Carcinogenesis vol.27 no.1 pp.123–130, 2006
doi:10.1093/carcin/bgi176
Advance Access publication July 6, 2005

Breast cancer resistance protein (Bcrp1/Abcg2) reduces systemic exposure of the dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into breast milk

Antonius E.van Herwaarden, Els Wagenaar, Barbara Karnekamp, Gracia Merino, Johan W.Jonker and Alfred H.Schinkel*

Division of Experimental Therapy, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands

*To whom correspondence should be addressed.
Email: a.schinkel@nki.nl

The breast cancer resistance protein (BCRP/ABCG2) usually protects the body from a wide variety of environmental and dietary xenotoxins by reducing their net uptake from intestine and by increasing their hepatobiliary, intestinal and renal elimination. BCRP is also highly expressed in lactating mammary glands in mice, and this expression is conserved in cows and humans. As a result, BCRP substrates can be secreted into milk. We investigated whether different classes of dietary carcinogens are substrates of Bcrp1/BCRP and the implications for systemic exposure and breast milk contamination. Using polarized cell lines, we found that Bcrp1 transports the heterocyclic amines 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and the potent human hepatocarcinogen aflatoxin B1, and decreases their cellular accumulation up to 10-fold. *In vivo* pharmacokinetic studies showed that [^{14}C]IQ, [^{14}C]Trp-P-1 and [^{3}H]aflatoxin B1 plasma levels were substantially lower in wild-type compared with Bcrp1$^{-/-}$ mice, after both oral and intravenous administration, demonstrating that Bcrp1 restricts systemic exposure to these carcinogens. Moreover, Bcrp1 mediates transfer of [^{14}C]IQ, [^{14}C]Trp-P-1 and [^{3}H]aflatoxin into milk, with 3.4 ± 0.6, 2.6 ± 0.3 and 3.8 ± 0.5-fold higher milk to plasma ratios, respectively, in lactating wild-type versus Bcrp1$^{-/-}$ mice. We have thus identified Bcrp1/BCRP as one of the molecular mechanisms by which heterocyclic amines and aflatoxin are transferred into milk, thereby posing a health risk to breast-fed infants and dairy consumers. Paradoxically, Bcrp1/BCRP appears to have both protective and adverse roles with respect to exposure to dietary carcinogens.

Introduction

The breast cancer resistance protein (BCRP/ABCG2) and its murine homolog Bcrp1 belong to the ATP binding cassette (ABC) transmembrane drug transporter family. Because of the apical localization in epithelial tissues, such as the intestine, kidney, placenta and bile canalicular membrane, Bcrp1/BCRP reduces systemic and tissue uptake of its substrates, and mediates their extrusion from the body (1). We previously demonstrated that Bcrp1 restricts the oral uptake and systemic exposure of the heterocyclic amine (HA) and dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in mice (2). Since BCRP has broad substrate specificity, we were interested in the relevance of BCRP activity for other carcinogens to which humans are exposed through their diet.

The HAs are mutagenic and carcinogenic compounds formed during the heating of protein-containing foods such as meat and fish. HAs can be detected in the urine of healthy volunteers on a normal diet, and exposure occurs on a daily basis (3,4). In this study we focused on 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), representing two classes of HAs (IQ-type and non-IQ type, respectively) (Figure 1). PhIP is the most abundant HA present in food, whereas IQ has the highest carcinogenic potency. Administration of IQ to rats increases the incidence of tumors in liver, small intestine and mammary gland, whereas Trp-P-1 increases the incidence of liver carcinomas (5). Although HAs are present in nanogram per gram quantities in cooked meat, this exposure is often chronic. The incremental cancer risk due to dietary intake of HAs in humans in the developed world has been estimated at 1.1×10^{-4} (6).

Another dietary carcinogen, aflatoxin B1 (Figure 1), is one of the most potent mycotoxins known to science and an important risk factor for the development of hepatocellular carcinoma in humans (7,8). Certain *Aspergillus* species produce aflatoxins, and infection of stored corn, grains and nuts by these fungi leads to contamination with aflatoxin, thus

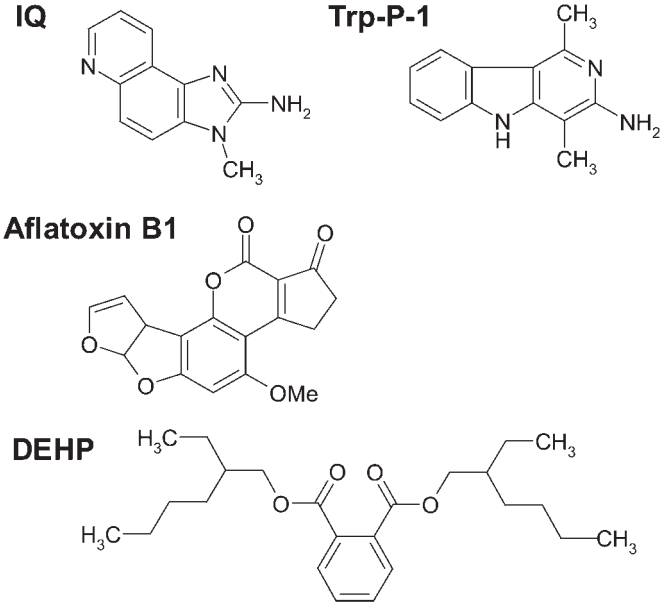

Fig. 1. Structures of IQ, Trp-P-1, aflatoxin B1 and DEHP.

Abbreviations: BCRP/ABCG2, breast cancer resistance protein; DEHP, di(2-ethylhexyl)phthalate; HA, heterocyclic amine; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole.

exposing humans to this hepatocarcinogen (8). Epidemiological studies in parts of Africa and Asia, where high levels of exposure occur, link aflatoxin B1 exposure and hepatocellular carcinomas, and the risk is strongly synergistic with viral hepatitis (8).

Contamination of milk with dietary and environmental carcinogens is a major concern for breast-feeding mothers and dairy consumers. For instance, the carcinogen di(2-ethylhexyl) phthalate (DEHP), present in polyvinylchloride polymers as a plasticizer and widely spread in the environment, is transferred into breast milk in rats (9,10). The dietary carcinogen PhIP, present in a normal western diet, can be detected in the milk of healthy women (11). Moreover, Paulsen *et al*. (12) demonstrated that the transfer of this HA from the lactating mother to the suckling through breast milk can induce intestinal tumors in rodent neonates. When dairy cattle eat aflatoxin B1 contaminated feeds, the hydroxylated metabolite aflatoxin M1 is transferred into the milk and milk products (8). Aflatoxin M1 is carcinogenic for both liver and colon in rats and can be detected in the breast milk of healthy women (13,14).

