

**The Breast Cancer Resistance Protein (BCRP/ABCG2)
affects pharmacokinetics, hepatobiliary excretion and
milk secretion of the antibiotic nitrofurantoin**

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ABBREVIATIONS: ABC, ATP-binding cassette; AUC, area under the plasma concentration-time curve; BCRP, breast cancer resistance protein; HPLC, high performance liquid chromatography; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

ABSTRACT

Nitrofurantoin is a commonly used urinary tract antibiotic prescribed to lactating woman. It is actively transported into human and rat milk by an unknown mechanism. Recently, our group has demonstrated an important role of the breast cancer resistance protein (BCRP/ABCG2) in the secretion of xenotoxins into the milk. This ATP binding cassette drug efflux transporter extrudes xenotoxins from cells in intestine, liver, mammary gland and other organs, affecting the pharmacological and toxicological behavior of many compounds. We investigated whether Bcrp1 is involved in the pharmacokinetic profile of nitrofurantoin and its active secretion into the milk. Using polarized cell lines, we found that nitrofurantoin is efficiently transported by murine Bcrp1 and human BCRP. After oral administration of nitrofurantoin (10 mg/kg), the AUC in Bcrp1 knockout mice was almost 4-fold higher than in wild-type mice (148.8 ± 30.4 versus 37.5 ± 6.8 min. μ g/ml); and after i.v. administration (5 mg/kg), 2-fold higher (139.2 ± 22.0 versus 73.9 ± 9.0 min. μ g/ml). Hepatobiliary excretion of nitrofurantoin was almost completely abolished in Bcrp1 knockout mice (9.6 ± 3.2 % versus 0.2 ± 0.1 % in wild-type and Bcrp1 knockout mice, respectively). The milk to plasma ratio of nitrofurantoin was almost 80 times higher in wild-type compared to Bcrp1 knockout lactating females (45.7 ± 16.2 versus 0.6 ± 0.1). Nitrofurantoin elimination via milk was quantitatively even higher than hepatobiliary elimination. We conclude that Bcrp1 is an important determinant for the bioavailability of nitrofurantoin and the main mechanism involved in its hepatobiliary excretion and secretion into the milk.

Nitrofurantoin (1-[(5-nitro-2-furanyl)methylene]amino-2,4-imidazolidinedione) is a nitrofuran-derivative antibacterial agent widely used in human and veterinary medicine. In humans, it is mainly used to treat urinary tract infections, which are among the most common bacterial infections. Patients receiving nitrofurantoin may have rare but serious side effects such as chronic liver disease, cholestatic hepatitis, or hemolytic anemia in glucose-6-phosphate dehydrogenase-deficient patients (Gerk *et al.*, 2001a). Moreover, nitrofurantoin has been shown to be mutagenic and carcinogenic in animal models (Kari *et al.*, 1997). Further knowledge about the factors affecting the pharmacokinetics of nitrofurantoin is therefore of clinical and toxicological importance.

Nitrofurantoin is also prescribed to lactating women. Inadvertent transfer of drugs administered to the mother to milk is always a matter of concern in view of possible adverse effects in the infant. Nitrofurantoin is an inexpensive antibiotic that is often used in developing countries where formula feeding is not an alternative for breastfeeding (Kari *et al.*, 1997). However, preliminary results from a continuous breeding study with mice revealed that chronic nitrofurantoin treatment of lactating mice resulted in decreased pup growth rate (Kari *et al.*, 1997).

It has been demonstrated that nitrofurantoin is actively transported into human and rat milk, reaching milk-to-serum concentration ratios of 20 and 100 times those predicted by diffusion, respectively (Gerk *et al.*, 2001a,b). Furthermore, active, saturable transport of nitrofurantoin across a murine cell culture model of lactation has been demonstrated (Toddywalla *et al.*, 1997). However, the active transport mechanism involved has not been identified yet.

Breast cancer resistance protein (BCRP/ABCG2) is a member of the ATP-binding cassette (ABC) family of transporters (Doyle et al., 1998; Allen and Schinkel, 2002) that affects the pharmacological and toxicological behavior of many drugs and toxins. This 655 amino acid transmembrane protein transports a range of anticancer drugs, dietary compounds and food carcinogens like PhIP (Jonker et al., 2002; van Herwaarden et al., 2003). It actively extrudes its substrates from cells and it is localized in the apical membranes of intestinal and placental epithelia, and in the biliary canalicular membrane of hepatocytes, and in the blood-brain barrier. Several *in vivo* studies indicated that Bcrp1 limits the oral bioavailability and fetal and brain penetration, and mediates the hepatobiliary excretion and intestinal elimination of its (drug) substrates (Jonker et al., 2000; van Herwaarden et al., 2003; Cisternino et al., 2004).

Our group has recently demonstrated expression of mouse Bcrp1 and human BCRP in the lactating mammary gland and established an important role of Bcrp1 in the active secretion and concentration of several drugs and carcinogenic xenotoxins into milk (Jonker et al., 2005). Since nitrofurantoin is actively secreted into the milk (Gerk et al., 2001a,b), Bcrp1 is a good candidate for the mechanism involved in this phenomenon. In addition, there are indications for involvement of Bcrp1 in the renal excretion of some of its substrates (Mizuno et al., 2004). Since nitrofurantoin is extensively excreted into the urine of humans and is used to treat urinary infections, it was further interesting to investigate the role of Bcrp1 in this excretory pathway for nitrofurantoin.

In this study, we have demonstrated that nitrofurantoin is transported by Bcrp1/BCRP and that Bcrp1 is involved in the pharmacokinetic profile of nitrofurantoin

and its active secretion into the milk, applying *in vitro* and *in vivo* studies using Bcrp1 knockout mice.

