Wagenaar E, *et al.* The breast cancer resistance protein (BCRP/ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. Nat Med. 2005;11:127–9.
7. Merino G, Alvarez AI, Pulido MM, Molina AJ, Schinkel AH, Prieto JG. Breast cancer resistance protein (BCRP/ABCG2) transports fluoroquinolone antibiotics and affects their oral availability, pharmacokinetics, and milk secretion. Drug Metab Dispos. 2006;34:690–5.
8. van Herwaarden AE, Wagenaar E, Merino G, Jonker JW, Rosing H, Beijnen JH, *et al.* Multidrug transporter ABCG2/breast cancer resistance protein secretes riboflavin (vitamin B2) into milk. Mol Cell Biol. 2007;27:1247–53.
9. Merino G, Jonker JW, Wagenaar E, van Herwaarden AE, Schinkel AH. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic Nitrofurantoin. Mol Pharmacol. 2005;67:1758–64.
10. Wang L, Leggas M, Goswami M, Empey PE, McNamara PJ. 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 ATP-binding cassette transporter family G member 2 (Abcg2) knockout model to study nitrofurantoin transfer into milk. Drug Metab Dispos. 2008;36:2591–6.
11. Zhang Y, Zhou L, Unadkat JD, Mao Q. Effect of pregnancy on nitrofurantoin disposition in mice. J Pharm Sci. 2009;98:4306–15.
12. Zhang Y, Wang H, Unadkat JD, Mao Q. Bcrp1 Limits Fetal Distribution of Nitrofurantoin in the Pregnant Mouse. Drug Metab Dispos. 2007;35:2154–8.

13. Merino G, van Herwaarden AE, Wagenaar E, Jonker JW, Schinkel AH. Sex-dependent expression and activity of the ATP-binding cassette transporter breast cancer resistance protein (BCRP/ABCG2) in liver. Mol Pharmacol. 2005;67:1765–71.
14. Jacobs A, Wegewitz U, Sommerfeld C, Grossklaus R, Lampen A. Efficacy of isoflavones in relieving vasomotor menopausal symptoms - A systematic review. Mol Nutr Food Res. 2009;53:1084–97.
15. Hu M. Commentary: Bioavailability of Flavonoids and Polyphenols: Call to Arms. Mol Pharm. 2007;4:803–6.
16. Morris ME, Zhang S. Flavonoid-drug interactions: effects of flavonoids on ABC transporters. Life Sci. 2006;78:2116–30.
17. Alvarez AI, Real R, Perez M, Mendoza G, Prieto JG, Merino G. Modulation of the activity of ABC transporters (P-glycoprotein, MRP2, BCRP) by flavonoids and drug response. J Pharm Sci. 2010;99:598–617.
18. Wang X, Morris ME. Effects of the flavonoid chrysin on nitrofurantoin pharmacokinetics in rats: potential involvement of ABCG2. Drug Metab Dispos. 2007;35:268–74.
19. Zhang S, Yang X, Morris ME. Combined effects of multiple flavonoids on breast cancer resistance protein (ABCG2)-mediated transport. Pharm Res. 2004;21:1263–73.
20. Zhang S, Wang X, Sagawa K, Morris ME. Flavonoids chrysin and benzoflavone, potent breast cancer resistance protein inhibitors, have no significant effect on topotecan pharmacokinetics in rats or mdr1a/1b (-/-) mice. Drug Metab Dispos. 2005;33:341–8.
21. Kawase A, Matsumoto Y, Hadano M, Ishii Y, Iwaki M. Differential effects of chrysin on nitrofurantoin pharmacokinetics mediated by intestinal breast cancer resistance protein in rats and mice. J Pharm Pharm Sci. 2009;12:150–63.
22. Perez M, Real R, Mendoza G, Merino G, Prieto JG, Alvarez AI. Milk secretion of nitrofurantoin, as a specific BCRP/ABCG2 substrate, in assaf sheep: modulation by isoflavones. J Vet Pharmacol Ther. 2009;32:498–502.
23. Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, *et al.* Potent and specific inhibition of the breast cancer resistance protein multidrug transporter *in vitro* and in mouse intestine by a novel analogue of fumitremorgin C. Mol Cancer Ther. 2002;1:417–25.
24. Perez M, Blazquez AG, Real R, Mendoza G, Prieto JG, Merino G, *et al. In vitro* and *in vivo* interaction of moxidectin with BCRP/ABCG2. Chem Biol Interact. 2009;180:106–12.
25. Imai Y, Tsukahara S, Asada S, Sugimoto Y. Phytoestrogens/flavonoids reverse breastcancer resistance protein/ABCG2-mediated multidrug resistance. Cancer Res. 2004;64:4346–52.