We have shown previously that the transfer of several xenotoxins into milk can be attributed to the highly induced levels of Bcrp1/BCRP in the luminal membrane of alveoli of the mammary gland during lactation (15). Bcrp1 is highly expressed in lactating, but not in virgin or non-lactating, mammary glands in mice and this expression pattern is conserved in cows and humans. As a result, many BCRP substrates can be secreted into breast milk. In this study we identify several potent dietary carcinogens as Bcrp1/BCRP substrates, and demonstrate their Bcrp1-mediated secretion into breast milk. On the other hand we show that Bcrp1 also reduces the systemic exposure to these dietary carcinogens.

Materials and methods

Animals

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

Chemicals

IQ, [^{14}C]IQ, Trp-P-1 and [^{14}C]Trp-P-1 (10 mCi/mmol) were from Toronto Research Chemicals (Ontario, Canada); [^{14}C]DEHP (11.7 mCi/mmol), DEHP and aflatoxin B1 were from Sigma Chemical (St Louis, MO); [^{3}H]aflatoxin B1 (16.6 Ci/mmol) was from Moravek Biochemicals (Brea, CA); [^{3}H]inulin and [^{14}C]inulin were from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK); oxytocin (Syntocinon 5 IU/ml) was from Novartis Pharma B.V. (Arnhem, The Netherlands); methoxyflurane (Metofane) was from Medical Developments Australia (Springvale, Victoria, Australia); GF120918 was from GlaxoSmithKline (Research Triangle Park, NC); and Ko143 was described before (16).

Transport assay

In the transwell transport assay we used the previously described polarized pig kidney epithelial cell line LLC-PK1, human multidrug resistance protein (*MDR*)*1*$^{-}$, murine *Mdr1a*- and *Bcrp1*-transfected LLC-PK1 subclones, the polarized canine kidney cell line MDCK-II, and murine *Bcrp1*-, human multidrug resistance associated protein (*MRP*)*2*- and human *BCRP*-transduced MDCK-II subclones (2,17). LLC-PK1 cells and derivatives are especially suitable to study P-glycoprotein-mediated transport due to the very low endogenous porcine P-glycoprotein in these cells, whereas MDCK-II cells and derivatives are very suitable to study Bcrp1/BCRP-mediated transport due to their negligible endogenous BCRP-like transport activity (1,2). Transport assays and cell culture conditions were carried out as previously described (2). Cells were grown to confluent polarized monolayers on porous membrane filters and vectorial transport of [^{14}C]IQ, [^{14}C]Trp-P-1 and [^{3}H]aflatoxin B1 across the monolayer was determined, either with or without GF120918 or Ko143. At the end of the experiment, cell monolayers were rinsed with cold phosphate-buffered saline and radioactivity in the filter was determined. The tightness of the monolayer was measured by monitoring the paracellular flux of inulin to the opposite compartment, which had to remain <1.5% of the total radioactivity per hour.

Pharmacokinetic experiments

Radiolabeled compounds were administered by intravenous (i.v.) injection into the tail vein or by gavage into the stomach of mice lightly anesthetized with methoxyflurane. IQ or Trp-P-1 was formulated in 20% (v/v) dimethyl sulfoxide and 4% (w/v) D-glucose solution. Aflatoxin B1 or DEHP was formulated in Cremophor EL–ethanol (1:1, w/v) and diluted in 5% (w/v) D-glucose solution (1:3). A 5 μl drug solution per gram body weight (1 mg/kg) was administered to male wild-type and Bcrp1$^{-/-}$ mice, and plasma samples were taken at several time points by cardiac puncture under anesthesia with methoxyflurane. Mice were subsequently killed by cervical dislocation. For tissue distribution organs were removed and solubilized in 'Solvable' (Perkin Elmer, MA). Intestinal contents were separated from intestinal tissue. For milk secretion experiments, lactating wild-type or Bcrp1$^{-/-}$ females with ~10-day-old pups were used. Radiolabeled compounds (1 mg/kg) were i.v. administered together with a subcutaneous administration of oxytocin (200 μl of a 1 IU/ml solution) to stimulate milk secretion. After 30 min, milk samples were collected bilaterally from all five (thoracic and inguinal) mammary glands by gentle vacuum suction, and blood samples were taken by cardiac puncture under anesthesia with methoxyflurane. Levels of radioactivity in plasma, tissue homogenates, feces and milk were determined by liquid scintillation counting.

Pharmacokinetic calculations and statistical analysis

All values are given as average ± standard deviation (SD), unless indicated otherwise. AUC from time = 0 to the last sampling point was calculated by the linear trapezoidal rule, and standard errors (SE) are indicated. A two-tailed unpaired Student's *t*-test was used to assess the significance of difference between two sets of data. Differences were considered to be statistically significant when $P < 0.05$.

Results

In vitro transport of aflatoxin B1, IQ and Trp-P-1 by Bcrp1 and BCRP

To assess whether Bcrp1/BCRP is involved in the transport of aflatoxin B1, IQ, Trp-P-1 and DEHP *in vitro*, we used the polarized canine kidney cell line MDCK-II and its subclones transduced with murine *Bcrp1* or human *BCRP* cDNAs. The cell lines were grown to confluent polarized monolayers on porous membrane filters, and vectorial transport of [^{3}H]aflatoxin B1, [^{14}C]IQ, [^{14}C]Trp-P-1 and [^{14}C]DEHP across the monolayer was determined.

In the MDCK-II parental cell line, apically and basolaterally directed translocation of [^{3}H]aflatoxin B1 (2 μM) were similar (Figure 2A) and this was also the case for the *MRP2*-transduced MDCK-II subclone (data not shown). In the *Bcrp1*-transduced MDCK-II cell line, apically directed translocation was highly increased and basolaterally directed translocation drastically decreased (Figure 2B). Although human BCRP is comparatively poorly expressed in the *BCRP*-transduced MDCK-II cell line, the results with this cell-line showed a qualitatively similar picture as in *Bcrp1*-transduced MDCK-II cells (Figure 2D). This transport by Bcrp1 and BCRP was completely inhibited by Ko143, a selective Bcrp1 inhibitor (16), and by the Bcrp1 and P-gp inhibitor GF120918 (Figure 2C) (data not shown), resulting in a vectorial translocation pattern similar to that of the MDCK-II parent cell line.