Materials and Methods

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used were male or lactating female *Bcrp1*^{-/-} and wild-type mice, all of >99% FVB genetic background between 9 and 14 weeks of age. Animals were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle and received a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

Chemicals. Nitrofurantoin, furazolidone and xylazine were from Sigma Chemical Co. (St-Louis, MO); [³H]inulin was from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, United Kingdom); ketamine (Ketanest-S[®]) was from Parke-Davis (Hoofddorp, The Netherlands); oxytocin (Sintocinon[®]) was from Novartis (Basel, Switzerland); methoxyflurane (Metofane[®]) was from Medical Developments Australia Pty, Ltd. (Springvale, Victoria, Australia); Ko143 was described previously (Allen et al., 2002). All other compounds used were reagent grade.

Cells and tissue culture. The polarized canine kidney cell line MDCK-II was used in the transport assays. Human MDR1-, MRP2-, BCRP- and murine *Bcrp1*-transduced MDCK-II subclones were described previously (Jonker et al., 2000; Evers et al., 1998; Pavsek et al., 2004). The MDCK-II cells and transduced subclones were cultured in DMEM supplied with glutamax (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% CO₂. The cells were trypsinized every 3 to 4 days for subculturing.

Transport assays. Transport assays were carried out as described earlier (Huisman et al., 2001), 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 medium was replaced every day. Two hours before the start of the experiment, medium at both the apical and basolateral side of the monolayer was replaced with 2 ml of Optimem medium (Life Technologies, Inc.), without serum, either with or without 5 μM Ko143. 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 5 μM Ko143 and containing 10 μM nitrofurantoin and 192 nM [^3H]inulin. Cells were incubated at 37°C in 5% CO_2 and 50- μl aliquots were taken at $t = 2$ and 4 hours, and stored at -20°C until the time of analysis. The appearance of nitrofurantoin in the opposite compartment was measured by HPLC as described below, and presented as the fraction of total nitrofurantoin added at the beginning of the experiment. The tightness of the monolayer was measured by monitoring the paracellular flux of [^3H]inulin to the opposite compartment, which had to remain $<1.5\%$ of the total radioactivity/hour.

Pharmacokinetic experiments. For oral administration of nitrofurantoin (10 mg/kg), 3.3 μl of drug solution [appropriate concentration in 50% (v/v) ethanol, 50% (v/v) polyethyleneglycol 400]/g body weight were dosed by gavage into the stomach. For i.v. administration of nitrofurantoin (5 mg/kg), 5 μl of drug solution [appropriate concentration in 10% (v/v) ethanol, 40% (v/v) polyethyleneglycol 400, 50% PBS]/g body weight were injected into the tail of mice lightly anesthetized with methoxyflurane. Animals were sacrificed by cardiac puncture after anesthesia with methoxyflurane, and

blood was collected. Heparinized blood samples were centrifuged immediately at 3000 x g for 15 min and plasma collected and stored at -20°C until the time of HPLC analysis. 3-5 animals were used for each time point.

Gall bladder cannulation experiments. For gall bladder cannulation experiments, mice were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (6.7 mg/kg). The gall bladder was cannulated as described previously (van Herwaarden et al., 2003). Bile was collected in 15 min fractions for 60 min after injection of nitrofurantoin (5 mg/kg) into the tail vein. At the end of the experiment, blood was collected by cardiac puncture. Bile and plasma were stored at -20°C until the time of HPLC analysis. 5-6 animals were used for each group.

Metabolic cage experiment. Mice were housed in a Ruco Type m/1 metabolic cage (Valkenswaard, The Netherlands). They were allowed to get accustomed to the cages for 2 days, before receiving nitrofurantoin (5 mg/kg) injected into the tail vein. Feces and urine were collected in fractions of 0-4, 4-8, 8-24 h after drug administration, feces were homogenized in 4% (w/v) bovine serum albumin solution, and feces and urine were stored at -20°C until the time of HPLC analysis. 4 animals were used for each group.

Milk secretion experiments. For milk experiments, pups of approximately 10 days old were separated from the mother approximately 4 hours before starting the experiment. To stimulate milk secretion, oxytocin (200 μl of 1 I.U./ml solution) was administered subcutaneously to lactating dams. Nitrofurantoin (5 mg/kg) was injected into the tail vein at 30 min before milk was collected. At the indicated time, 50 μl of milk was collected from the 4th and 5th pairs of mammary glands by gentle vacuum suction.

Immediately after milk collection, animals were sacrificed by cardiac puncture after anesthesia with methoxyflurane, and heparinized blood was collected. Milk and plasma were stored at -20°C until the time of HPLC analysis. 3-4 animals were used for each group.

HPLC analysis. The conditions for HPLC analysis of nitrofurantoin were modified based on a previously published method (Gerk et al., 2001b). The methodology for the extraction of the samples was based on the use of an organic solvent, such as cold methanol, for protein precipitation (Bollag and Edelstein, 1991; Prieto et al., 2003). 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 $\mu\text{g}/\text{ml}$ furazolidone solution were 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 16000 x g for 5 min at 4°C and 50 μl of the organic phase was injected into the HPLC system. Samples from the transport assays were not processed and 100 μl of the culture media were directly injected into the HPLC system. The system consisted of a Waters 616 pump, a Waters 717plus autosampler and a UV detector (model UV2000, ThermoSeparation products). Separation was performed at 30°C on a reversed-phase column (Nucleosil 120 C18, 10- μm particle size, 250 mm x 4 mm), preceded by a precolumn cartridge (3.9 x 20 mm) packed with the same packing material. The composition of the mobile phase was 25 mM potassium phosphate buffer (pH 3.00)/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. The integration was performed using the software Millennium³² (Waters, Etten-Leur, The Netherlands). Standard samples in the appropriate drug-free matrix were prepared yielding a concentration range from 0.125 µg/ml up to 8 µg/ml.