26. Wang SW, Chen J, Jia X, Tam VH, Hu M. Disposition of flavonoids via enteric recycling: structural effects and lack of correlations between *in vitro* and *in situ* metabolic properties. Drug Metab Dispos. 2006;34:1837–48.
27. Zhang S, Sagawa K, Arnold RD, Tseng E, Wang X, Morris ME. Interactions between the flavonoid biochanin A and P-glycoprotein substrates in rats: *In vitro* and *in vivo* J. Pharm Sci. 2010;99:430–41.
28. Muenster U, Grieshop B, Ickenroth K, Gnoth MJ. Characterization of substrates and inhibitors for the *in vitro* assessment of BCRP mediated drug-drug interactions. Pharm Res. 2008;25(10):2320–6.
29. Telang U, Ji Y, Morris ME. ABC transporters and isothiocyanates: potential for pharmacokinetic diet-drug interactions Biopharm. Drug Dispos. 2009;30(7):335–44.
30. Allen JD, Brinkhuis RF, Wijnholds J, Schinkel AH. The mouse Bcrp1/Mxr/Abcp gene:amplification and overexpression in cell lines selected for resistance totopotecan, mitoxantrone, or doxorubicin. Cancer Res. 1999;59:4237–41.
31. Xu H, Kulkarni KH, Singh R, Yang Z, Wang SW, Tam VH, *et al.* Disposition of Naringenin via Glucuronidation Pathway Is Affected by Compensating Efflux Transporters of Hydrophilic Glucuronides. Mol Pharm. 2009;6:1703–15.
32. Pulido MM, Molina AJ, Merino G, Prieto JG, Alvarez AI. Interaction of enrofloxacin with breast cancer resistance protein (BCRP/ABCG2): Influence of flavonoids and role in milk secretion in sheep. J Vet Pharmacol Ther. 2006;29:279–87.
33. Enokizono J, Kusuhara H, Sugiyama Y. Effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens. Mol Pharmacol. 2007;72(4):967–75.
34. Zhang L, Zuo Z, Lin G. Intestinal and hepatic glucuronidation of flavonoids. Mol Pharm. 2007;4(6):833–45.
35. Zhou S, Hu Y, Zhang B, Teng Z, Gan H, Yang Z, *et al.* Dose-dependent absorption, metabolism, and excretion of genistein in rats. J Agric Food Chem. 2008;56(18):8354–9.
36. Moon YJ, Sagawa K, Frederick K, Zhang S, Morris ME. Pharmacokinetics and bioavailability of the isoflavone biochanin A in rats. AAPS J. 2006;8(3):E433–442.
37. Kishida T, Nagamoto M, Ohtsu Y, Watakabe M, Ohshima D, Nashiki K, *et al.* Lack of an inducible effect of dietary soy isoflavones on the mRNA abundance of hepatic cytochrome P-450 isozymes in rats. Biosci Biotechnol Biochem. 2004;68(3):508–15.
38. Williamson G, Manach C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. Am J Clin Nutr. 2005;81:243S–55S.
39. Tamaki H, Satoh H, Hori S, Ohtani H, Sawada Y. Inhibitory effects of herbal extracts on breast cancer resistance protein (BCRP) and structure-inhibitory potency relationship of isoflavonoids. Drug Metab Pharmacokinet. 2010;25:170–9.
40. Bolca S, Urpi-Sarda M, Blondeel P, Roche N, Vanhaecke L, Possemiers S, *et al.* Disposition of soy isoflavones in normal human breast tissue. Am J Clin Nutr. 2010;91:976–84.
41. Reagan-Shaw S, Nihal N, Ahmad M. Dose translation from animal to human studies revisited. FASEB J. 2007;22:659–61.
42. Andrade JE, Twaddle NC, Helferich WG, Doerge DR. Absolute bioavailability of isoflavones from soy protein isolate-containing food in female BALB/c mice. J Agric Food Chem. 2010;58:4529–36.
43. Setchell KD KD, Brown NM, Desai P, Zimmer-Nechemias L, Wolfe BE, Brashear WT, *et al.* Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. J Nutr. 2001;131:1362S–75S.
44. Gu L, House SE, Prior RL, Fang N, Ronis MJ, Clarkson TB, *et al.* Metabolic phenotype of isoflavones differ among female rats, pigs, monkeys, and women. J Nutr. 2006;136:1215–21.
45. Cimafranca MA, Davila J, Ekman GC, Andrews RN, Neese SL, Peretz J, *et al.* Acute and chronic effects of oral genistein administration in neonatal mice. Biol Reprod. 2010;83:114–21.