Transport experiments with the HAs [^{14}C]IQ and [^{14}C]Trp-P-1 (2 μM) yielded qualitatitively the same results, i.e. no vectorial transport in the MDCK-II parental cell line (Figure 2E and I) and the *MRP2*-transduced MDCK-II subclone (data not shown), and increased apically directed translocation and decreased basolaterally directed translocation in

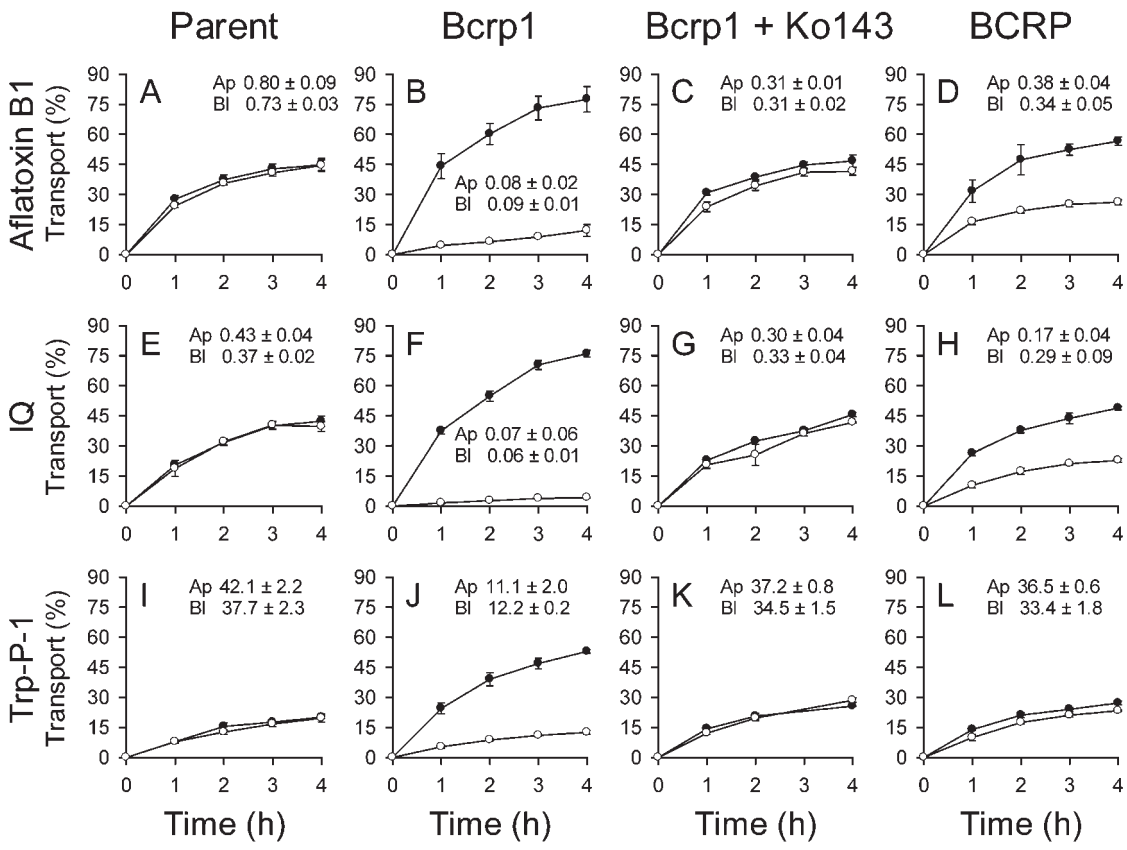

Fig. 2. Transepithelial transport of [^{3}H]aflatoxin B1, [^{14}C]IQ and [^{14}C]Trp-P-1 (each 2 μM) in MDCK-II cells, either non-transduced (**A**, **E**, **I**) or transduced with murine *Bcrp1* (**B**, **C**, **F**, **G**, **J**, **K**) or human *BCRP* (**D**, **H**, **L**) cDNAs. Ko143 was added where indicated (C, G, K). At $t = 0$, the radiolabeled carcinogens were applied in one compartment (basolateral or apical), and the percentage of radioactivity appearing in the opposite compartment at $t = 1$, 2, 3 and 4 h was measured and plotted ($n = 3$). Translocation from the basolateral to the apical compartment (closed circles); translocation from the apical to the basolateral compartment (open circles).Values within figures represent the percent of radioactivity found in the monolayer after 4-h incubation after initial compound application to the apical (Ap) or basolateral (Bl) compartments. Error bars (often smaller than the symbols) indicate SD. In K ($t = 3$) sample points were not measured.

the *Bcrp1*-transduced MDCK-II cell line (Figure 2F and J). [^{14}C]IQ transport was also present in the human *BCRP*-transduced MDCK-II cell lines, but [^{14}C]Trp-P-1 transport in the *BCRP*-transduced subclone was negligible (Figure 2H and L). Transport of [^{14}C]IQ by BCRP and [^{14}C]Trp-P-1 by Bcrp1 was completely inhibited by Ko143 and GF120918 (Figure 2G and K) (data not shown). For [^{14}C]DEHP, vectorial transport could not be assessed in the transwell transport assay due to very low uptake into the cells (data not shown).

Cellular accumulation of [^{3}H]aflatoxin B1, [^{14}C]IQ and [^{14}C]Trp-P-1 in the monolayers was determined at the end of the 4-h transport assay (Figure 2). In Bcrp1-overexpressing MDCK-II cells, intracellular concentrations of aflatoxin B1 decreased up to 10-fold compared with parental cells (Figure 2A and B), for IQ and Trp-P-1 this was 6- and ∼4-fold, respectively (Figure 2 E, F, I and J).

To assess P-glycoprotein-mediated transport of the dietary carcinogens, we tested [^{3}H]aflatoxin B1 (2 μM), [^{14}C]IQ (100 μM) and [^{14}C]Trp-P-1 (2 μM) transport in polarized LLC-PK1 cells expressing murine Mdr1a or human MDR1. There was no marked difference in apically or basolaterally directed translocation of [^{3}H]aflatoxin B1, [^{14}C]IQ and [^{14}C]Trp-P-1 in the LLC-PK1 parental or *Mdr1a*- or *MDR1*-transduced cell lines (data not shown). In contrast, in a *Bcrp1*-transfected LLC-PK1 subclone, apically directed translocation of [^{3}H]aflatoxin B1, [^{14}C]IQ and [^{14}C]Trp-P-1 was highly increased and basolaterally directed translocation was highly decreased compared with the parental LLC-PK1 parent cell line (data not shown). We conclude that Trp-P-1 is transported by Bcrp1, and that both human BCRP and murine Bcrp1 transport IQ. The hepatocarcinogen aflatoxin B1 is also a transported substrate of murine Bcrp1 and human BCRP. In contrast, human MRP2, human MDR1 and murine Mdr1a do not efficiently transport these carcinogens.