Pharmacokinetic calculations and statistical analysis. The two-sided unpaired Student's *t* test was used throughout to assess the statistical significance of differences between the two sets of data. Results are presented as the means ± standard deviations. Differences were considered to be statistically significant when $p < 0.05$. AUC from time = 0 to the last sampling point was calculated by the linear trapezoidal rule, and oral availability was determined by $(\text{AUC p.o.}/ \text{dose p.o.})/(\text{AUC i.v.}/\text{dose i.v.})$, assuming linearity of dose-dependence.

Results

***In vitro* transport of nitrofurantoin.** To determine whether murine Bcrp1 and human BCRP were involved in nitrofurantoin transport *in vitro*, we made use of the polarized canine kidney cell line MDCK-II and its subclones transduced with murine Bcrp1 and human BCRP cDNAs. In addition, human MDR1- and human MRP2-transduced MDCK-II subclones were also tested for the possible role of these other ABC transporters in the *in vitro* transport of nitrofurantoin. The parental and transduced cell lines were grown to confluent polarized monolayers on porous membrane filters, and vectorial transport of nitrofurantoin (10 μ M) across the monolayers was determined. In the MDCK-II parental cell line, nitrofurantoin was consistently translocated somewhat more efficiently in the basolateral direction than in the apical direction (Fig. 1A), suggesting low endogenous basally directed transport. In the Bcrp1- and BCRP-transduced MDCK-II cell lines, apically directed translocation was highly increased and basolaterally directed translocation drastically decreased (Fig. 1, B and D). When the selective Bcrp1 inhibitor Ko143 was used (Allen et al., 2002), the Bcrp1/BCRP mediated transport was completely inhibited (Fig. 1, C and E), resulting in a vectorial translocation pattern equal to that of the MDCK-II parental cell line. In the MDR1- and MRP2-transduced MDCK-II cell lines, the vectorial translocation was similar to the MDCK-II parental cell line (Fig. 1, F and G).

These results show highly efficient transport of nitrofurantoin by murine Bcrp1 and human BCRP, but not by MDR1 or MRP2.

Plasma pharmacokinetics of nitrofurantoin in Bcrp1^{-/-} and wild-type mice. To assess whether the *in vitro* Bcrp1-mediated transport of nitrofurantoin was also

relevant *in vivo*, we studied nitrofurantoin bioavailability in Bcrp1^{-/-} and wild-type mice. We determined the plasma concentration of nitrofurantoin as a function of time, after oral and intravenous administration of nitrofurantoin in both types of mice (Fig. 2A and B). After oral administration of nitrofurantoin (10 mg/kg), the AUC of the Bcrp1^{-/-} mice was increased almost 4-fold compared with the wild-type mice (148.8 ± 30.4 versus 37.5 ± 6.8 min.µg/ml, $p < 0.05$). In the case of i.v. administration (5 mg/kg), the AUC of the Bcrp1^{-/-} was almost 2-fold higher compared with the wild-type mice (139.2 ± 22.0 versus 73.9 ± 9.0 min.µg/ml, $p < 0.05$). The calculated oral availability was significantly increased in Bcrp1^{-/-} compared with wild-type mice (53.4 ± 13.8 % versus 25.4 ± 9.6 %, $p < 0.05$). Bcrp1 thus appears to be both an important determinant for the oral availability and the elimination of nitrofurantoin.

Hepatobiliary, fecal and urinary excretion of nitrofurantoin in Bcrp1^{-/-} and wild-type mice. To investigate the role of Bcrp1 in the hepatobiliary excretion of nitrofurantoin, we administered nitrofurantoin (5 mg/kg) i.v. to Bcrp1^{-/-} and wild-type mice with a cannulated gall bladder and ligated common bile duct. Biliary excretion of nitrofurantoin was measured in fractions of 15 min during 1 h. Bcrp1^{-/-} mice showed a dramatically decreased excretion of nitrofurantoin into bile compared with wild-type mice (Fig. 3). At 1 h after i.v. administration, the cumulative nitrofurantoin excretion, as percentage of the dose, was 9.7 ± 3.2 % versus 0.2 ± 0.1 % in wild-type and Bcrp1^{-/-} mice, respectively. This indicates that more than 98% of the biliary excretion over 1 h can be attributed to Bcrp1 activity. Plasma concentrations at the end of the experiment were 0.13 ± 0.02 and 0.29 ± 0.13 µg/ml ($p < 0.05$) for wild-type and Bcrp1^{-/-} mice, respectively. We conclude that the hepatobiliary excretion of nitrofurantoin in mice is

virtually completely due to Bcrp1 activity, as biliary nitrofurantoin was nearly abolished in Bcrp1^{-/-} mice.

To assess the involvement of Bcrp1 in fecal and urinary excretion of nitrofurantoin in nonanesthetized mice, nitrofurantoin (5 mg/kg) was administered i.v. to Bcrp1^{-/-} and wild-type mice housed in metabolic cages. Fecal and urinary excretion of the unchanged drug was measured. Most of the nitrofurantoin in feces and urine was excreted during the first 0-8 h. The percentage of the dose excreted in feces over the 24 h after administration was negligible (<1%) and there was no difference between wild-type and Bcrp1^{-/-} mice. Regarding urinary excretion of nitrofurantoin over the 24 h after administration, there was no significant difference between wild-type and Bcrp1^{-/-} mice (11.3 ± 7.1 % *versus* 7.7 ± 3.7 % of dose). This suggests that Bcrp1 does not have a significant role in the fecal and urinary excretion of nitrofurantoin. Note that enzymatic degradation by body tissues or intestinal flora probably accounts for the remaining fraction of the dose, since nitroreduction by these mechanisms has a major contribution in the elimination of the drug (Buzard et al., 1961).