In vivo plasma pharmacokinetics of [^{14}C]IQ, [^{14}C]Trp-P-1 and [^{3}H]aflatoxin B1 in Bcrp1$^{-/-}$ and wild-type mice

To assess whether the *in vitro* observed Bcrp1-mediated transport is also relevant *in vivo*, we administered [^{3}H]aflatoxin B1, [^{14}C]IQ or [^{14}C]Trp-P-1 (each 1 mg/kg) either orally or i.v. to male *Bcrp1*$^{-/-}$ and wild-type mice, and measured plasma radioactivity levels after 30 min (Figure 3). There was a 1.7-fold increase in plasma [^{3}H]aflatoxin B1 in *Bcrp1*$^{-/-}$ mice compared with the wild-type after oral administration, and a 1.4-fold increases after i.v. administration. There were 1.7- and 2.3-fold increases in plasma [^{14}C]IQ and [^{14}C]Trp-P-1 in *Bcrp1*$^{-/-}$ mice compared with the wild-type after oral administration, and 1.8- and 2.2-fold increases after i.v. administration (Figure 3). At a dose of 1 mg/kg [^{14}C]IQ, the $AUC_{7.5\text{-}30}$ for i.v. administration was 1.8-fold higher in *Bcrp1*$^{-/-}$ compared with wild-type mice, further supporting that Bcrp1 activity substantially decreases the systemic exposure of its substrates

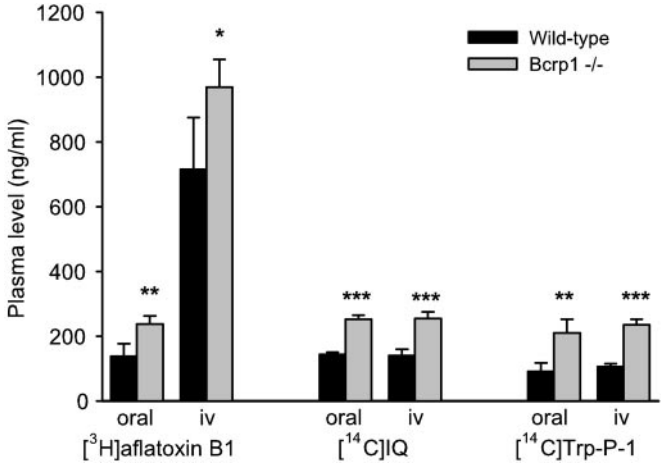

Fig. 3. Plasma concentrations (ng-equivalent/ml) at $t = 30$ min after oral and i.v. administration of [^{3}H]aflatoxin B1, [^{14}C]IQ or [^{14}C]Trp-P-1 (1 mg/kg each) to male wild-type and *Bcrp1*$^{-/-}$ mice. Data are expressed as average; error bars indicate SD ($n = 3$–4; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

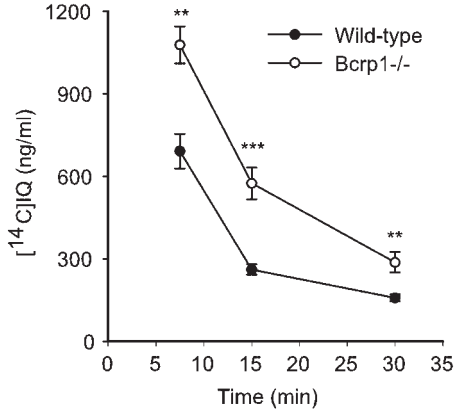

Fig. 4. Plasma concentrations (ng-equivalent/ml) at 7.5, 15 and 30 min after i.v. administration of [^{14}C]IQ (1 mg/kg) to male wild-type and *Bcrp1*$^{-/-}$ mice. Data are expressed as average; error bars indicate SD ($n = 3$–4; $^{**}P < 0.01$, $^{***}P < 0.001$).

(278 ± 8.2 versus 155 ± 5.0 h·ng/ml, $P < 0.01$; Figure 4). Plasma [^{14}C]DEHP radioactivity was not different between *Bcrp1*$^{-/-}$ and wild-type mice after oral (1.04 ± 0.62 versus 0.93 ± 0.06 μg/ml; $P = 0.77$) or i.v. (2.18 ± 0.28 versus 2.04 ± 0.02 μg/ml; $P = 0.55$) administration (at 1 mg/kg) suggesting that DEHP is not a Bcrp1 substrate. We conclude that Bcrp1 activity substantially decreases the systemic exposure of aflatoxin B1, IQ and Trp-P-1, but not of DEHP.

Tissue distribution of [^{14}C]IQ and [^{3}H]aflatoxin B1 in Bcrp1$^{-/-}$ *versus wild-type mice*

To investigate the role of Bcrp1 in the tissue distribution and elimination of [^{14}C]IQ in more detail we i.v. administered [^{14}C]IQ (1 mg/kg) to male wild-type and Bcrp1$^{-/-}$ mice. Radioactivity was measured in several tissues and intestinal contents 7.5 and 30 min after i.v. [^{14}C]IQ administration (data not shown and Table I). Overall, comparing the levels in various compartments between 7.5 and 30 min, differences between Bcrp1$^{-/-}$ and wild-type mice were qualitatively similar. There was a relatively high accumulation of radioactivity

Table I. Levels of radioactivity in male wild-type and *Bcrp1*$^{-/-}$ mice 30 min after i.v. administration of 1 mg/kg [^{14}C]IQ

Tissue	Wild-type	Bcrp1$^{-/-}$	ratio$^{-/-}$/wt
Plasma	140.4 ± 19.8	254.6 ± 20.9**	1.8
Brain	26.2 ± 2.45	36.8 ± 8.2*	1.4
Testis	157.1 ± 57.7	161.2 ± 23.1	1.0
Spleen	210.4 ± 17.0	243.4 ± 30.7	1.2
Lung	136.9 ± 1.55	189.7 ± 30.3*	1.4
Kidney	763.2 ± 62.8	2275.7 ± 221***	3.0
Liver	1113.8 ± 143	981.2 ± 90.9	0.9
Small intestine (tissue)	3479.5 ± 371	1725.1 ± 275***	0.5
Cecum (tissue)	299.1 ± 20.6	354.4 ± 27.9*	1.2
Colon (tissue)	165.3 ± 15.2	226.9 ± 48.4	1.4
Content small intestine	41525.4 ± 10878	18266.7 ± 5140**	0.4
Content cecum	533.2 ± 81.8	348.0 ± 27.2**	0.7
Content colon	720.4 ± 146	584.6 ± 95.0	1.4

Results are expressed as average ^{14}C concentrations (ng-equivalent/g or ml) ± SD ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

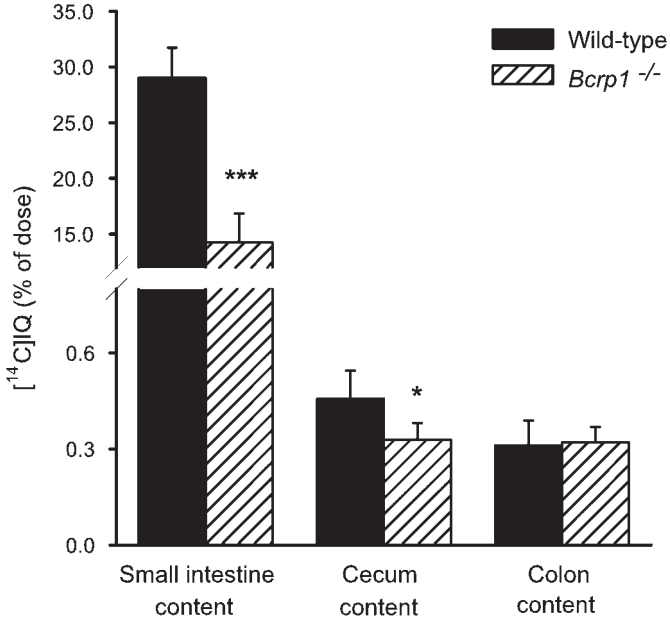

Fig. 5. Intestinal contents of radioactivity in male wild-type and *Bcrp1*$^{-/-}$ mice 30 min after i.v. administration of [^{14}C]IQ (1 mg/kg). Data are expressed as percentage of the administered dose ± SD of radioactivity measured in the contents of small intestine, cecum and colon ($n = 4$, $^{*}P < 0.05$, $^{***}P < 0.001$).

in liver, kidney and small intestine for both genotypes (Table I). Despite higher plasma levels of [^{14}C]IQ in Bcrp1$^{-/-}$ mice, for both time points radioactivity levels in liver between wild-type and Bcrp1$^{-/-}$ mice were similar (data not shown and Table I). In contrast to the liver, the percentage of the dose of [^{14}C]IQ retrieved from the content of small intestine after 30 min was 2.2-fold higher in wild-type compared with Bcrp1$^{-/-}$ mice (29.0 ± 2.7 versus 13.3 ± 2.6%, $P < 0.001$), indicating Bcrp1-mediated hepatobiliary and possibly direct intestinal transport of [^{14}C]IQ into the lumen of this organ (Figure 5). Radioactivity measured in kidneys of Bcrp1$^{-/-}$ mice 30 min after administration was 3.0-fold higher compared with wild-type mice (Table I), a significant increase even when corrected for the higher plasma levels (8.96 ± 0.80 versus 5.48 ± 0.57 tissue/plasma ratio, $P < 0.001$), indicating a role for

Bcrp1 in preventing accumulation of [^{14}C]IQ in this organ, possibly by tubular secretion and/or preventing renal reabsorption of glomerularly filtrated [^{14}C]IQ.

In addition to IQ we were interested in the effects of Bcrp1 on the tissue distribution of aflatoxin B1. One hour after oral administration of [^{3}H]aflatoxin B1 (1 mg/kg) to wild-type and Bcrp1$^{-/-}$ mice, mice were killed and radioactivity was measured in liver and kidney. In liver, no difference in radioactivity was measured between wild-type and Bcrp1$^{-/-}$ mice (0.75 $\pm$ 0.06 versus 0.85 $\pm$ 0.25 μg/g, $P = 0.44$), whereas the kidney [^{3}H]aflatoxin B1 concentrations in Bcrp1$^{-/-}$ mice were 2-fold higher compared with wild-type (0.61 $\pm$ 0.20 versus 0.31 $\pm$ 0.03 μg/g, $P < 0.05$).

The tissue distribution of [^{14}C]IQ and [^{3}H]aflatoxin B1 further substantiates that these compounds are substrates of Bcrp1 and that Bcrp1 can affect the systemic exposure to these carcinogens *in vivo*.

[^{14}C]IQ, [^{14}C]Trp-P-1 and [^{3}H]aflatoxin B1 are transported into breast milk by Bcrp1

Xenotoxin and carcinogen accumulation in breast milk and dairy products is a major public health concern. During lactation, Bcrp1/BCRP is expressed in the mammary gland (15). We investigated the consequences of this expression for the transfer to breast milk of IQ, Trp-P-1, aflatoxin B1 and DEHP circulating in the blood of the mother. We administered [^{14}C]IQ, [^{14}C]Trp-P-1, [^{3}H]aflatoxin B1 or [^{14}C]DEHP (i.v., 1 mg/kg) to female lactating wild-type and *Bcrp1*$^{-/-}$ mice. Milk and maternal plasma samples were obtained 30 min after i.v. administration and milk to plasma (*M*/*P*) concentration ratios were calculated. *M*/*P* ratios of [^{14}C]IQ, [^{14}C]Trp-P-1 and [3H]aflatoxin B1 were 3.4 $\pm$ 0.6, 2.6 $\pm$ 0.3 and 3.8 $\pm$ 0.5 (ratio $\pm$ SE)-fold higher in wild-type versus Bcrp1$^{-/-}$ mice, respectively (Figure 6). In contrast, the *M*/*P* ratio of DEHP was not different between wild-type and Bcrp1$^{-/-}$ mice (Figure 6). We conclude that Bcrp1 secretes [^{14}C]IQ, [^{14}C]Trp-P-1 and [^{3}H]aflatoxin B1, but not [^{14}C]DEHP into the breast milk.