Secretion of nitrofurantoin into the milk in Bcrp1^{-/-} and wild-type mice. To test whether Bcrp1 plays a role in the secretion of nitrofurantoin into the milk, nitrofurantoin (5 mg/kg) was administered i.v. to lactating Bcrp1^{-/-} and wild-type females, and 30 min after administration, milk and blood were collected. The data obtained from the analysis of milk and plasma are shown in Fig. 4. Despite the higher plasma level (5-fold), the concentration of nitrofurantoin was substantially lower in the milk of Bcrp1^{-/-} mice (almost 15-fold). Our data clearly show that nitrofurantoin is actively transported into the milk of mice, since the observed milk-to-plasma ratio for wild-type mice (45.7 ±

16.2) was much higher than the theoretical milk-to-plasma ratio of 0.5 that physicochemical principles would suggest (Kari et al., 1997). In addition, we found that this parameter was almost 80 times higher in wild-type compared to Bcrp1^{-/-} lactating females (45.7 ± 16.2 versus 0.6 ± 0.1).

The percentage of the dose that is secreted into the milk in 30 min was estimated assuming that the total milk volume present in the mammary glands at the time of milk collection was 1 - 2 ml. In this case, the values are around 7.5 - 15 % for wild-type mice and 0.5 - 1 % for Bcrp1^{-/-} mice. These results indicate that Bcrp1 plays a major role in the secretion of nitrofurantoin into the milk and that milk can be a major excretory pathway for this drug, even higher than the hepatobiliary excretion (Fig. 3).

Discussion

Our data clearly show that the antibiotic nitrofurantoin is a very good substrate of Bcrp1/BCRP, and that mouse Bcrp1 restricts the oral bioavailability of nitrofurantoin. Bcrp1 also has a predominant role in the hepatobiliary excretion of the drug and in its secretion into the milk. Somewhat surprisingly, we did not observe a substantial effect of Bcrp1 on renal nitrofurantoin excretion.

The contribution of Bcrp1 to the hepatobiliary excretion of nitrofurantoin (98 %) is remarkable, if we compare it with other Bcrp1 substrates, like topotecan (75 %; Jonker et al., 2000) and PhIP (less than 50 %; van Herwaarden et al., 2003). One explanation for the very large effect of Bcrp1 on nitrofurantoin hepatobiliary excretion is that this drug is not a P-glycoprotein or MRP2 substrate (Fig. 1, F and G), whereas PhIP is also a MRP2 substrate *in vitro* (Dietrich et al., 2001a) and rat Mrp2 plays a role in its hepatobiliary excretion (Dietrich et al., 2001b). Note that in the case of topotecan (a P-glycoprotein substrate), the contribution of Bcrp1 to its hepatobiliary excretion was investigated using the Bcrp1-inhibitor GF120918 in P-glycoprotein knockout mice (Jonker et al., 2000), and the inhibition may not have been complete.

Our data suggest that intestinal Bcrp1 restricts nitrofurantoin oral bioavailability by reducing its intestinal absorption, but this could not be definitively demonstrated, since fecal excretion of unchanged nitrofurantoin was negligible, and also not different between wild-type and Bcrp1^{-/-} mice. The very small percentage of the dose recovered from feces could be explained by extensive degradation of nitrofurantoin in the intestinal tract, since rapid breakdown of the drug occurs during fecal incubation (Paul et al., 1960). Note that nitroreduction by intestinal flora (together with nitroreduction by body tissues)

has a major contribution in the elimination of the drug (Buzard et al., 1961). Active short-lived intermediates are formed, which are further rapidly converted to non-active molecules (Shahverdi et al., 2003). Furthermore, we cannot exclude the possibility of extensive reabsorption of the nitrofurantoin excreted into the bile (enterohepatic cycle), providing additional opportunity for metabolic degradation.

Our results also suggest that Bcrp1 does not play a substantial role in the urinary excretion of nitrofurantoin. Probably, next to glomerular filtration, other transporters are involved in the urinary excretion of nitrofurantoin (Moller and Sheikh, 1983). The percentage of the dose excreted into the urine we found (around 10%) is quite low compared to the data in the literature (40-50%) (Paul et al., 1960). Note that in the present study, we only detect unchanged nitrofurantoin. However, it was not always clear from other studies whether all the nitrofurantoin detected was unchanged. In the case of rats, about 50% of an administered dose of nitrofurantoin was recovered as metabolites in the urine (Braunlich et al., 1978). In addition, it is known that there are species differences with regard to excretion of nitrofurans in the urine (Paul et al., 1960).

Expression of BCRP in the mouse and human lactating mammary gland and the important role of Bcrp1 in the milk secretion of its substrates has recently been demonstrated by our group (Jonker et al., 2005). Our data clearly show that Bcrp1 plays a major role in the secretion of nitrofurantoin into the milk, as indicated by the large difference in milk-to-plasma ratio between wild-type and Bcrp1^{-/-} mice (80-fold higher in the wild-type mice). This difference is even higher than the values obtained previously in our group for other substrates like PhIP or topotecan (28-fold and 10-fold higher in wild-type mice, respectively) (Jonker et al., 2005). The nitrofurantoin milk-to-plasma ratio for

Bcrp1^{-/-} mice (0.6 ± 0.1) is similar to the theoretical milk-to-plasma ratio of 0.5 that physicochemical principles would predict (Kari et al., 1997), suggesting that, in the absence of Bcrp1, the only remaining component of the transport is passive diffusion.