Discussion

In this study, we demonstrate that the dietary carcinogens IQ, Trp-P-1 and aflatoxin B1 are transported substrates of murine Bcrp1. Bcrp1 knockout mouse studies show that this transport can restrict the systemic exposure to these carcinogens, indicating a protective role for Bcrp1. Paradoxically, we also found that [^{14}C]IQ, [^{14}C]Trp-P-1 and [^{3}H]aflatoxin B1 are all secreted into milk by Bcrp1. HAs and aflatoxins can be found in human milk and milk transfer of HAs has been shown to cause intestinal tumors in rodent neonates (11,12,14). Because milk is the sole food for young mammals and an often important constituent of the human diet long after weaning, Bcrp1/BCRP-mediated transfer of carcinogens into the milk poses a health risk to breast-fed infants and dairy consumers. Bcrp1/BCRP thus appears to have both protective and adverse roles with respect to exposure to dietary carcinogens.

Because of its broad substrate specificity and strategic tissue distribution, a main function of BCRP is protection of the body against harmful xenotoxins (1,2,18,19); we previously described that Bcrp1 restricts the exposure of the HA carcinogen PhIP in mice (2). In this study, we show that in addition to PhIP two other HAs and the human hepatocarcinogen aflatoxin B1 are substrates of Bcrp1. We demonstrate lower plasma levels in wild-type compared with *Bcrp1*$^{-/-}$ mice after i.v. and oral administration of aflatoxin B1, as well as IQ and Trp-P-1. This indicates that also for these newly identified and structurally diverse substrates, representing two different classes of dietary carcinogens, Bcrp1 has a protective role in limiting their systemic exposure. Inhibition of BCRP by drug intake, as well as the existence of BCRP polymorphisms, can cause inter- and intraindividual variation in BCRP activity (20,21). Possibly, induction and stimulation of BCRP by food and drug intake may also occur. We recently found that hepatic Bcrp1 expression is gender dependent in mice and humans (22). Therefore, systemic exposure to HAs and aflatoxins in humans, and thus overall susceptibility to these carcinogens, may well be affected by inter- and intraindividual differences in BCRP activity.

In addition, Bcrp1 is highly expressed in stem cells in a range of different tissues (23,24). In our transwell transport experiments we demonstrate that intracellular concentrations of aflatoxin B1, IQ and Trp-P-1 were markedly decreased when Bcrp1 was over-expressed in cells. Bcrp1 might thus well be able to directly limit exposure of stem cells to these dietary carcinogens *in vivo*. Given their extensive proliferative capacity, stem cells could be an important target for initial carcinogenic events. Bcrp1 might thus reduce the susceptibility to carcinogens at both systemic and (stem) cell level.

In van Herwaarden *et al*. (2) the hepatobiliary and direct intestinal excretion of [^{14}C]PhIP 1 h after i.v. administration was 40.7 versus 10.5% of dose in wild-type versus Bcrp1$^{-/-}$ mice, of which 36.5 versus 9% of dose in wild-type versus Bcrp1$^{-/-}$ could be attributed to hepatobiliary excretion. This demonstrated a major role for Bcrp1 in hepatobiliary excretion of [^{14}C]PhIP into the lumen of the intestine. Qualitatively similar to those results, in this study we found a very substantial 2-fold higher accumulation of [^{14}C]IQ in the lumen of the small intestine of wild-type versus Bcrp1$^{-/-}$ mice ($\sim$30% of the dose, measured only 30 min after the administration of [^{14}C]IQ). The higher levels of [^{14}C]IQ in the intestinal lumen of wild-type compared with Bcrp1$^{-/-}$ mice are consistent with an increased hepatobiliary and perhaps intestinal excretion and reduced net uptake of [^{14}C]IQ from the intestine caused by Bcrp1 activity.

Contrary to our expectation, liver concentrations of i.v administered [^{14}C]IQ or orally administered [^{3}H]aflatoxin B1 were not different between wild-type and Bcrp1$^{-/-}$ mice 30 and 60 min after administration, respectively. It should be noted though that despite the observation that overall radioactivity in liver (including the biliary tree) between wild-type and Bcrp1$^{-/-}$ mice did not differ, Bcrp1 activity may still decrease intracellular carcinogen concentrations in hepatocytes. While the hepatic expression of Bcrp1 clearly contributes to decrease of systemic exposure to substrate carcinogens such as IQ, Trp-P-1 and aflatoxin B1, effects on the liver itself appear to be complex.

While for both aflatoxin B1 and IQ, transport by both murine Bcrp1 and human BCRP is evident, we have not been able to convincingly show transport of Trp-P-1 by human BCRP in our transwell assay, in contrast to its transport by murine Bcrp1. We note that also transport of aflatoxin B1 and IQ is more pronounced in the Bcrp1-transduced cells compared with the BCRP-transduced cells. This might be the result of differences in substrate specificity/affinity between the murine and human homologues, but it might also arise from a relatively lower expression of the human BCRP construct compared with