Extrapolating our data to humans, the estimated values for the percentage of the dose excreted into the milk in the wild-type mice (7.5-15%) are quite close to what has been estimated in the case of humans. Gerk et al. (2001a) estimated that a breastfed infant would consume 0.2 mg/kg (6 % of the maternal dosage) of nitrofurantoin each day. These authors also showed that, in humans, the observed milk-to-serum concentration ratio was 22-fold greater than the one predicted from passive distribution. In the present work, we have shown that nitrofurantoin is effectively transported by human BCRP. It is thus very likely that the same predominant role of Bcrp1 in the milk secretion (and intestinal uptake and hepatobiliary excretion) of nitrofurantoin in mice also applies to human BCRP. Though it has been considered that the 6% of the maternal dose of nitrofurantoin that would be consumed by the infant is a low exposure, it can be of clinical relevance for suckling infants younger than 1 month, with a glucose-6-phosphatase dehydrogenase deficiency or with sensitivity to nitrofurantoin (Gerk et al., 2001a). Note that the effective exposure of the infant could vary due to differences in BCRP activity or genetic BCRP polymorphisms (Kondo et al., 2004; Sparreboom et al., 2004), not only in the mother but also in the infant.

Some of the previously reported interactions between nitrofurantoin and other drugs could be explained by competitive inhibition of BCRP-mediated transport. Gerk et al. (2001b) showed that nitrofurantoin reduced milk-to-serum concentration ratios of cimetidine by 33%. Since our group has recently shown that cimetidine is a Bcrp1

substrate and that this transporter also plays a prominent role in its milk secretion (Jonker et al., 2005), it could be that the nitrofurantoin-cimetidine interaction observed is BCRP-mediated.

From the present study, we cannot exclude the presence of additional basolateral transport mechanisms for nitrofurantoin, as has been suggested (Gerk et al., 2001b, 2003). Transport in parental MDCK cells even suggests existence of such transporter (Fig. 1A). However, these other transport mechanisms would probably not have a great influence on the exposure to nitrofurantoin, since our data clearly show that Bcrp1 plays a predominant role in the pharmacokinetics, hepatobiliary excretion and milk secretion of nitrofurantoin. Moreover, the most obvious apical ABC transporters P-glycoprotein and MRP2 do not appear to transport nitrofurantoin. In this perspective, we can consider that nitrofurantoin is an excellent marker drug for BCRP/Bcrp1 activity.

References

- Allen JD and Schinkel AH (2002) Multidrug resistance and pharmacological protection mediated by the breast cancer resistance protein. *Mol Cancer Ther* 1:427-434.
- Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JHM, Koomen G-J and Schinkel AH (2002) 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* 1:417-425.
- Bollag DM and Edelman ST (1991) *Protein Methods*. Wiley-Liss, Inc., New York.
- Braunlich H, Bonow A and Schroter S (1978) Age-dependence of renal tubular reabsorption of nitrofurantoin. *Arch Int Pharmacodyn* 232:92-101.
- Cisternino S, Mercier C, Bourasset F, Roux F and Scherrmann JM (2004) Expression, upregulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. *Cancer Res* 64:3296-3301.
- Dietrich CG, de Waart DR, Ottenhoff R, Schoots IG and Oude Elferink RPJ (2001a) Increased bioavailability of the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in MRP2-deficient rats. *Mol Pharm* 59:974-980.
- Dietrich CG, de Waart DR, Ottenhoff R, Bootsma AH, van Gennip AH and Oude Elferink RPJ (2001b) Mrp2-deficiency in the rat impairs biliary and intestinal excretion and influences metabolism and disposition of the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis* 22:805-811.
- Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK and Ross DD (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* 95:15665-15670.

- Evers R, Kool M, van Deemter L, Janssen H, Calafat J, Oomen LC, Paulusma CC, Oude Elferink RP, Baas F, Schinkel AH and Borst P (1998) Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. *J Clin Investig* 1:1310-1319.
- Gerk PM, Kuhn RJ, Desai NS and McNamara PJ (2001a) Active transport of nitrofurantoin into human milk. *Pharmacotherapy* 21:669-675.
- Gerk PM, Oo CY, Paxton EW, Moscow JA and McNamara PJ (2001b) Interactions between cimetidine, nitrofurantoin, and probenecid active transport into rat milk. *J Pharmacol Exp Ther* 296:175-180.
- Gerk PM, Moscow JA and McNamara PJ (2003) Basolateral active uptake of nitrofurantoin in the CIT3 cell culture model of lactation. *Drug Metab Dispos* 31:691-693.
- Huisman MT, Smit JW, Wiltshire HR, Hoetelmans RMW, Beijnen JH and Schinkel AH (2001) P-glycoprotein limits oral availability, brain, and fetal penetration of saquinavir even with high doses of ritonavir. *Mol Pharmacol* 59:806-813.
- Jonker JW, Smit JW, Brinkhuis RF, Maliepaard M, Beijnen JH, Schellens JHM and Schinkel AH (2000) Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J Natl Cancer Inst* 92:1651-1656.
- Jonker JW, Buitelaar M, Wagenaar E, Van Der Valk MA, Scheffer GL, Scheper RJ, Plosch T, Kuipers F, Elferink RP, Rosing H, Beijnen JH and Schinkel AH (2002) The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci USA* 26:15649-15654.