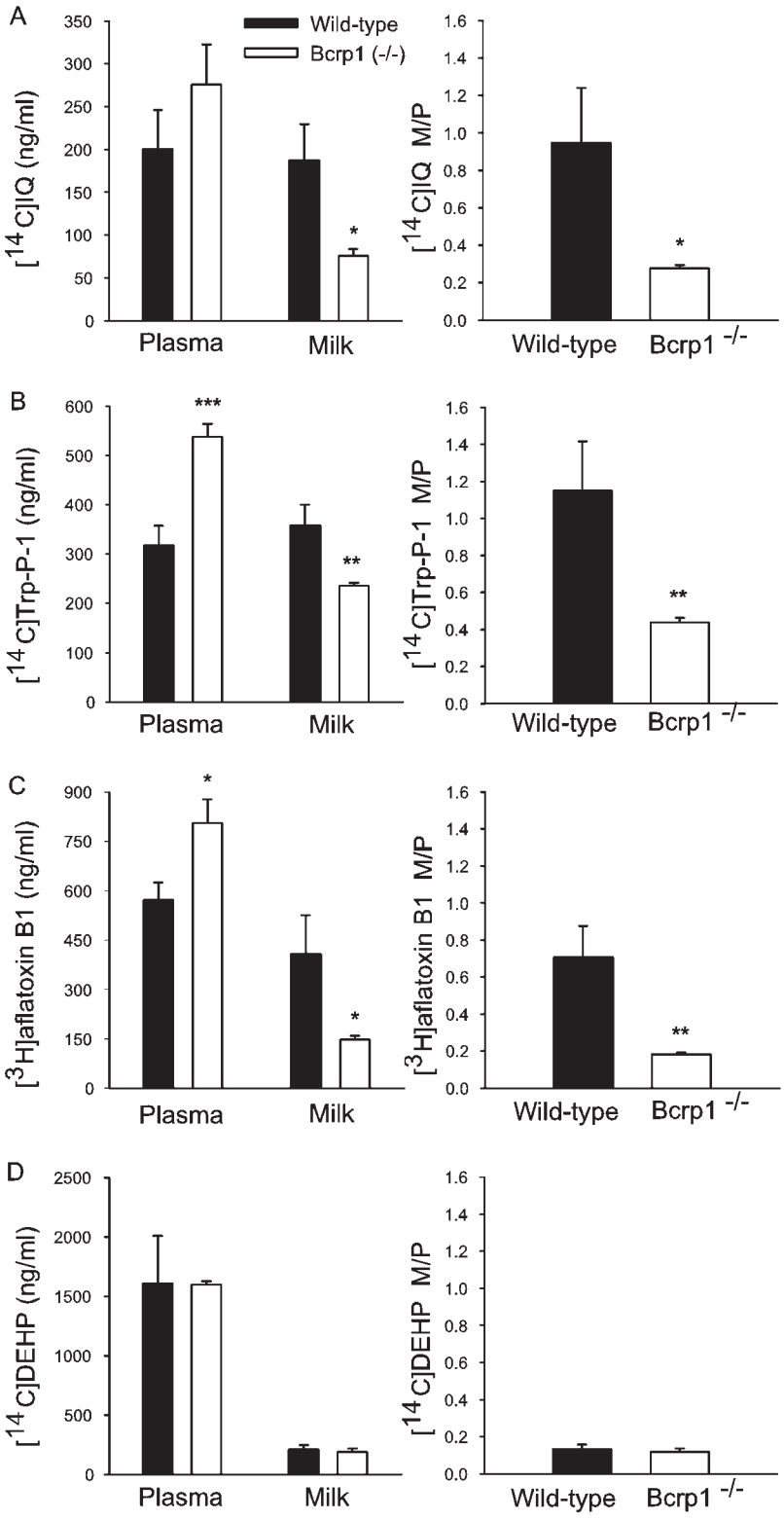

Fig. 6. Bcrp1 mediates secretion of [^{3}H]aflatoxin B1, [^{14}C]IQ and [^{14}C]Trp-P-1, but not [^{14}C]DEHP into the milk. Radiolabeled compounds (1 mg/kg) were administered i.v. to lactating wild-type or *Bcrp1*$^{-/-}$ females, and after 30 min plasma and milk concentrations and milk/plasma (*M*/*P*) ratios were determined for (**A**) [^{14}C]IQ, (**B**) [^{14}C]Trp-P-1, (**C**) [^{3}H]aflatoxin B1 and (**D**) [^{14}C]DEHP. Data are expressed as average concentration ± SD and ratio ± SE ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Bcrp1 in the cell lines used; for instance, mitoxantrone accumulation was 3.7-fold higher in the BCRP-transduced cells compared with Bcrp1-transduced cells (25). Unfortunately, currently available antibodies do not allow a straightforward comparison of murine and human Bcrp1/BCRP protein levels. Thus far, there are no indications for pronounced differences in substrate specificity between wild-type human BCRP and murine Bcrp1 (26). Moreover, structurally diverse substrates

such as topotecan, mitoxantrone, pheophorbide a and PhIP are all transported by both human BCRP and murine Bcrp1, suggesting a strongly conserved substrate spectrum. However, detailed differences in transport kinetics and/or substrate affinity are yet unexplored.

Until recently, transfer of xenotoxins into the breast milk has been considered to be the result of physicochemical properties of the excreted compound such as ionization, molecular weight, lipophilicity and plasma protein binding, and also carrier-mediated transport (27). In this study we demonstrate that in addition to the factors mentioned above, the presence of an apically located ATP-dependent transporter can be an important factor in the transfer of carcinogens into the milk. We convincingly demonstrated this for the dietary carcinogens [^{14}C]IQ, [^{14}C]Trp-P-1 and [^{3}H]aflatoxin B1 using wild-type versus Bcrp1$^{-/-}$ mice. The plasticizer [^{14}C]DEHP is also transferred (and perhaps even concentrated) into the breast milk, as reported by Dostal *et al*. (10). However, this secretion into the milk is apparently not mediated by the presence of Bcrp1 in the mammary gland. Furthermore, the milk to plasma ratio for DEHP that we found in wild-type mice is comparatively low (Figure 6), which is not indicative of a pronounced concentration of this compound in mouse milk.

We note that the carcinogen doses used in this study are high, ~20-fold higher than incidental human exposure to aflatoxin B1 and 10 000-fold higher than chronic daily human exposure to HAs (6,8,28). However, the main aim of this study is to elucidate principles of the influence of BCRP/Bcrp1 on carcinogen pharmacokinetics, such as its role in milk secretion. As always, extrapolation of these preclinical data to the human situation should be done with caution.

We have previously shown that BCRP is highly expressed in the lactating mammary gland in mice, cows and humans, and mediates xenotoxin transfer into the milk (15). We demonstrate in this study that as a consequence there is a Bcrp1-mediated transfer of the dietary carcinogens [^{3}H]aflatoxin B1, [^{14}C]IQ and [^{14}C]Trp-P-1 from the mother to the vulnerable suckling infant via the milk. This makes it difficult to reconcile with a protective role of BCRP. It may be that under normal circumstances Bcrp1/BCRP in the intestine, liver and kidney is sufficiently efficient to keep dietary levels of these contaminants virtually completely out of the maternal circulation, thus limiting the risk of their accumulation in milk. However, several studies demonstrate the presence of HAs and aflatoxins in human breast milk and dairy products of cattle (11,14,29). Transfer of these dietary carcinogens by BCRP into milk may therefore still have relevant adverse consequences for exposure of newborns.

Acknowledgements

This project was in part financially supported by the Dutch Cancer Society, projects NKI 2000-2271 and NKI 2000-2143.

Conflict of Interest Statement: Bcrp1 knockout mice are commercially distributed by the company Taconic. Alfred H. Schinkel's research group benefits from the revenues.