- Jonker JW, Merino G, Musters S, van Herwaarden AE, Bolscher E, Wagenaar E, Mesman E, Dale TC and Schinkel AH (2005) The Breast Cancer Resistance Protein (BCRP/ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. *Nat Med* 11:127-129.
- Kari FW, Weaver R and Neville MC (1997) Active transport of nitrofurantoin across the mammary epithelium *in vivo*. *J Pharmacol Exp Ther* 280:664-668.
- Kondo C, Suzuki H, Itoda M, Ozawa S, Sawada J, Kobayashi D, Ieiri I, Mine K, Ohtsubo K and Sugiyama Y (2004) Functional analysis of SNPs variants of BCRP/ABCG2. *Pharm Res* 21:1895-1903.
- Mizuno N, Suzuki M, Kusuhara H, Suzuki H, Takeuchi K, Niwa T, Jonker JW and Sugiyama Y (2004) Impaired renal excretion of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) sulfate in breast cancer resistance protein (Bcrp1/Abcg2) knockout mice. *Drug Metab Dispos* 32:898-901.
- Moller JV and Sheikh MI (1983) Renal organic anion transporter system: pharmacological, physiological and biochemical aspects. *Pharm Rev* 34:315-358.
- Paul MF, Paul HE, Bender RC, Kopko F, Harrington CM, Ells ER and Buzard JA (1960) Studies on the distribution and excretion of certain nitrofurans. *Antibiotics and Chemotherapy* 10:287-302.
- Pavek P, Merino G, Wagenaar E, Bolscher E, Novotna M, Jonker JW and Schinkel AH (2005) Human breast cancer resistance protein (BCRP/ABCG2): interactions with steroid drugs, hormones, the dietary carcinogen PhIP, and transport of cimetidine. *J Pharmacol Exp Ther* 312:144-152.

- Prieto JG, Merino G, Pulido MM, Estevez E, Molina AJ, Vila L and Alvarez AI (2003)
Improved LC method to determine ivermectin in plasma. *J Pharm Biomed Anal*
31:639-645.
- Shahverdi AR, Fazeli MR, Raffi F, Kakavand M, Jamalifar H and Hamed J (2003)
Inhibition of nitrofurantoin reduction by menthol leads to enhanced antimicrobial
activity. *J Chemother* 15:449-453.
- Sparreboom A, Gelderblom H, Marsh S, Ahluwalia R, Obach R, Principe P, Twelves C,
Verweij J and McLeod HL (2004) Diflomotecan pharmacokinetics in relation to
ABCG2 421 C>A genotype. *Clin Pharmacol Ther* 76:38-44.
- Toddywalla VS, Kari FW and Neville MC (1997) Active transport of nitrofurantoin
across a mouse mammary epithelial monolayer. *J Pharmacol Exp Ther* 280:669-676.
- van Herwaarden AE, Jonker JW, Wagenaar E, Brinkhuis RF, Schellens JH, Beijnen JH
and Schinkel AH (2003) The breast cancer resistance protein (Bcrp1/Abcg2) restricts
exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.
Cancer Res 63:6447-6452.

FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Transepithelial transport of nitrofurantoin (10 μ M) in MDCKII (parent) (A), MDCKII-Bcrp1 (B and C), MDCKII-BCRP (D and E), MDCKII-MDR1 (F) and MDCKII-MRP2 (G) monolayers. The experiment was started with the addition of nitrofurantoin to one compartment (basolateral or apical). After 2 and 4 hours, the percentage of drug appearing in the opposite compartment was measured by HPLC and plotted. BCRP inhibitor Ko143 (C and E) was present as indicated. Results are the means; error bars (sometimes smaller than the symbols) indicate the standard deviations (n = 3). Closed circles: translocation from the basolateral to the apical compartment; open circles: translocation from the apical to the basolateral compartment.

Fig. 2. Plasma concentration *versus* time curve after oral (A) and i.v. (B) administration of nitrofurantoin (10 mg/kg p.o., 5 mg/kg i.v.) to wild-type (open circles) and Bcrp1^{-/-} (closed circles) mice. Plasma samples were taken during 120 min (A) or 60 min (B). Plasma levels of nitrofurantoin were determined by HPLC. Results are the means; error bars indicate the standard deviations (n = 3-6, *, P < 0.05, **, P < 0.01)

Fig. 3. Cumulative hepatobiliary excretion of nitrofurantoin in wild-type and Bcrp1^{-/-} mice. Nitrofurantoin (5 mg/kg) was administered i.v. to mice with a cannulated gall bladder. Levels of nitrofurantoin were measured in bile fractions of Bcrp1^{-/-} (closed circles) and wild-type mice (open circles) by HPLC. Data are expressed as percentage of the dose. Results are the means; error bars (sometimes smaller than the symbols) indicate the standard deviations (n = 5-8). At all time points P < 0.005.

Fig. 4. Plasma and milk concentration (A) and milk-to-plasma ratio (B) of nitrofurantoin in wild-type and *Bcrp1*^{-/-} lactating females. Nitrofurantoin (5 mg/kg) was administered i.v. to mice and milk and plasma were collected after 30 min and analyzed by HPLC. Results are the means; error bars (sometimes smaller than the symbols) indicate the standard deviations (n = 3-4). At all parameters $P < 0.005$, comparing wild-type and *Bcrp1*^{-/-} data .

Fig. 1

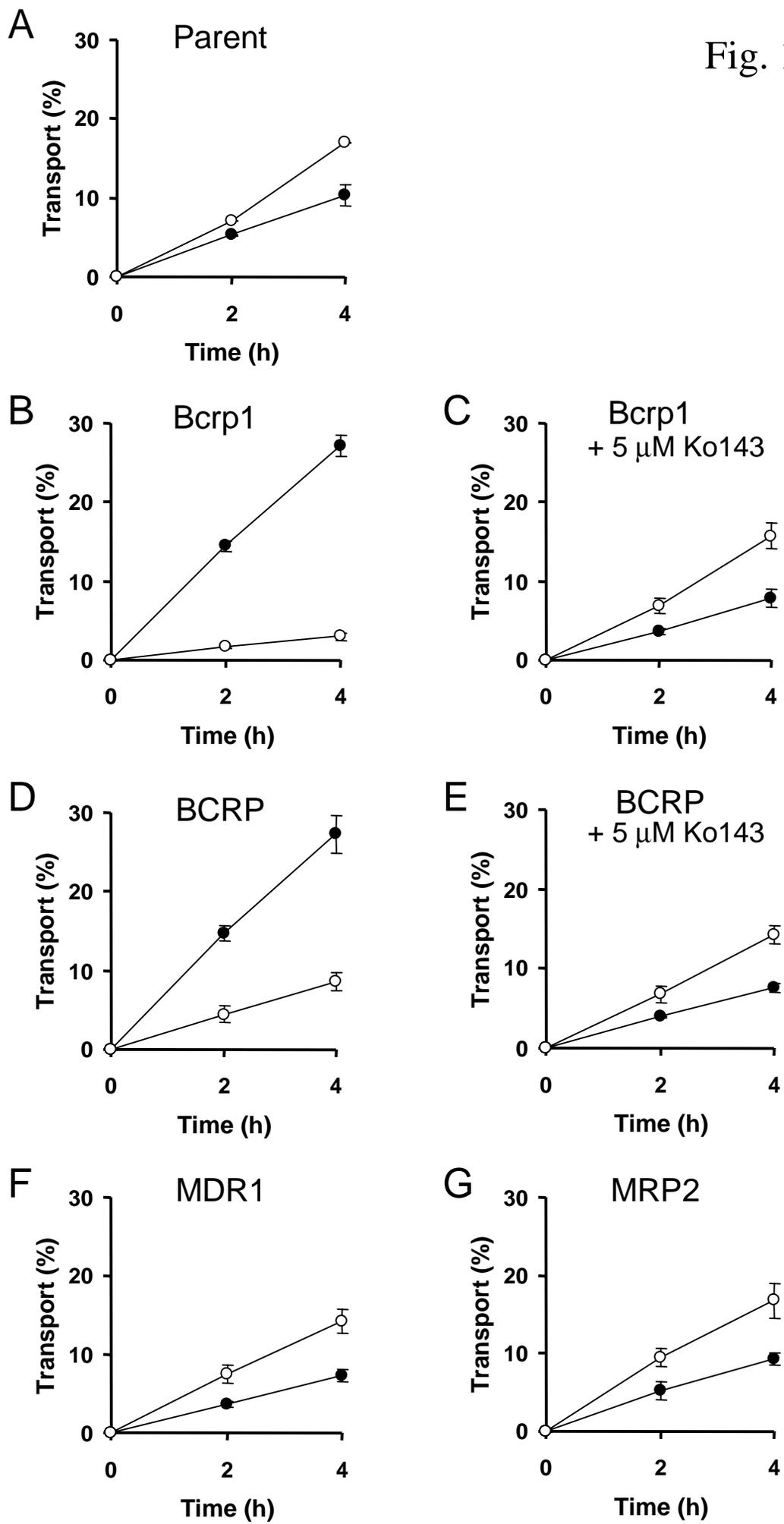


Fig. 2

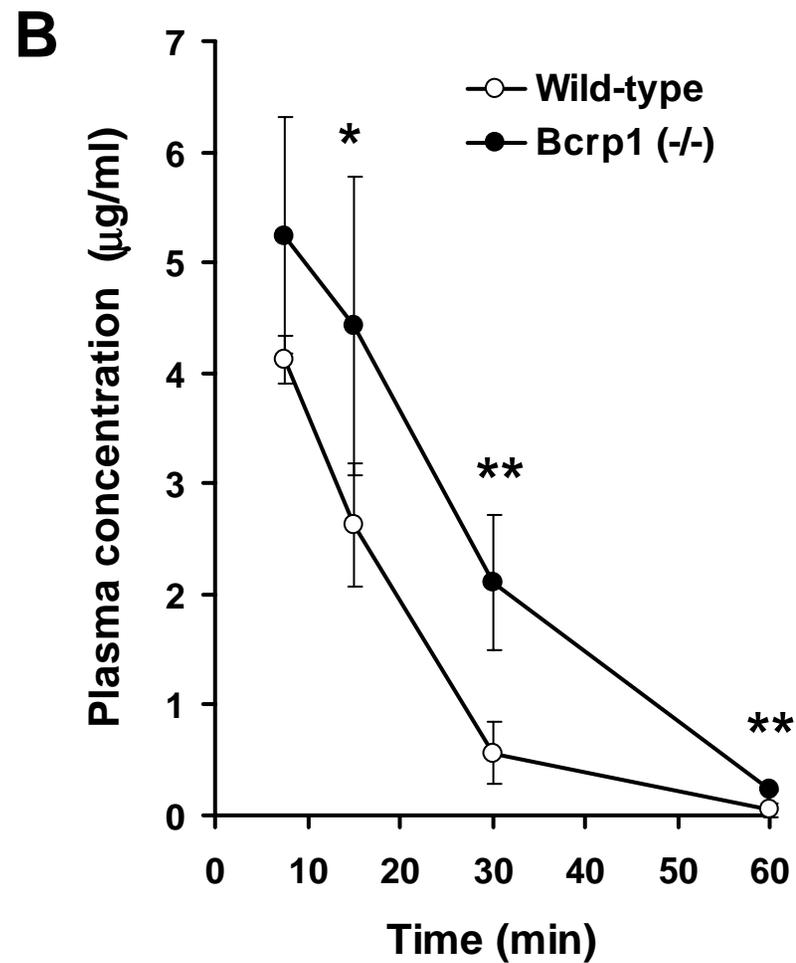
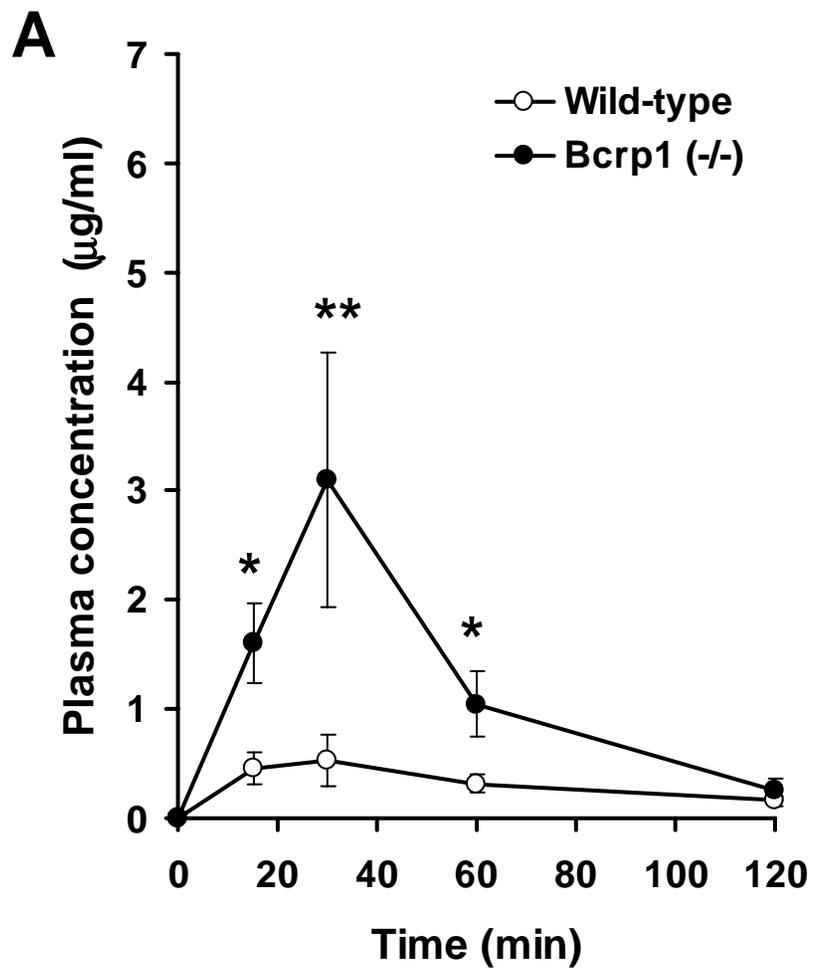