References

1. Jonker,J.W., Smit,J.W., Brinkhuis,R.F., Maliepaard,M., Beijnen,J.H., Schellens,J.H. and Schinkel,A.H. (2000) Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J. Natl Cancer Inst*., **92**, 1651–1656.
2. van Herwaarden,A.E., Jonker,J.W., Wagenaar,E., Brinkhuis,R.F., Schellens,J.H., Beijnen,J.H. and Schinkel,A.H. (2003) The breast cancer resistance protein (Bcrp1/Abcg2) restricts exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Cancer Res*., **63**, 6447–6452.
3. Ushiyama,H., Wakabayashi,K., Hirose,M., Itoh,H., Sugimura,T. and Nagao,M. (1991) Presence of carcinogenic heterocyclic amines in urine of healthy volunteers eating normal diet, but not in patients receiving parenteral alimentation. *Carcinogenesis*, **12**, 1417–1422.
4. Sugimura,T. (2000) Nutrition and dietary carcinogens. *Carcinogenesis*, **21**, 387–395.
5. Eisenbrand,G. and Tang,W. (1993) Food-borne heterocyclic amines. Chemistry, formation, occurrence and biological activities. A literature review. *Toxicology*, **84**, 1–82.
6. Layton,D.W., Bogen,K.T., Knize,M.G., Hatch,F.T., Johnson,V.M. and Felton,J.S. (1995) Cancer risk of heterocyclic amines in cooked foods: an analysis and implications for research. *Carcinogenesis*, **16**, 39–52.
7. Henry,S.H., Bosch,F.X., Troxell,T.C. and Bolger,P.M. (1999) Policy forum: public health. Reducing liver cancer—global control of aflatoxin. *Science*, **286**, 2453–2454.
8. Bennett,J.W. and Klich,M. (2003) Mycotoxins. *Clin. Microbiol. Rev*., **16**, 497–516.
9. Becker,K., Seiwert,M., Angerer,J., Heger,W., Koch,H.M., Nagorka,R., Rosskamp,E., Schluter,C., Seifert,B. and Ullrich,D. (2004) DEHP metabolites in urine of children and DEHP in house dust. *Int. J. Hyg. Environ. Health*, **207**, 409–417.
10. Dostal,L.A., Weaver,R.P. and Schwetz,B.A. (1987) Transfer of di(2-ethylhexyl) phthalate through rat milk and effects on milk composition and the mammary gland. *Toxicol. Appl. Pharmacol*., **91**, 315–325.
11. De Bruin,L.S., Martos,P.A. and Josephy,P.D. (2001) Detection of PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) in the milk of healthy women. *Chem. Res. Toxicol*., **14**, 1523–1528.
12. Paulsen,J.E., Steffenson,I., Andreasssen,A., Vikse,R. and Alexander,J. (1999) Neonatal exposure to the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine via breast milk or directly induces intestinal tumors in multiple intestinal neoplasia mice. *Carcinogenesis*, **20**, 1277–1282.
13. Cullen,J.M., Ruebner,B.H., Hsieh,L.S., Hyde,D.M. and Hsieh,D.P. (1978) Carcinogenicity of dietary aflatoxin M1 in male Fischer rats compared to aflatoxin B1. *Cancer Res*., **47**, 1913–1917.
14. Zarba,A., Wild,C.P., Hall,A.J., Montesano,R., Hudson,G.J. and Groopman,J.D. (1992) Aflatoxin M1 in human breast milk from The Gambia, West Africa, quantified by combined monoclonal antibody immunoaffinity chromatography and HPLC. *Carcinogenesis*, **13**, 891–894.
15. Jonker,J.W., Merino,G., Musters,S., van Herwaarden,A.E., Bolscher,E., Wagenaar,E., Mesman,E., Dale,T.C. and Schinkel,A.H. (2005) The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. *Nat. Med*., **11**, 127–129.
16. Allen,J.D., van Loevezijn,A., Lakhai,J.M., van der Valk,M., van Tellingen,O., Reid,G., Schellens,J.H., Koomen,G.J. and Schinkel,A.H. (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.
17. Pavek,P., Merino,G., Wagenaar,E., Bolscher,E., Novotna,M., Jonker,J.W. and Schinkel,A.H. (2004) 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.
18. Doyle,L.A. and Ross,D.D. (2003) Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene*, **22**, 7340–7358.
19. Jonker,J.W., Buitelaar,M., Wagenaar,E. *et al*. (2002) The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc. Natl Acad. Sci USA*, **99**, 15649–15654.
20. Imai,Y., Nakane,M., Kage,K., Tsukahara,S., Ishikawa,E., Tsuruo,T., Miki,Y. and Sugimoto,Y. (2002) C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance. *Mol. Cancer Ther*., **1**, 611–616.
21. Zamber,C.P., Lamba,J.K., Yasuda,K., Farnum,J., Thummel,K., Schuetz,J.D. and Schuetz,E.G. (2003) Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics*, **13**, 19–28.
22. Merino,G., van Herwaarden,A.E., Wagenaar,E., Jonker,J.W. and Schinkel,A.H. (2005) Sex-dependent expression and activity of the

ATP-binding cassette transporter breast cancer resistance protein (BCRP/ABCG2) in liver. *Mol. Pharmacol*., **67**, 1765–1771.
23. Zhou,S., Schuetz,J.D., Bunting,K.D. *et al*. (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat. Med*., **7**, 1028–1034.
24. Asakura,A. and Rudnicki,M.A. (2002) Side population cells from diverse adult tissues are capable of *in vitro* hematopoietic differentiation. *Exp. Hematol*., **30**, 1339–1345.
25. Merino,G., Jonker,J.W., Wagenaar,E., Pulido,M.M., Molina,A.J., Alvarez,A.I. and Schinkel,A.H. (2005) Transport of anthelmintic benzimidazole drugs by breast cancer resistance protein (BCRP/ABCG2). *Drug Metab. Dispos*., **33**, 614–618.
26. Schinkel,A.H. and Jonker,J.W. (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv. Drug Deliv. Rev*., **55**, 3–29
27. Ito,S. and Lee,A. (2003) Drug excretion into breast milk—overview. *Adv. Drug Deliv. Rev*., **55**, 617–627.
28. Wakabayashi,K., Ushiyama,H., Takahashi,M., Nukaya,H., Kim,S., Hirose,M., Ochiai,M., Sugimura,T. and Nagao,M. (1993) Exposure to heterocyclic amines. *Environ. Health Perspect*., **99**, 129–133.
29. Martins,M.L. and Martins,H.M. (2004) Aflatoxin M1 in yoghurts in Portugal. *Int. J. Food Microbiol*., **91**, 315–317.

Received April 28, 2005; revised June 15, 2005; accepted June 28, 2005