Fig. 3

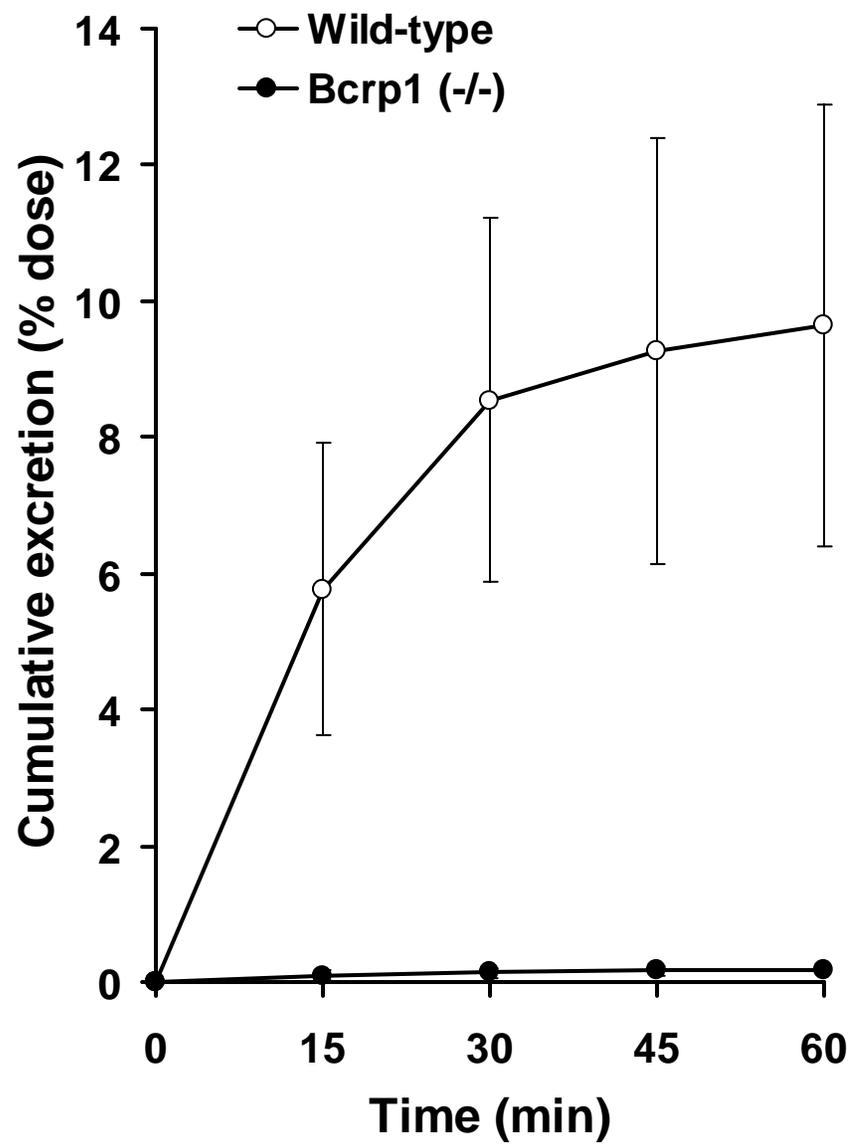


Fig. 4

